

Light and temperature regulated terpene biosynthesis: hepatoprotective monoterpene picroside accumulation in *Picrorhiza kurrooa*

Tabasum Kawoosa · Harsharan Singh · Amit Kumar · Sunil Kumar Sharma · Kiran Devi · Som Dutt · Surender Kumar Vats · Madhu Sharma · Paramvir Singh Ahuja · Sanjay Kumar

Received: 31 August 2009 / Revised: 1 December 2009 / Accepted: 5 December 2009 / Published online: 14 January 2010
© Springer-Verlag 2010

Abstract *Picrorhiza (Picrorhiza kurrooa)* is an endangered medicinal plant with well-known hepatoprotective activity attributed to monoterpenoid picrosides. The present article details on regulatory genes of terpenoid metabolism, 3-hydroxy-3-methylglutaryl coenzyme A reductase (*pkhmgr*) and 1-deoxy-D-xylulose-5-phosphate synthase (*pkdxs*) from

picrorhiza. Since no molecular information was available, these genes were cloned to full-length by degenerate primers and rapid amplification of cDNA ends, followed by cloning of the upstream sequences that showed the presence of core sequences for light and temperature responsiveness. Electrophoretic mobility shift assay confirmed binding of protein to these motifs. Expression of *pkhmgr* and *pkdxs* was up-regulated at 15°C as compared to at 25°C as well as under light as compared to dark conditions. Picrosides content exhibited the trend similar to gene expression. To rule out the possible limitation of carbon pool under dark condition, plantlets of *picrorhiza* were raised in vitro in Murashige and Skoog medium supplemented with 3% sucrose. Results showed similar up-regulation of both the genes and the higher picrosides content in in vitro raised plantlets in the presence of light. Data suggested the important roles played by light and temperature in regulating *pkhmgr* and *pkdxs*, and the picrosides level in *picrorhiza*.

Electronic supplementary material The online version of this article (doi:10.1007/s10142-009-0152-9) contains supplementary material, which is available to authorized users.

T. Kawoosa · H. Singh · A. Kumar · S. K. Sharma · K. Devi · S. Dutt · M. Sharma · P. S. Ahuja · S. Kumar (✉)
Biotechnology Division, Institute of Himalayan Bioresource Technology (CSIR), P.O. Box No. 6, Palampur 176061, Himachal Pradesh, India
e-mail: sanjayplp@rediffmail.com
e-mail: sanjaykumar@ihbt.res.in

S. K. Vats
Biodiversity Division, Institute of Himalayan Bioresource Technology (CSIR), P.O. Box No. 6, Palampur 176061, Himachal Pradesh, India

Present Address:
H. Singh
Biotechnology Division, Lyallpur Khalsa College, Jalandhar, Punjab, India

Present Address:
A. Kumar
Department of Experimental Medicine and Biotechnology, Post Graduate Institute of Medical Education and Research, Chandigarh, India

Present Address:
S. K. Sharma
Biotechnology Division, Jaypee University of Information Technology, Waknaghat, Distt., Solan, Himachal Pradesh, India

Keywords Gene expression · Light · Mevalonate · MEP · Methylerythritol phosphate · MVA · *Picrorhiza kurrooa* · Picroside · Promoters · Terpenoid · Temperature

Abbreviations

aa	amino acid
DXS	1-deoxy-D-xylulose-5-phosphate synthase
EMSA	electrophoretic mobility shift assay
GDP	geranyl diphosphate
HMGR	3-hydroxy-3-methylglutaryl coenzyme A reductase
MEP	methylerythritol phosphate
MVA	mevalonate
PLACE	plant <i>cis</i> -acting regulatory elements

Introduction

Terpenoids constitute the largest family of natural plant products with over 30,000 members possessing important biological and physiological functions (Sacchettini and Poulter 1997). Terpenoids are derived from geranyl diphosphate (GDP) which is synthesized by sequential head to tail addition of isopentenyl pyrophosphate and its allelic isomer dimethylallyl diphosphate (Wise and Croteau 1998). Synthesis of isopentenyl pyrophosphate and dimethylallyl diphosphate proceeds via cytosolic mevalonate (MVA) and plastid methylerythritol phosphate (MEP) pathways (Mahmoud and Croteau 2002; Hampel et al. 2006; Fig. 1). A cross-talk between these two pathways has also been reported (Hampel et al. 2005). Supply of GDP is critical in realizing the yield of terpenoids (Nogués et al. 2006), therefore, studies on regulation of genes in GDP biosynthesis assume central importance.

Regulation of gene expression at the level of transcription is a major control point in many biological processes. Transcriptional regulation is achieved through binding of transcription factors to short consensus sequences of DNA known as *cis*-acting regulatory elements or motifs, usually located in the promoter regions, upstream of the coding sequences (Udvardi et al. 2007). It is generally believed that genes having similar expression patterns contain common motifs in their promoter (Klok et al. 2002). Thus, a common set of transcription factors co-regulate expression of genes and hence the pathway.

Picosides, the medicinally important hepatoprotectants (Floersheim et al. 1990), are synthesized by picrorhiza (*Picrorhiza kurrooa* Royle ex Benth.), an endangered plant species of family Scrophulariaceae. The species is distributed between 3,000–5,000 m above mean sea level in Himalaya (Chettri et al. 2005). Indiscriminate and extensive harvesting and lack of organized cultivation of the 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).

Picosides are terpenoids with an iridane skeleton of monoterpene origin (Fig. 1). Previous studies have shown that *3-hydroxy-3-methylglutaryl coenzyme A reductase (hmgr)* and *1-deoxy-D-xylulose-5-phosphate synthase (dxs)* are regulatory genes in terpenoids biosynthesis (Korth et al. 2000; Hsieh and Goodman 2005) for which no information is available in picrorhiza. In the present work, we cloned these genes from picrorhiza, analyzed the *cis*-acting elements, and the motifs for light and temperature regulation were studied. Electrophoretic mobility shift assay (EMSA) confirmed binding of proteins to the identified motifs. Gene expression analysis supported the results on transcriptional regulation of *hmgr* and *dxs* by light and temperature. A positive relationship between

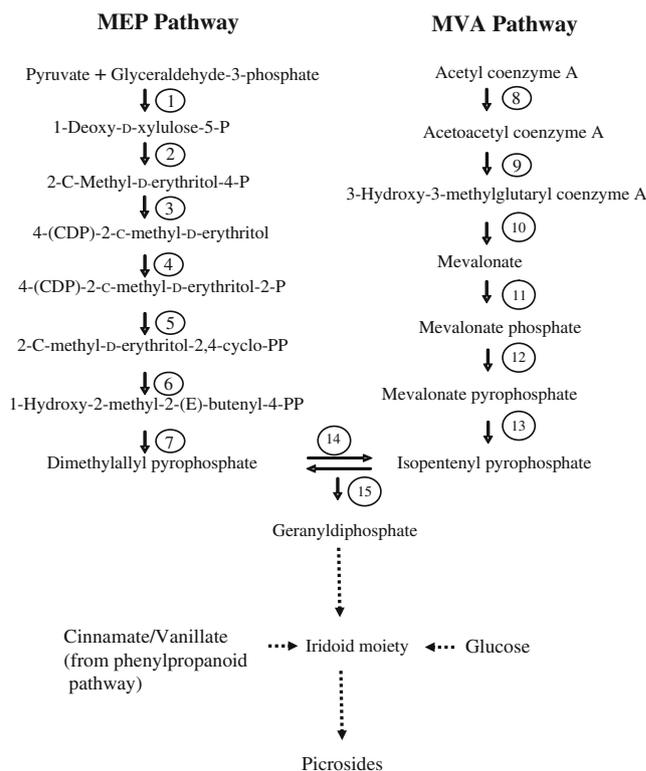


Fig. 1 Schematic pathway for picosides biosynthesis (adapted from Mahmoud and Croteau 2002). Geranyldiphosphate (GDP) can be derived from mevalonate (MVA) or methylerythritol phosphate (MEP) pathway. GDP yields iridoid, which is converted into picosides in the presence of glucose and cinnamic acid or vanillic acid. *Encircled numbers* represent enzyme catalyzing the corresponding reaction step as follows: 1 1-deoxy-D-xylulose-5-phosphate synthase; 2 1-deoxy-D-xylulose-5-phosphate reductoisomerase; 3 2-C-methylerythritol 4-phosphate cytidyl transferase; 4 4-(cytidine-5'-diphospho)-2-C-methylerythritol kinase; 5 2-C-methylerythritol-2,4-cyclophosphate synthase; 6 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase; 7 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase; 8 acetoacetyl CoA thiolase; 9 3-hydroxy-3-methylglutaryl coenzyme A synthase; 10 3-hydroxy-3-methylglutaryl coenzyme A reductase; 11 mevalonate kinase; 12 phosphomevalonate kinase; 13 mevalonate-5-pyrophosphate decarboxylase; 14 isopentenyl pyrophosphate isomerase; 15 geranyldiphosphate synthase. *Solid arrows* indicate known steps, whereas *dotted arrows* indicate unknown steps

expression of these genes and picosides content was obtained.

