MOLECULAR CHARACTERIZATION OF PICROSIDES BIOSYNTHETIC MACHINERY COMPONENTS IN POPULATIONS OF A MEDICINAL HERB, *Picrorhiza kurroa Royle ex Benth*

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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Dedicated to family, friends, and the research community

DECLARATION BY THE SCHOLAR

I hereby declare that the work reported in the Ph.D. thesis entitled "**Molecular characterization of picrosides biosynthetic machinery components in populations of a medicinal herb, *Picrorhiza kurroa Royle ex Benth***" submitted at Bennett University, Greater Noida, India, is an authentic record of my work carried out under the supervision of **Dr. Rajinder Singh Chauhan**. I have not submitted this work elsewhere for any other degree or diploma.

I am fully responsible for the contents of my Ph.D. Thesis.

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SUPERVISOR'S CERTIFICATE

This is to certify that the work reported in the Ph.D. thesis entitled "**Molecular characterization of picrosides biosynthetic machinery components in populations of a medicinal herb, *Picrorhiza kurroa Royle ex Benth***", submitted by **Roma Pandey** at **Bennett University, Greater Noida, India**, is a bonafide record of her original work carried out under my supervision. This work has not been submitted elsewhere for any other degree or diploma.

Dr. Rajinder Singh Chauhan
Dean (Research & Consultancy) &
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Date: 11.08.2022

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
4CL	4 -coumarate CoA ligase
C4H	Cinnamic acid-4-hydroxylase
cDNA	Complementary DNA
CDS	Coding DNA sequences
ChIP	Chromatin immunoprecipitation
CITES	Convention on International Trade in Endangered Species
CRISPR	Clustered regularly interspaced short palindromic repeats
DXPS	1-deoxy-D-xylulose-5-phosphate synthase
EMSA	Electrophoretic mobility shift assays
G10H	Geraniol-10-hydroxylase
HFRI	Himalayan Forest Research Institute
HMGR	Hydroxymethylglutaryl-CoA reductase
HP	Himachal Pradesh
HPLC	High Performance Liquid Chromatography
IUCN	International Union for Conservation of Nature
Kb	Kilobase pairs
m a.s.l	Meter above sea level
MEP	Methyl erythritol phosphate
Min	Minute
MVA	Mevalonate
NBD	Nucleotide-binding domain
NGS	Next Generation Sequencing
NR	Non redundant
PAL	Phenylalanine ammonia lyase
pkdoubleWRKY	Double WRKY type transcription factor sequenced from *Picrorhiza kurroa*
PKR	*Picrorhiza kurroa* roots
PKS	*Picrorhiza kurroa* shoots

PKST	*Picrorhiza kurroa* stolons
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
RNAi	RNA interference
RP	Reverse Phase
RT	Room Temperature
TF	Transcription factor
TFA	Trifluoroacetic acid
TMD	Transmembrane domains
UV	Ultraviolet
VIGS	Virus induced gene silencing
WHO	World Health Organization
µg	Microgram

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ABSTRACT

Picrorhiza kurroa is a North-western Himalayan herb, known for its many medicinal properties particularly, hepatoprotective activity. It contains two major pharmacological iridoid glycosides: Picroside-I and Picroside-II, used in the preparation of several herbal drugs including hepatoprotective, anti-inflammatory, cardio-tonic, immunomodulator and so on. Picrosides are valuable compounds commercially isolated from underground parts of *Picrorhiza kurroa* resulting in its inclusion in the endangered species category. This necessitates search for a more optimal mechanism for meeting increasing demands of Picrosides. Comprehensive knowledge on regulatory machinery of Picrosides biosynthesis (pathway gene paralogues, transcription factors and ABC transporters) can help in devising a bio-molecular mechanism to enhance Picrosides production. Characterisation of different tissues of eighty-five accessions of *Picrorhiza kurroa* revealed significant variations for Picroside-I and Picroside-II contents. Thus, the current study focused on i) unravelling possible correlations between TFs and gene paralogues across a range of *Picrorhiza kurroa* accessions which might be contributing to differential contents of Picroside-I and Picroside-II in different tissues/accessions ii) identification of putative ABC transporters possibly involved in long distance and local transport of Picrosides in *Picrorhiza kurroa* tissues/organs. This was accomplished by comparative transcriptome analysis followed by expression analysis. Further a robust correlation study was undertaken to uncover their coordinated behaviour in association with Picrosides contents in different tissues of *Picrorhiza kurroa.* The analysis revealed four major TFs namely *PkWRKY71*, *P*k*WRKY12*, *PkNAC25*, and *PkMyb46* which possibly regulate different pathway gene paralogues in different tissues of *Picrorhiza kurroa*. Also, members of ABCB, ACCC and ABCG family showed elevated expression in various tissues of *Picrorhiza kurroa.* Moreover, ABC genes were used as hubs in a co-expression network analysis that revealed additional crucial interactions with other biosynthetic machinery components. The results of this study have provided potential leads, which through further functional validation can provide suitable targets, either for pathway engineering or as gene markers for selection of genetically superior populations of *Picrorhiza kurroa*.