Materials and methods

Plant material

Plants of picrorhiza (*P. kurrooa* Royle ex Benth.) were collected from its natural habitat at Rohtang pass (4,000 m altitude, 32°23' N, 77°15' E, India) and brought to the Institute at Palampur (1,300 m altitude; 32°06' N, 76°33' E, India). These were transplanted in plastic pots (20 cm height × 20 cm top diameter × 12 cm bottom diameter)

containing soil, sand, and farm yard manure mixture in a ratio of 2:1:1, and were maintained in the experimental farm of the Institute. Plants were allowed to acclimatize for 3 months before start of the experiment. Experiments were performed on fifth leaf (position with respect to the top apical leaf designated as first leaf), which was harvested at the designated time, followed by freezing in liquid nitrogen and storage at -80°C for further use.

Raising of in vitro shoot cultures of picrorhiza

Young leaves of picrorhiza were collected from plants grown in the experimental farm, washed with tween-20, and surface-disinfected with solution containing 0.05% (w/v) streptomycin sulfate and 0.05% (w/v) bavistin for 20 min. The explants were further washed with distilled water and surface sterilized with sequential treatment of 70% ethanol for 45 s and mercuric chloride 0.05% (w/v) for 10 min. Subsequently, these were washed with autoclaved distilled water and inoculated on Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% sucrose. Prior to autoclaving, pH of the medium was adjusted to 5.7 followed by addition of 0.8% agar. Unless indicated, regenerated plantlets were incubated at $25\pm 2^{\circ}\text{C}$ with 16 h photoperiod (photosynthetic photon flux density, $70\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$).

Exposure of picrorhiza to temperature and light

Potted plants were shifted from the experimental farm to the plant growth chamber maintained at $25\pm 2^{\circ}\text{C}$ (16 h photoperiod; photosynthetic photon flux density, $350\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$). After 5 days, a few pots were shifted to another growth chamber maintained at $15\pm 2^{\circ}\text{C}$. Data was collected on day 12th after shifting as detailed in the figure legends.

Our data on the effect of temperature showed poor expression of the targeted genes at 25°C , which led us to study the effect of light in plants maintained at 15°C . For dark treatment, plants were covered with a cardboard box to restrict entry of light and the samples were harvested at 12 h from start of the treatment. Any long-term experiment with the plants exposed to dark could lead to possible starvation thus limiting the carbon pool. In order to differentiate the impact of light through effect on carbon pool or the light per se, plants were raised in vitro through tissue culture as detailed above. Four-week-old plantlets raised at $25\pm 2^{\circ}\text{C}$ were transferred to test tubes containing MS medium supplemented with 3% sucrose and maintained at $15\pm 2^{\circ}\text{C}$ in light and complete darkness, separately. Samples were harvested on day 6 of the treatment for analysis of gene expression and picrosides content. Beyond day 6, plants kept in dark showed etiolation and yellowing.

Cloning of cDNAs of *pkhmgr* and *pkdxs*

RNA was isolated from leaf tissue using the method of Ghawana et al. (2007) and digested with DNase 1 (RNase-free) using Message Clean[®] Kit (GenHunter[®] Corporation, USA). Complementary DNA (cDNA) was synthesized as described by Singh et al. (2004). Degenerate primers for *hmgr* and *dxs* were designed from the conserved regions of corresponding genes reported for different plant sources and the partial gene sequences were amplified by PCR as detailed in Table 1. The amplicons were cloned in pGEM-T Easy Vector (Promega, USA), plasmids were isolated using Qiagen Plasmid Mini-isolation Kit (Qiagen, GmbH), and sequencing was performed using BigDye terminator cycle sequencing mix (Version 3.1; Applied Biosystems, USA) using an automated DNA sequencer (ABI PRISM[™] 310 and 3130 *xl* Genetic Analyzer, Applied Biosystems, USA). Protocols were followed essentially as described by the respective manufacturers.

Full-length cDNAs were cloned by performing rapid amplification of cDNA ends (RACE; SMART[™] RACE cDNA Amplification Kit; Clontech, USA) as per the manufacturer's instructions using the gene specific primers (*hmgr5'R*, *hmgr3'R*, *dxs5'R* and *dxs3'R*; Table 1). These primers were designed based upon the partial sequences of the genes as cloned above. After aligning the sequences obtained by 5' and 3' RACE, full-length cDNA was amplified using the end sequences, cloned in pGEM-T Easy Vector (Promega, USA) and confirmed by sequencing.

Wherever needed, cDNA-sequences were analyzed using BLASTN, BLASTX, and BLASTP programs of National Center for Biotechnology Information (NCBI) with default parameters (<http://www.ncbi.nlm.nih.gov/>). Secondary structure of the deduced amino acid sequence was analyzed using Self-Optimized Prediction Method with Alignment (SOPMA; <http://www.npsa-pbil.ibcp.fr/>).

Construction of genome-walking libraries and cloning of promoters

DNA was isolated from leaf tissue using cetyltrimethylammonium bromide-based procedure (Doyle and Doyle 1987), treated with RNase (DNase-free) and purified with phenol/chloroform for constructing libraries for genome walking (GenomeWalker[™] Universal Kit; BD Bioscience, Clontech, USA). Genomic DNA was digested for 16 h with restriction enzymes *DraI*, *EcoRV*, *PvuII*, and *StuI*, separately to create blunt-end fragments and purified using phenol/chloroform. The restricted fragments were ligated to adaptors supplied by the manufacturer to produce four separate libraries. Promoter sequences were amplified using the Advantage Genomic Polymerase Mix (BD Bioscience, Clontech, USA) in a thermocycler (i-cycler, Bio-Rad,

Table 1 Oligonucleotide sequences and PCR conditions used in the present work

Name ^a	Sequence (5'-3') ^b	PCR conditions
Degenerate primers for amplification of the target genes		
<i>hmgrF</i>	ATWYTNGGRCARTGYTYGARATG	Initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C, 30 s; 43°C, 1 min; 72°C, 2 min. Final extension at 72°C for 7 min
<i>hmgrR</i>	AGRITYTTRAGCRTGTTYAGCTCBAC	
<i>dxsF</i>	GCTCYCCAYATGDTCGMCCT	Initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C, 30 s; 52°C, 1 min; 72°C, 2 min. Final extension at 72°C for 7 min
<i>dxsR</i>	TGSTAYAGCTGAHCAGCATGC	
Primers for RACE PCR		
<i>hmgr5'R</i>	AGTGGCCCTCTTAACCAAGGAAAACC	Primary PCR
<i>hmgr3'R</i>	CCAGAGCTCCGGTGGTTAGGTTTTT	5 cycles of 94°C, 30 s; 72°C, 3 min, followed by 5 cycles of 94°C, 30 s; 70°C, 30 s; and 30 cycles of 94°C, 30 s; 68°C, 30 s; 72°C, 4 min. Final extension at 72°C for 7 min
<i>dxs5'R</i>	CTTACCACACCACGCATTTTATTTG	
<i>dxs3'R</i>	TAGACACTTTTGAAGCGGATTGATG	Secondary PCR
		30 cycles of 94°C, 30 s; 68°C, 30 s; 72°C, 3 min. Final extension at 72°C for 7 min
Primers for expression studies		
<i>hmgrF</i>	ACTGATTACCACGCCCTTC	Initial denaturation at 94°C for 3 min, followed by 32 cycles of 94°C, 30 s; 60°C, 1 min; 72°C, 1 min. Final extension at 72°C for 7 min
<i>hmgrR</i>	TGGTTTCAAAGGTGTTTCAG	
<i>dxsF</i>	CGTCGATTGTAGCTGTAGGC	Initial denaturation at 94°C for 3 min, followed by 31 cycles of 94°C, 30 s; 60°C, 1 min; 72°C, 1 min. Final extension at 72°C for 7 min
<i>dxsR</i>	TATCGGTGATTGGAGATGGA	
Primers for genome walking		
<i>hmgrR-p</i>	ATTGATGCTTGATCTGTGAAGGTCTAGGG	Primary PCR
<i>hmgrR-s</i>	GGTGGTGTGGAGAGAAAATGGGGTAATTTT	5 cycles of 94°C, 25 s; 72°C, 3 min, followed by 32 cycles of 94°C, 25 s; 67°C, 3 min. Final extension at 67°C for 7 min
<i>dxsR-p</i>	CAGCTGGACAGCGTATGAAGCAATTAT	
<i>dxsR-s</i>	CATATCGGTGATTGGAGATGGAGCCATG	Secondary PCR
		5 cycles of 94°C, 25 s; 72°C, 3 min, followed by 30 cycles of 94°C, 30 s; 67°C, 3 min. Final extension at 67°C for 7 min

^a Primers name with “F”, “R”, “p” and “s” represent forward primers, reverse primers, primers used in primary PCR, and secondary PCR, respectively

^b R=A/G; M=A/C; W=A/T; Y=C/T; S=C/G; D=A/G/T; H=A/C/T; B=G/C/T; N=A/T/G/C

USA). Primary PCR was performed with gene-specific primers (*hmgrR-p* and *dxsR-p*; Table 1) and the outer adaptor primer (AP1, supplied by the manufacturer) using all the four genomic libraries, followed by secondary PCR with nested gene-specific primers (*hmgrR-s*, *dxsR-s*; Table 1) and the nested adaptor primer (AP2, supplied by the manufacturer). The PCR fragments were analyzed on a 1.2% agarose gel. Amplicons were purified using QIAEX[®]II Gel Extraction Kit (Qiagen, GmbH), cloned into pGEM-T Easy Vector (Promega, USA), and sequenced. Sequence information was also used for subsequent rounds of genome walking (if needed).

Sequences were analyzed in silico for detection of various motifs using “plant *cis*-acting regulatory DNA elements” (PLACE) database (<http://www.dna.affrc.go.jp/PLACE>; Higo et al. 1999). PLACE has advantage over the other eukaryotic databases such as TFD and TRANSFAC in terms of containing larger numbers of eukaryotic *cis*-elements from vascular plants (Higo et al. 1999). Multiple Expectation-Maximization for Motif Elicitation was used for the detection of common motifs in both the promoters

with motif width set to 6-9 and the number of motifs to be detected was set to 10 (<http://meme.sdsc.edu/meme/meme>). Since varying length of promoters was obtained for the two genes, the analysis of plus-strands was confined to 1,000 nucleotides upstream to the translation start site.

Reverse transcriptase-PCR analysis

DNA-free RNA was used to synthesize cDNA using Accuscript[™] High Fidelity 1st strand cDNA Synthesis Kit (Stratagene, USA). This cDNA was to be used as template for Reverse transcriptase-PCR reaction using gene specific primers (*hmgrF*, *hmgrR*; *dxsF*, *dxsR*) as mentioned in Table 1. Cycling conditions were optimized to obtain amplification under the exponential phase. *26S rRNA* based primer pair was used as internal control for expression studies (Singh et al. 2004). Amplicons were analyzed and quantified using the Alpha DigiDoc Gel Documentation and Image analysis system (Alpha Innotech, USA). Each experiment was repeated at least twice with three biological

replicates each time and the representative figure of one experiment is shown in the manuscript.

Extraction and estimation of picrosides

Picrosides were estimated as described by Dutt et al. (2004). Leaf tissue was harvested, washed with distilled water, blotted dry, weighed, then frozen in liquid nitrogen and stored at -80°C . The frozen samples (20 mg) were ground to fine powder in liquid nitrogen using pestle and mortar followed by addition of 1.0 ml of 80% methanol with intermittent grinding for 1 min. Extract was transferred to a centrifuge tube and the pestle and mortar was rinsed with 1.0 ml of 80% methanol to recover the leftover sample. Extracts were pooled, centrifuged at $15,000\times g$ for 15 min and the supernatant was used for picrosides estimation. Samples were filtered through 0.45 micron filter (Millipore, USA) for high pressure liquid chromatography analysis using LC 4000 module, 2487 dual λ absorbance detector (both from Waters, UK), and LiChrosorb[®] RP-18, 250 X 4.0 mm (Hibar) column (Merck & Co., Inc., USA). The mobile phase (flow rate, 1.0 ml/min) consisted of a mixture of trifluoroacetic acid (0.05%) and methanol-acetonitrile (1:1) in 70:30 ratio. Picrosides were monitored at 270 nm and quantified using pure picroside-I (P-I) and picroside-II (P-II) as standards.

Nuclear proteins extraction and electrophoretic mobility shift assay

Leaf tissue (20 g) was harvested from the plants growing at temperature of 15°C and a photoperiod of 16 h, and ground in pre-chilled pestle-mortar using extraction buffer [10 mM HEPES (pH 7.8), 10 mM KCl, 10 mM MgCl_2 , 5 mM EDTA, 1 mM DTT, 250 mM Sucrose, 0.2 mM PMSF and 0.5% Triton X-100] to isolate nuclear proteins essentially as described by Busk and Pages (1997). Oligonucleotides (30 mer) possessing low temperature responsive element (LTRE; CCGAA) and light responsive element (GATA), as present in promoters of *pkhmgr* and *pkdxs*, were designed having sequences: 5'-GAATTCAAAACCTTAACTGAAA CCGAAAATTGAGAAA-3' (LTRE-1); 5'-GAA TTCTTTCTCAATTTTCGGTTTCAGTTAAAGTTTT-3' (LTRE-2; complementary to LTRE-1); 5'-GTGCCAGTG TTGATATTATAAGTGCCAGTG-3' (GATA-1); 5'-CACTGGCACTTATAATATCAACACTGGCAC-3' (GATA-2; complementary to GATA-1). Equal moles of complementary oligonucleotides were mixed and annealed by incubating at 95°C for 5 min followed by slow cooling to room temperature. For preparation of probe for EMSA (Hennighausen and Lubon 1985), double-stranded oligonucleotides were radio-labeled with α - ^{32}P -dATP (3,000 Ci/mmol) using Klenow DNA polymerase I enzyme (USB,

USA) that filled up the recessive ends. Recessive ends were created by incorporating GAATTC at 5' end of the oligonucleotides. Binding of protein was carried out in $1\times$ binding buffer [30 mM HEPES (pH 7.8), 60 mM KCl, 0.3 mM EDTA, 0.5 mM DTT and 10% glycerol] containing $4\ \mu\text{g}$ of nuclear protein. For specific competition, unlabeled competitor DNA was added in excess to the binding mixture. After 10 min incubation at room temperature, probe (10,000 cpm) was added and incubated further for 20 min. Samples were loaded onto a 6% non-denaturing polyacrylamide gel and run in $0.5\times$ TBE buffer [$1\times$ TBE is 50 mM Tris-borate (pH 8.2), 1 mM EDTA] at 15 V/cm. Gel was blotted onto 3MM filter paper (Whatman, Clifton, NJ), dried under vacuum at 80°C for 2 h, and subjected to autoradiography.

Results and discussion

Hepatoprotective activity of picrorhiza owes to the presence of monoterpenoid picrosides (P-I and P-II; Luper 1998). Since terpenoids are derived from GDP that can be synthesized both from cytoplasmic MVA and plastidic MEP pathways (Croteau et al. 2005), it is important to study the regulation of these two pathways as feeders of GDP. Enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (encoded by *hmgr*) catalyzes reduction of 3-hydroxy-3-methylglutaryl coenzyme A into mevalonate and is regarded as rate-limiting step in the MVA pathway (Wang et al. 2007), for example in potato (*Solanum tuberosum*) and *Arabidopsis* (Yang et al. 1991; Leivar et al. 2005). Enzyme 1-deoxy-D-xylulose-5-phosphate synthase (encoded by *dxs*) catalyzes the first rate-limiting step involving condensation of pyruvate and glyceraldehyde-3-phosphate to produce 1-deoxy-D-xylulose-5-phosphate in the MEP pathway. The gene has been found to be regulatory (Muñoz-Bertomeu et al. 2006; Estevez et al. 2001), such as in tomato (*Lycopersicon esculentum*), periwinkle (*Catharanthus roseus*) and *Arabidopsis* (Lois et al. 2000; Chahed et al. 2000; Juan et al. 2001). Since *hmgr* and *dxs* are regarded as important genes of terpenoid biosynthesis pathway (Fig. 1), the present work studied these genes, their promoters, and the picrosides content to identify the gene regulation and its possible relationship with the picrosides content in picrorhiza.