CHAPTER 1
INTRODUCTION

Picrorhiza kurroa Royle ex Benth also known as Kutki is an endangered, ethnobotanically valuable medicinal herb that grows in region of the North-Western Himalayas. It is a perennial herb found in the high-altitude at an elevation of 3000-5000 m [1]. The plant is part of the family Plantaginaceae (formerly Scrophulariaceae). *Picrorhiza kurroa* is valued since ancient times for hepatoprotective activity and for treatment of stomach, liver, allergic and respiratory disorders [2]. The modern as well as traditional medicinal system in Asian countries including India, China and Nepal have used *Picrorhiza kurroa* in various preparations [3]. The underground tissues of *Picrorhiza kurroa* (roots/rhizhome) are primarily used in various herbal formulations like Katuki, Arogya, Livplus and Livocare for treatment of liver ailments.

Picrorhiza kurroa favours vegetative propagation by stolons that later develop into rhizomes. The mature rhizomes serve as a source of shoot buds that eventually give rise to a new plant [4]. The ability of the herb to regenerate itself has been harmed by the indiscriminate collection of mature rhizomes from the wild by various entities involved in herbal drug industries. With increasing popularity of products with "herbal" tag, the demand for quality and authentic raw material is high resulting in overexploitation of the herb.

Narrow distribution, lack of coordinated cultivation practices, low seed viability, extensive and illegal collection of underground tissues from natural habitat have thereby, resulted in the plant being listed as an endangered species by the (IUCN) International Union for Conservation of Nature and Natural Resources and has also been included in the Appendix II of CITES (the Convention on International Trade in Endangered Species of Wild Fauna and Flora) list [5]. Further, due to the vulnerable status of this therapeutic herb, legislative limits on its collection from natural habitats have been imposed, resulting in a shortage of authentic raw material for the herbal drug and pharmaceutical industries.

1.1 Market value of *Picrorhiza kurroa*

Picrorhiza kurroa is among top 15 medicinal herbs in terms of revenue generated by traded material in India. Roots/rhizomes of *Picrorhiza kurroa* are sold in dried form under the local

name Kutki with price ranging from Rs. 1900-2200 per Kg[1]. Around 375 tonnes of *Picrorhiza kurroa* are produced worldwide, with about 70 tonnes emanating from India [6].

1.2 Chemical constituents of *Picrorhiza kurroa*

In *Picrorhiza kurroa,* about 200 primary and secondary metabolites have been identified [7]. The dried rhizomes and roots of this threatened plant have been found to contain more than 50 secondary metabolites [6]. Presence of Picroside-I and Picroside-II, two significant iridoid glycosides, has been primarily recognised for the hepatoprotective properties of *Picrorhiza kurroa*. Additionally, Picroside-III, Picroside-IV and Picroside-V, 6-feruloyl catalpol, minecoside, kutkoside, vernicoside, bartioside, aucubin, pikuroside are other iridoids present in *Picrorhiza kurroa* [2].

1.3 Pharmacological properties of Picroside-I and Picroside-II

Picroside-I and Picroside-II are the most valued iridoid glycosides present in *Picrorhiza kurroa.* These two major bioactive constituents have immense therapeutic significance which are based on their hepatoprotective, nephroprotective, anti-inflammatory, anti-allergic, anti-hyperglycaemia, and anti-cancer properties [6], [8], [9]. These are also used in the treatment of arthritis and vitiligo. In some studies, Picroside-I has been reported to have antimicrobial activity and effective against Hepatitis-B while Picroside-II has antioxidant, tumour suppressor, neuroprotective activity and is used in the treatment of renal ischemia, myocardial ischemia, hind limb ischemia and cerebral ischemia [10]–[16] as illustrated in Figure 1.