Degenerate primers (Table 1) followed by RACE led to amplification of 2,245 bp and 2,317 bp of *pkhmgr* (accession no. DQ347962) and *pkdxs* (accession no. EU561005), respectively. The cDNAs consisted of 106 and 50 bp of 5' untranslated region; 453 bp, and 203 bp of 3' untranslated regions; and open reading frames of 1,686 and 2,064 bp for *pkhmgr* and *pkdxs*, respectively. Deduced proteins (hereinafter referred to as pKHMGR and pKDXS, respectively) had molecular masses/isoelectric points of

Table 2 Location of motifs with respect to the translation start site detected in *Upkmggr* and *Upkdxs* using PLACE database (<http://www.dna.affrc.go.jp/PLACE>). Motifs at serial number 25-73 are specific to either *Upkmggr* or *Upkdxs*

S.N.	Motif ^a	Role ^b	Motif location		S.N.	Motif ^a	Role ^b	Motif location	
			<i>Upmggr</i>	<i>Upkdxs</i>				<i>Upmggr</i>	<i>Upkdxs</i>
1	AAACAAA (S000477)	OTR	-147, -151	-423	38	RGTGACNNGC (S000022)	OTR/FS	-400	
2	NGATT (S000454)	CY	-650	-317, -588, -679	39	TGTCA (S000498)	BF	-434, -617, -596, -694, -881	
3	CAAT (S000028)	BT	-394, -600, -740, -794, -879	-302, -160, -40, -997, -762, -720, -935, -832	40	YTCANTYY (S000395)	L/BT	-94, -87	
4	YACT (S000449)	LS	-29, -38, -66, -80, -103, -291, -309, -465, -669, -728, -815, -837, -843, -864, -885, -906, -921, -927, -942, -948, -963, -990	-101, -172, -255, -368, -524, -802	41	CAACA (S000314)	FS	-908, -992	
5	CNAACAC (S000148)	SS	-991	-256	42	GCCAC (S000482)	L	-39, -816	
6	CCAAT (S000030)	T	-740	-160, -302, -720, -832	43	TACTATT (S000184)	OTR	-725	
7	GTAC (S000493)	S	-423, -670	-173, -498, -765, -803	44	CCGAAA (S000250)	T	-179	
8	AAAG (S000265)	GB	-1, -334, -643, -888, -893, -972	-5, -20, -60, -83, -146, -342	45	TATAAAT (S000109)	BT	-281	
9	ACACNNG (S000292)	SS	-862, -904, -925, -946	-769	46	TATTAAT (S000110)	BT	-235	
10	CANNTG (S000144)	T/D/AB/SS	-145, -925	-99, -366	47	TTWTWTTWTT (S000067)	OTR	-226, -238	
11	GATA (S000039)	L	-222, -510, -959	-13, -244, -612, -661	48	AAACCCTAA (S000308)	RM	-802	
12	GRWAAW (S000198)	L	-177, -197, -957, -364	-13, -242, -610, -659	49	AAACCCTA (S000472)	AM	-803	
13	GATAA (S000199)	L	-957	-243, -611, -660	50	TGACT (S000442)	S	-160, -532	
14	WAACCA (S000408)	T/D/AB	-68	-795, -834	51	ATAGAA (S000296)	LS	-128, -953	
15	CNGTTR (S000176)	T/D	-551	-945	52	CAAACAC (S000143)	SS		-256
16	AATAAA (S000080)	FS	-204, -391, -894	-10, -343	53	ACGT (S000415)	L/AB		-624, -727
17	AGAAA (S000245)	FS	-127, -133, -169, -952	-2, -61, -80, -84	54	CAACTC (S000421)	GB		-127
18	ACTCAT (S000450)	HR	-834, -939	-521	55	CWWWWWWWWG (S000431)	SS		-459
19	ATATT (S000098)	RS	-312, -508, -538, -606, -746	-292	56	TATTAG (S000491)	CY		-546
20	RTTTTTR (S000103)	SS	-257, -544	-226, -389, -841, -567	57	WTTSSCS (S000476)	CD		-325
21	TAAAG (S000387)	LS	-888, -893, -972	-342	58	GANTTNC (S000494)	OTR		-336
22	TTATTT (S000203)	L/BT	-230, -548	-250	59	TTGACC (S000142)	BF		-262, -313
23	TGACY (S000457)	BF	-160, -404, -532	-262, -313	60	GGTCANNAGTC (S000155)	BF		-139
24	TGAC (S000447)	GB	-161, -405, -532, -840, -966, -987	-263, -314	61	TAACGTA (S000420)	GB/SS		-623
25	TGTAAAG (S000001)	SS	-972		62	GGTTAA (S000125)	L		-449
26	TGHAAARK (S000122)	SS	-334		63	CCAATGT (S000432)	OTR		-718, -830
27	AACAAAC (S000353)	SS	-150		64	CCGAC (S000153)	T		-949
28	TAACGT (S000177)	T/D	-188		65	GGATA (S000180)	T/D		-244
29	MACGYGB (S000507)	AB	-358		66	CCTTTT (S000259)	GB		-92, -350, -642
30	VCGCGB (S000501)	S	-58, -400, -414, -416		67	AGGTCA (S000254)	FS		-142
31	TGTAAAAGT (S000034)	SS	-333		68	AACCAA (S000362)	PR		-833

Table 2 (continued)

S.N.	Motif ^a	Role ^b	Motif location		S.N.	Motif ^a	Role ^b	Motif location	
			<i>Uphmgr</i>	<i>Upkdxs</i>				<i>Uphmgr</i>	<i>Upkdxs</i>
32	AAAGAT (S000461)	RS	-1		69	CGGATA (S000363)	L		-244
33	CTCTT (S000462)	RS	-48, -100		70	TAAAG (S000387)	LS		-342
34	ACTTTA (S000273)	AU	-192		71	TATTTAA (S000400)	SM		-627
35	AAAGAT (S000461)	RS	-1		72	TTGAC (S000390)	S/BF		-263, -314
36	YAACKG (S000409)	T/D/AB	-188		73	TTAATGG (S000433)	RS		-490
37	GAAAAA (S000453)	L/BF	-177, -197, -364						

^aR=A/G; M=A/C; W=A/T; Y=C/T; S=C/G; D=A/G/T; H=A/C/T; B=G/C/T; N=A/T/G/C; K=G/T; V=A/C/G

^bMotifs responsive to: *L* light, *T* temperature, *D* drought/dehydration, *SS* seed/embryo specificity, *LS* leaf/plastid specificity, *RS* root specificity, *FS* flower/pollen specificity, *AB* abscisic acid, *GB* gibberellins, *CY* cytokinin, *AU* auxin, *BF* biotic factors, *S* signaling, *BT* basic transcription, *SM* secondary metabolism, *CD* cell division, *HR* hypo-osmolarity, *RM* root meristem, *AM* apical meristem, *PR* phytochrome regulation, and *OTR* other processes

60.65 kDa/7.24 and 73.63 kDa/8.60, respectively. BLASTX analysis showed extensive identity (>70%) of the deduced proteins with those from other plant sources (Electronic Supplementary Fig. S1).

In silico analysis showed the presence of corresponding conserved domains in both of the deduced proteins (Electronic Supplementary Fig. S2). HMG coenzyme A reductase class I domain was found to be present between amino acid (aa) positions 162 and 551 in pkHMGR. In pkDXS, three characteristic domains were recorded. These were: thiamine pyrophosphate binding domain (between aa positions 77 and 334), transketolase pyrimidine binding domain (between aa positions 364 and 527), and transketolase-c terminal domain (between aa positions 545 and 667).

SOPMA analysis revealed 46.52% α -helices, 4.99% β -turns, 14.62% extended strands and 33.87% random coils in pkHMGR. In pkDXS, α -helices, β -turns, extended strands, and random coils were 36.97%, 6.84%, 14.56%, 41.63%, respectively (Electronic Supplementary Table S1, Fig. S3). Deduced secondary structures were in agreement with those reported for functional genes in other plant systems (Electronic Supplementary Table S1). Various in silico analyses suggested the cloned genes to be functional as has been reported by the other studies (Walter et al. 2002; Wang et al. 2007).

Cloning and analysis of promoters of *pkhmgr* and *pkdxs*

The promoters of *pkhmgr* (*Upkhmgr*, 1,065 bp; accession no. FJ228691) and *pkdxs* (*Upkdxs*, 1,146 bp; accession no. FJ228692) were cloned by genome walking. In silico analysis was limited to 1 kb sequences upstream of translation start site for consistency in comparative analysis of the two promoters under study. Table 2 lists various

motifs along with their function and location in the two promoters analyzed. A total of 73 types of motifs were identified in *Upkhmgr* and *Upkdxs*, out of which 24 motifs were common to both, whereas 27 and 22 motifs were specific to *Upkhmgr* and *Upkdxs*, respectively (Table 2). In *Upkhmgr*, number of motifs identified for basal transcription, light responsiveness, low temperature responsiveness, tissue specificity (leaf, seed, root, flower, meristems), hormone (gibberellins, cytokinins, abscisic acid, and auxin) responsiveness and biotic factors were 11, 17, 7, 61, 19, and 11, respectively. These figures for *Upkdxs* were 9, 16, 8, 28, 23, and 7 for the above motifs in the same order.

Ecological niche of picrorhiza is characterized by an environment of low temperature and high light intensity (Chandra 2004). Light responsive motifs in both the promoters were spread throughout the promoter (Fig. 2) and these include: GATA box (WGATAR; Gilmartin et al. 1990), GT-1 (GRWAAW; Villain et al. 1996), TATA box (TTATTT; Tjaden et al. 1995), and I box (GATAA; Terzaghi and Cashmore 1995). GATA factors were first identified as proteins that interact with conserved WGATAR (W = T or A; R = G or A) motifs involved in erythroid-specific gene expression in vertebrates (Evans et al. 1988) and have been mostly implicated in light-dependent gene regulation in plants (Reyes et al. 2004). In *Arabidopsis*, Teakle et al. (2002) established that different families of GATA-binding factors bind to DNA sequence elements containing GATA or GAT sequence. It was suggested that GATA core sequence in the GATA motif was important for binding relevant transcription factors and the flanking sequences are non-consequential.

GT-1 box is a regulatory motif usually found in tandem repeats in the promoter region of many different plant genes regulated by light (Villain et al. 1996). Apart from light, GT-1 is also shown to be regulating salt and

Fig. 2 Nucleotide sequences of *Upkdmgr* and *Upkdxs* promoters cloned from picrorhiza. One thousand nucleotides upstream to translation start site were analyzed. The nucleotide just upstream to the first nucleotide of translation start codon is numbered as -1 and subsequent upstream numbering is in descending order. Light and temperature responsive motifs are *boxed* and *underlined*, respectively

<i>pkhmgr</i>	-1000	GCACCAACACTGCACACATAATGTAAAGACTGACACTGATAATAGAAACACTGGCACTCATAATATAAACACTTGCACT
<i>pkdxs</i>	-1000	CAATTCACCCCATTTTGTGTTGGGGAATCCTTCGTATAAAAATTAACCCGCGTTAAAATGGCAATTAACCTFGAAATTTAA
<i>pkhmgr</i>	-920	TATAATATCAACACTGGCACTAATAAAGTAAAGACTGTCAATTTATAATATAAACACTGGCATTATAGCATTAACTACTGA
<i>pkdxs</i>	-920	TTCCATTAAATCCCACTAAGGCGTGTTCATTATCGCGTAAACCTGGAGGAGGCCCTTTAATAGTTAGAACAGTTTTTAC
<i>pkhmgr</i>	-840	CACTCATAATGTTTAGCCGCGCCACTGTCTAAACCTAAATCACAATGTATTATGAGTGCCAGTGTTCCTTTACATTGT
<i>pkdxs</i>	-840	AAAACCAATGTTTCGTCTTAAATCGTCAAGGTTGTACTTTAAACCACGAAGTTTTATGTTAAATACACAAGGTACAAATG
<i>pkhmgr</i>	-760	GCCAGTGTATTATATACCAATGCCAGTGTACTATTATGAGTGCCAGTGTATTATTAATTTGFTGAGTGTTCCTATTAC
<i>pkdxs</i>	-760	TTTCGTCTTAAATCATCAAAATTTTATCTTACGTTACCAATGTTTCATGTTAAATCACCAAAGATAACAAACATTGATGA
<i>pkhmgr</i>	-680	ATTATGAGTACTATAAGTGCCAGTGTGATTTTAAAAGTCCAGTGTGAGTATTATGAGTGTGAGTGTTCCTATTATCAA
<i>pkdxs</i>	-680	TTTAAGATGAACATTTGATAATTTAAAACGAAACCTTTTACCCTGTTGATTTTAAACGTAACCTTTGATAATTTAACCGAA
<i>pkhmgr</i>	-600	TGTCAGTGTTCCTATTATGAGTGCCAGTGTTCCTTTTATACATTGTGCCGTTATTTTATTATATTATGACTATTATGAGTGC
<i>pkdxs</i>	-600	AACCTTGATGATTTAATACGAAACCTGTTCCTTTGTAACAAACCTTAACTATTAGACGGTCTCCCTCTAGTTTTACTCAT
<i>pkhmgr</i>	-520	CAGTGTGATAATTTATAAGTGCCAGTGTGAGTAGTAGTGCCAGTGTTCCTTATACTATAATTGCCAGTGTTCCTATTATG
<i>pkdxs</i>	-520	AAATGATAGGCTTTAGTTGTACATTAATGAAATTAATTCGAAGTTCATTACCATTTTAATGATCGGGTTAAATTTTATCA
<i>pkhmgr</i>	-440	AGTGTGAGTGTGAGTACCCCGCGCTACAGTGACCGCGCTACAATAAATCCGGTTTTCTTAATGTTATGAAAAACCG
<i>pkdxs</i>	-440	CAAGAATCCCAACAAATGGGTTGAAGCATGTTCTATTGTTCTGTTTTGCTAGAGCTATCTCGTCTCACTTCGCCATG
<i>pkhmgr</i>	-360	CGTCTTTCTGAGTAATTTGTAAGTGTTCCTATTTTACATCATATTAATTTTCAATTTTCATCATATACTTATTATAAAT
<i>pkdxs</i>	-360	GCTGCCCTTTTAAATAAAGAAATTCCTTATTGGCGGCTAGATTGACCTTTTATCCAAATAGGCCATATGTTTGAAGTTA
<i>pkhmgr</i>	-280	ACATTACATTTTTTTTAAATTTTTATCACACATTTTTTATTATTAATTTTATTTGATAATCCGATCCCGAATTAATTA
<i>pkdxs</i>	-280	TTATGCTAATTTCTTGACCAAAACACTTATTTCCGATAATAGTTGTTGATTTTTTGAATTTTTTGGGTAACATATACAGTA
<i>pkhmgr</i>	-200	AAAACTTTAACTGAAACGAAAAATTTGAGAAATTTATGACTACAAACAAACAAATGCCGGCACAGAAATAGAAATTCACA
<i>pkdxs</i>	-200	ATTATGTCATCAACGCTTGCTTTTGTACTCTAGTGCCCAATCTTTCTATAAAAAGGTCATCAGTCTTCAACTCCTCATA
<i>pkhmgr</i>	-120	GGCTCTCTCGCTTACTCTTCACTTCTTCAGTCTTTCCACTATAACGAAACCACTCCCGTAGGCATCGCTCTTCCAGGCC
<i>pkdxs</i>	-120	CCAAGTAGCAACCATCACTTGCTTTTTCAGAAAAGAAAACCTTGAGCTCTTCAGAAAAGAGCTTGATCACAATCCCC
<i>pkhmgr</i>	-40	ACTAAAAATTAATCTTCTTCCACACCACCCGAAAGAT -1
<i>pkdxs</i>	-40	TAATACAAAAAAGAGGAAATAAAAAAGAAAA -1

pathogen induced gene expression (Park et al. 2004) and hence is not considered very specific in relation to regulation by light.

Apart from the above motifs common in the two promoters, promoter-specific light responsive motifs were also detected. In *Upkdmgr*, GCCAC (SORLIP) box was identified which is found to be over-represented in the phytochrome A-induced promoters (Hudson and Quail 2003). *Upkdxs* possessed CGGATA (REbeta) box, which is shown to be required for phytochrome regulation in *Lemma gibba Lhcb21* gene promoter (Degenhardt and

Tobin 1996). Yet another light responsive box GGTTAA was identified in *Upkdxs*, which has been reported to be present in upstream sequences of the light-responsive pea *rbcS-3A* gene (Green et al. 1988).

Among the various light responsive motifs analyzed in silico, GATA core sequence was specific to light and was present in *Upkdmgr* and *Upkdxs*. Therefore, GATA core was selected to study its interaction with putative transcription factors by EMSA. Results showed prominent shifts for GATA motif (Fig. 3 lane 5) suggesting binding of putative transcription factors to the motif in vitro.

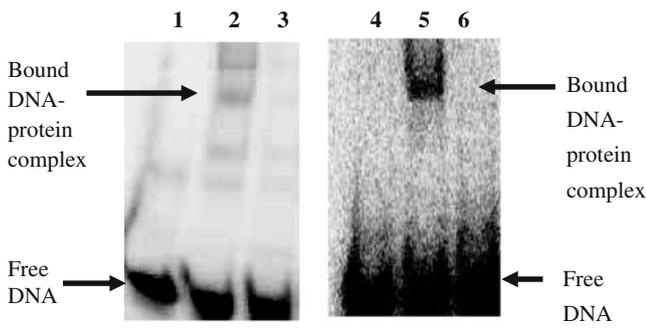


Fig. 3 Electrophoretic mobility shift assay showing DNA-protein interaction. *Lane 1* radio-labeled low temperature responsive sequence (LTRE; 5'-GAATTCAA^{ACTTTAACTGAA}ACCGAAAAAATTGA GAAA-3'); *lane 2* radio-labeled LTRE sequence incubated with nuclear protein; *lane 3* radio-labeled LTRE sequence incubated with nuclear protein and unlabeled (functioning as specific competitor) LTRE sequence; *lane 4* radio-labeled light responsive (GATA) sequence (5'-GTGCCAGTGTGATATTATAAGTGCCAGTG-3'); *lane 5* radio-labeled GATA sequence incubated with nuclear protein; and *lane 6* radio-labeled GATA sequence incubated with nuclear protein and unlabeled (functioning as specific competitor) GATA sequence. Core LTRE and GATA sequences are *underlined*

Low temperature responsive motifs were investigated to examine various *cis*-acting regulatory elements and *trans*-acting factors that have been reported to be involved in plants responses to temperature. Presence of several motifs involved in low temperature responses were detected in both the promoters analyzed (Table 2, Fig. 2). These included low temperature responsive elements (LTRE, CCGAC/CCGAA) and dehydration responsive elements [DREs: CANNTG (MYC), WAACCA, CNGTTR, TAACTG, YAACKG (MYB), ACGT, and MACGYGB (ABRE)]. LTREs containing pentanucleotide CCGAC/CCGAA have been identified in a number of promoters of a low-temperature-responsive gene such as in *Brassica napus* (BN115) and *Hordeum vulgare* (ABA-regulated barley gene, *HVA1*; Dunn et al. 1998). EMSA showed the importance of CCGA core sequence within the pentanucleotide LTRE for DNA protein interaction. Motifs related to water stress/drought viz; MYC, MYB, and ABRE like boxes are involved in ABA-mediated responses to various adverse environmental cues including cold (Agarwal et al. 2006; Choi et al. 2000).

LTRE core sequence CCGA was present in *Upkdmgr* and *Upkdxs* and was used in DNA-protein interaction studies. Results showed prominent shifts for CCGA motif (Fig. 3 lane 2) suggesting binding of putative transcription factors to the motif *in vitro*.

Data on *in silico* analysis and EMSA suggested the possible functionality of light and temperature responsive motifs in promoters of both the genes. The next logical question was if these genes are also regulated by light and temperature? And how does it correlate with the picosides content under these conditions.

Effect of light and temperature on expression of *pkdmgr* and *pkdxs*, and picosides content

Expression of *pkdmgr* and *pkdxs* was higher by 216% and 286%, respectively, at 15°C as compared to those at 25°C (Fig. 4a, b). Also, total picosides increased by 22% at 15°C as compared to the plants maintained at 25°C (Fig. 4c). Since expression of *pkdmgr* and *pkdxs* was very poor at 25°C, experiment on the effect of dark was performed at 15°C. In a span of 12 h, expression of *pkdmgr* and *pkdxs* was higher by 70% and 112% in light as compared to that observed under dark (Fig. 5a, b). Picosides content in light was higher by 163% of that observed under dark conditions (Fig. 5c).

Dark conditions might starve the plants and it is likely that gene expression and picosides content in dark could be a reflection of the effect of carbon limitation. Light would affect carbon pool through photosynthesis and the

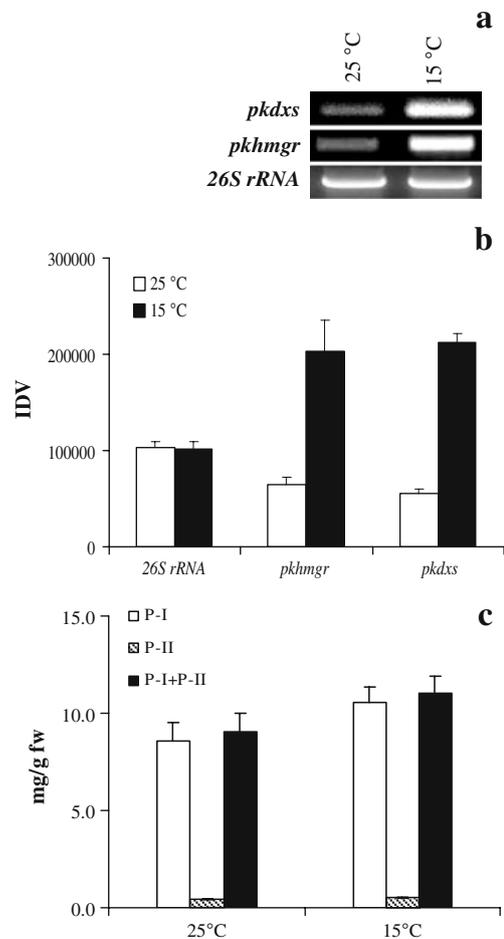


Fig. 4 Effect of temperature on expression of *pkdmgr* and *pkdxs*, and picosides content in fifth leaf of picrorhiza. Panel **a** shows gene expression, wherein *26S rRNA* was used as a marker for equal loading. Panel **b** shows integrated density value (IDV) of each amplicon as obtained in panel **a**. Panel **c** shows change in picosides content. Values in panel **b** and **c** are average of three separate biological replicates; *error bar* represents standard error of the mean

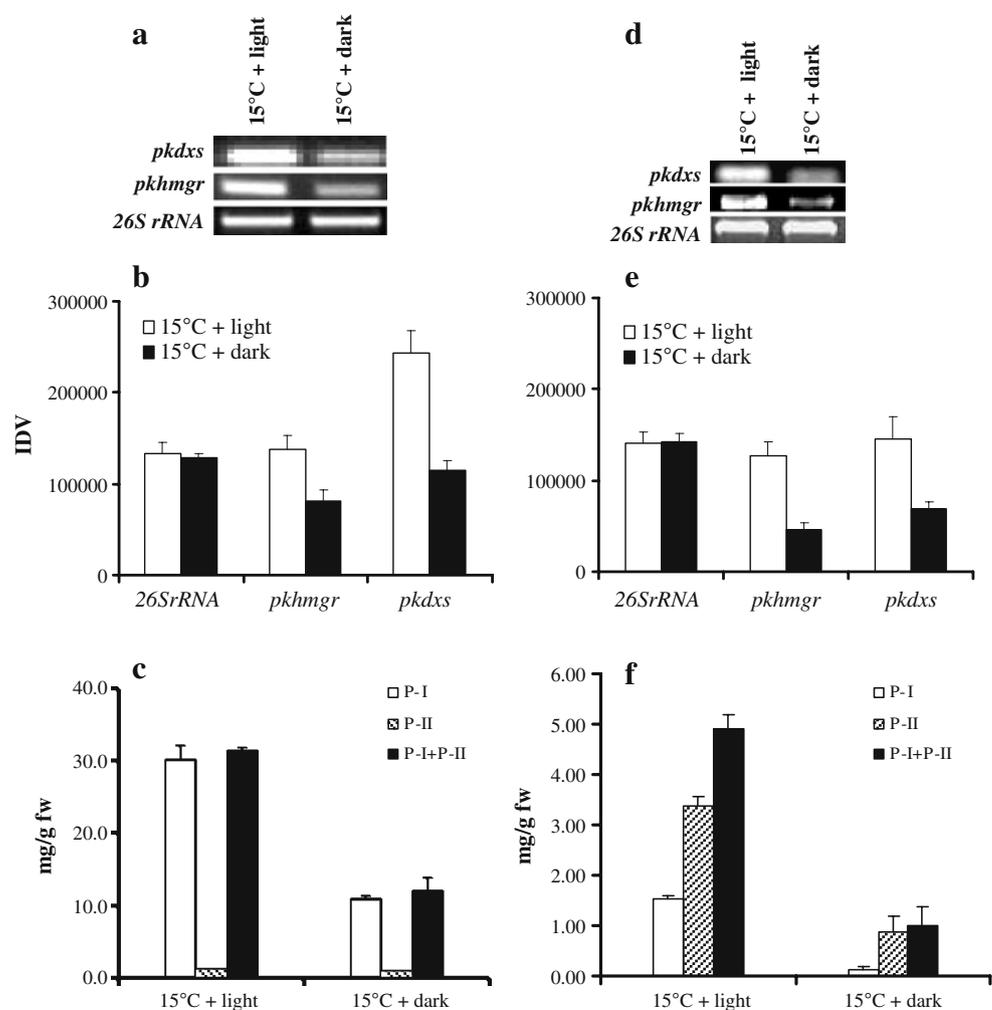
role of carbon pool in regulating secondary metabolites has been shown in *Hypericum perforatum* L. (Mosaleeyan et al. 2005). Also, the possibility exists that light modulated gene expression independent of carbon pool (Fey et al. 2005). Hence, the experiment was carried out using in vitro raised plantlets with the medium fortified with sucrose (3%). In this system also, expression of *pkhmgr* and *pkdxs* was higher by 173% and 112% in light as compared to that recorded under dark (Fig. 5d, e). Also, the picosides content in light was higher by 389% as compared to those in dark (Fig. 5f). Gene expression was in agreement with the promoter data wherein the motifs for light (e.g., GATA) and low temperature (e.g., LTRE) were present. EMSA also showed binding of the putative transcription factors to these motifs (Fig. 3).

The role of light and temperature in modulating a range of terpenoids and the corresponding transcripts has been documented, but there is no universal behavior and it varies depending upon the metabolite under study and the plant species. The same gene, *hmgr* for example, is stimulated by light in *Triticum aestivum* (Aoyagi et al. 1993), pea (Wong

et al. 1982) and potato (Korth et al. 2000) but is downregulated by light in *Lithospermum erythrorhizon* (Lange et al. 1998). Effect of light on the activity of *hmgr* promoter has been reported that explained the light mediated alteration in *hmgr* transcripts (Learned and Connolly 1997). Gene *dxs* has also been reported to be regulated by light in *Arabidopsis* (Hsieh and Goodman 2005; Cordoba et al. 2009).

Soitamo et al. (2008) showed that light at low temperature induced expression of genes involved in synthesis of phenylpropanoids, carotenoids, and terpenoids. In *Arabidopsis*, light-dependent flavonoid (Fuglevand et al. 1996) and phenylpropanoid (Hemm et al. 2004) biosynthesis has been attributed to: (1) upregulation of relevant genes at transcriptional level, and (2) the involvement of the primary photoreceptors- phytochrome B and cryptochrome. Light-mediated accumulation of reducing equivalents in the stroma and reactive oxygen species has been reported to function as signals from chloroplasts to the nucleus leading to altered gene expression (Fey et al. 2005). Low temperature mediated increase in secondary metabolites in

Fig. 5 Effects of light and dark on expression of *pkhmgr* and *pkdxs*, and picosides content in fifth leaf of picrorhiza plants (a, b, c) and plantlets raised through tissue culture (d, e, f). Panels a and d show gene expression, wherein *26S rRNA* was used as a marker for equal loading. Panels b and e show integrated density value (IDV) of each amplicon as obtained in panels a and d. Panels c and f show changes in picosides content. Values in panel b, c, e, and f are average of three separate biological replicates; error bar represents standard error of the mean



Arabidopsis has mainly been attributed to the transcriptional upregulation of genes of secondary metabolism, which in turn, has been suggested due to the over-expression of relevant transcription factors at low temperature (Hannah et al. 2005). It is also possible that upregulation of the two regulatory genes of the picrosides biosynthesis pathway by light and low temperature increased carbon partitioning towards terpenoid metabolism resulting in higher picrosides content in picrorrhiza.

Our results that light and low temperature favor picrosides accumulation are in agreement with the picrosides levels in natural population of picrorrhiza, wherein it has been reported that picrosides content increased by 135% in the plants growing at high (4,145 m) as compared to the low (1,350 m) altitude (Singh et al. 2005). Increase in altitude accompanies decrease in temperature and increase in light quanta (Streb et al. 1998). Molecular data on promoter analysis, gene expression, and picrosides content validated the importance of light and temperature in regulating picrosides content in picrorrhiza.

Acknowledgments We acknowledge financial support from Council of Scientific and Industrial Research (CSIR) for work on gene cloning through Mission Mode program entitled “Development of medicinal plant chemotypes for enhanced marker and value added compounds (COR002)”. Financial support from the Department of Biotechnology (DBT), Govt. of India is duly acknowledged for funding the project entitled “Molecular cloning and characterization of regulatory genes involved in picrosides metabolism in *Picrorhiza kurroa*” sanctioned vide sanction order number BT/PR6614/PBD/17/425/2005 to work on promoters. TK thanks CSIR, India, for awarding CSIR Diamond Jubilee Research Intern Award; HS thanks CSIR for awarding junior and senior research fellowships. AK, KD, and SKS gratefully acknowledge DBT for providing assistantship. Technical help provided by Mr. Digvijay Singh in gene sequencing is duly acknowledged. The manuscript represents IHBT communication number 0812.

References

- Agarwal M, Hao Y, Kapoor A, Dong CH, Fujii H, Zheng X, Zhu JK (2006) A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. *J Biol Chem* 28:37636–37645
- Aoyagi K, Beyou A, Moon K, Fang L, Ulrich T (1993) Isolation and characterization of cDNAs encoding wheat 3-hydroxy-3-methylglutaryl coenzyme A reductase. *Plant Physiol* 102:623–628
- Busk PK, Pages M (1997) Microextraction of nuclear proteins from single maize embryos. *Plant Mol Biol Rep* 15:371–376
- Chahed K, Oudin A, Guivarc’h N, Hamdi S, Che’nieux J-H, Rideau M, Clastre M (2000) 1-Deoxy-d-xylulose 5-phosphate synthase from periwinkle: cDNA identification and induced gene expression in terpenoidindole alkaloid-producing cells. *Plant Physiol Biochem* 38:559–566
- Chandra S (2004) Effect of altitude on energy exchange characteristics of some alpine medicinal crops from central Himalayas. *J Agron Crop Sci* 190:13–20
- Chettri N, Sharma E, Lama SD (2005) Non-timber forest produces utilization, distribution and status in a trekking corridor of Sikkim, India. *Lyonia* 8:89–101
- Choi H, Hong J, Ha J, Kang J, Kim SY (2000) ABRE/ACGT binding sites ABFs, a family of ABA-responsive element binding factors. *J Biol Chem* 275:1723–1730
- Cordoba E, Salmi M, León P (2009) Unravelling the regulatory mechanisms that modulate the MEP pathway in higher plants. *J Exp Bot* 60:2933–2943
- Croteau RB, Davis EM, Ringer KL, Wildung MR (2005) (-)-Menthol biosynthesis and molecular genetics. *Naturwissenschaften* 92:562–577
- Degenhardt J, Tobin EM (1996) A DNA binding activity for one of two closely defined phytochrome regulatory elements in an Lhcb promoter is more abundant in etiolated than in green plants. *Plant Cell* 8:31–41
- Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochem Bull* 19:11–15
- Dunn MA, White AJ, Vural S, Hughes MA (1998) Identification of promoter elements in a low-temperature-responsive gene (*blt4.9*) from barley (*Hordeum vulgare* L.). *Plant Mol Biol* 38:551–564
- Dutt S, Kiddle G, Singh B, Khambay B, Foyer CH (2004) Differential accumulation of picrosides in *Picrorhiza kurroa* Royle ex Benth plants. http://www.rothamsted-international.org/publications_posters.shtml
- Estevez JM, Cantero A, Reindl A, Reichler S, Leon P (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastid isoprenoid biosynthesis in plants. *J Biol Chem* 25:22901–22909
- Evans T, Reitman M, Felsenfeld G (1988) An erythrocyte specific DNA-binding factor recognizes a regulatory sequence common to all chicken globin genes. *Proc Natl Acad Sci USA* 85:5976–5980
- Fey V, Wagner R, Brautigam K, Wirtz M, Hell R, Dietzmann A, Leister D, Oelmüller R, Pfannschmidt T (2005) Retrograde plastid redox signals in the expression of nuclear genes for chloroplast proteins of *Arabidopsis thaliana*. *J Biol Chem* 280:5318–5328
- Floersheim GL, Bieri A, Koenig R, Pletscher A (1990) Protection against *Amanita phalloides* by the iridoid glycoside mixture of *Picrorhiza kurroa* (kutkin). *Agents Actions* 29:386–387
- Fuglevand G, Jackson JA, Jenkins GI (1996) UV-B, UV-A, and blue light signal transduction pathways interact synergistically to regulate chalcone synthase gene expression in *Arabidopsis*. *Plant Cell* 8:2347–2357
- Ghawana S, Singh K, Raizada J, Rani A, Bhardwaj PK, Kumar S (2007) A method for rapid isolation of RNA and kit thereof. *WO* 2007/113614
- Gilmartin PM, Sarokin L, Memelink J, Chua NH (1990) Molecular light switches for plant genes. *Plant Cell* 2:369–378
- Green PJ, Yong MH, Cuouo M, Kano-Murakami Y, Silverstein P, Chua NH (1988) Binding site requirements for the pea GT-1 correlate with sequences required for light dependent transcriptional activation of the *rbcS-3A* gene. *EMBO J* 7:4035–4044
- Hampel D, Mosandl A, Wiist M (2005) Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways. *Phytochemistry* 66:305–311
- Hampel D, Mosandl A, Wiist M (2006) Biosynthesis of mono- and sesquiterpenes in strawberry fruits and foliage: ²H labeling studies. *J Agric Food Chem* 54:1473–1478
- Hannah MA, Heyer AG, Hinch DK (2005) A global survey of gene regulation during cold acclimation in *Arabidopsis thaliana*. *PLoS Genet* 1:e266
- Hemm MR, Rider SD, Ogas J, Murry DJ, Chapple C (2004) Light induces phenylpropanoid metabolism in *Arabidopsis* roots. *Plant J* 38:765–778
- Hennighausen L, Lubon H (1985) Interaction of protein with DNA in vitro. *Method Enzymol* 152:721–735

- Higo K, Ugawa Y, Iwamoto M, Korenaga T (1999) Plant *cis*-acting regulatory DNA elements (PLACE) database. *Nucleic Acids Res* 27:297–300
- Hsieh MH, Goodman HM (2005) The arabidopsis IspH homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis. *Plant Physiol* 138:641–653
- Hudson ME, Quail PH (2003) Identification of promoter motifs involved in the network of phytochrome A-regulated gene expression by combined analysis of genomic sequence and microarray data. *Plant Physiol* 133:1605–1616
- Juan ME, Araceli C, Andreas R, Stuart R, Patricia L (2001) 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J Biol Chem* 276:22901–22909
- Klok EJ, Wilson IW, Wilson D, Chapman SC, Ewing RM, Somerville SC, Peacock WJ, Dolferus R, Dennis ES (2002) Expression profile analysis of the low-oxygen response in *Arabidopsis* root cultures. *Plant Cell* 14:2481–2494
- Korth KL, Jaggard DAW, Dixon RA (2000) Developmental and light-regulated post-translational control of 3-hydroxy-3-methylglutaryl-CoA reductase levels in potato. *Plant J* 23:507–516
- Lange BM, Severin K, Bechthold A, Heide L (1998) Regulatory role of microsomal 3-hydroxy-3-methylglutaryl-coenzyme A reductase for shikonin biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Planta* 204:234–241
- Learned RM, Connolly EL (1997) Light modulates the spatial patterns of 3-hydroxy-3-methylglutaryl coenzyme A reductase gene expression in *Arabidopsis thaliana*. *Plant J* 11:499–511
- Leivar P, Gonzalez VM, Castel S, Trelease RN, Lesias C, Arro M, Boronat A, Campos N, Ferrer A, Busquets X (2005) Subcellular localization of arabidopsis 3-hydroxy-3-methylglutaryl-coenzyme A reductase. *Plant Physiol* 137:57–69
- Lois LM, Rodriguez-Concepcion M, Gallego F, Campos N, Boronat A (2000) Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase. *Plant J* 22:503–513
- Luper S (1998) A review of plants used in the treatment of liver disease: part 1. *Altern Med Rev* 3:410–421
- Mahmoud SS, Croteau RB (2002) Strategies for transgenic manipulation of monoterpene biosynthesis in plants. *Trends Plant Sci* 7:366–373
- Mosaleyanon K, Zobayed SMA, Afreen F, Kozai T (2005) Relationships between net photosynthetic rate and secondary metabolite contents in St. John's wort. *Plant Sci* 169:523–531
- Munoz-Bertomeu J, Arrillaga I, Ros R, Segura J (2006) Up-regulation of 1-deoxy-D-xylulose-5-phosphate synthase enhances production of essential oils in transgenic spike lavender. *Plant Physiol* 142:890–900
- Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol Plant* 15:473–497
- Nayar MP, Sastri ARK (1990) Red data plants of India. CSIR Publication, New Delhi, p 271
- Nogués I, Brillì F, Loreto F (2006) Dimethylallyl diphosphate and geranyl diphosphate pools of plant species characterized by different isoprenoid emissions. *Plant Physiol* 141:721–730
- Park HC, Kim ML, Kang YH, Jeon JM, Yoo JH, Kim MC, Park CY, Jeong JC, Moon BC, Lee JH, Yoon HW, Lee SH, Chung WS, Lim CO, Lee SY, Hong JC, Cho MJ (2004) Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol* 135:2150–2161
- Reyes JC, Muro-Pastor MI, Florencio FJ (2004) The GATA family of transcription factors in *Arabidopsis* and rice. *Plant Physiol* 134:1718–1732
- Sacchetti JC, Poulter CD (1997) Creating isoprenoid diversity. *Science* 277:1788–1789
- Singh K, Raizada J, Bhardwaj P, Ghawana S, Rani A, Singh H, Kaul K, Kumar S (2004) 26S rRNA-based internal control gene primer pair for reverse transcription-polymerase chain reaction-based quantitative expression studies in diverse plant species. *Anal Biochem* 335:330–333
- Singh N, Gupta AP, Singh B, Kaul VK (2005) Quantification of picroside-I and picroside-II in *Picrorhiza kurroa* by HPTLC. *J Liq Chromatogr & Rel Technol* 28:1679–1691
- Soitamo AJ, Piippo M, Allahverdiyeva Y, Battchikova N, Aro EM (2008) Light has a specific role in modulating *Arabidopsis* gene expression at low temperature. *BMC Plant Biol* 8:13
- Streb P, Shang W, Feierabend J, Blligny R (1998) Divergent strategies of photoprotection in high-mountain plants. *Planta* 207:313–324
- Teakle GR, Manfield IW, Graham JF, Philip M, Gilmartin PM (2002) *Arabidopsis thaliana* GATA factors: organisation, expression and DNA-binding characteristics. *Plant Mol Biol* 50:43–57
- Terzaghi WB, Cashmore AR (1995) Light-regulated transcription. *Annu Rev Plant Physiol Plant Mol Biol* 46:445–474
- Tjaden G, Edwards JW, Coruzzi GM (1995) *Cis* elements and trans-acting factors affecting regulation of a non-photosynthetic light-regulated gene for chloroplast glutamine synthetase. *Plant Physiol* 108:1109–1117
- Udvardi MK, Kakar K, Wandrey M, Montanari O, Murray J, Andriankaja A, Zhang JY, Benedito V, Hofer JM, Chueng F, Town CD (2007) Legume transcription factors: global regulators of plant development and response to the environment. *Plant Physiol* 144:538–549
- Villain P, Mache R, Zhou DX (1996) The mechanism of GT element-mediated cell type-specific transcriptional control. *J Biol Chem* 271:32593–32598
- Walter MH, Hans J, Strack D (2002) Two distantly related genes encoding 1-deoxy-d-xylulose 5-phosphate synthases: differential regulation in shoots and apocarotenoid-accumulating mycorrhizal roots. *Plant J* 31:243–254
- Wang Y, Guo B, Zhang F, Yao H, Miao Z, Tang K (2007) Molecular cloning and functional analysis of the gene encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase from hazel (*Corylus avellana* L. Gasaway). *J Biochem Mol Biol* 40:861–869
- Wise ML, Croteau R (1998) Comprehensive natural products chemistry: isoprenoid biosynthesis. In: Cane DE (ed) Pergamon, Oxford
- Wong RJ, McCormack DK, Russell DW (1982) Plastid 3-hydroxy-3-methylglutaryl coenzyme A reductase has distinctive kinetic and regulatory features: properties of the enzyme and positive phytochrome control of activity in pea seedlings. *Arch Biochem Biophys* 216:631–638
- Yang Z, Park H, Lacy GH, Cramer CL (1991) Differential activation of potato 3-hydroxy-3-methylglutaryl coenzyme A reductase genes by wounding and pathogen challenge. *Plant Cell* 3:397–405