[1] https://echarak.in/echarak/pricewise1.do

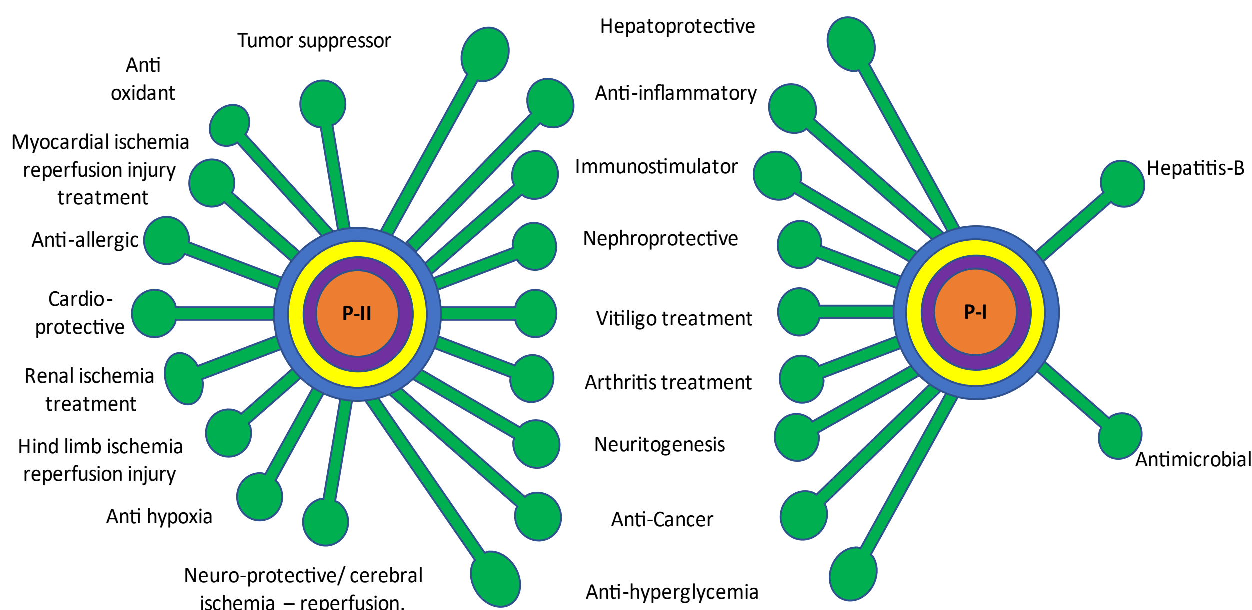

Figure 1.1: Medicinal properties of Picroside-I and Picroside-II

1.4 Biosynthetic machineries involved in the biosynthesis of Picroside-I and Picroside-II

Picroside-I and Picroside-II are biosynthesized through the combinatorial role of four different pathways (mevalonate, non-mevalonate, shikimate/phenylpropanoid and iridoid pathway) in different tissues of *Picrorhiza kurroa*. Various components of molecular biosynthetic machinery are known to play a major role in influencing Picroside-I and Picroside-II biosynthesis in different tissues of *Picrorhiza kurroa* [17]. These components include the pathway genes, gene paralogues, transcription factors (TFs), kinases and transporters. It is evident that better understanding of Picroside biosynthesis, transportation and their molecular regulatory mechanisms can introduce new possibilities in development of robust molecular and genetic tools towards metabolic engineering or genetic improvement; thereby, resulting in enhanced production of Picrosides.

1.5 Combinatorial role of Pathway gene paralogues and TFs in controlling of Picroside-I and Picroside-II biosynthesis

Several biosynthetic pathway genes have been reported to play a significant role in the biosynthesis of secondary metabolites [18], [19]. There are 14 rate-limiting enzymes in 4 pathways associated with terpenoids biosynthesis in various plant species [20], [21]. Several

of the genes in secondary metabolites biosynthetic pathways occur in multiple copies known as paralogues. Recently, pathway gene paralogues were identified by our group in *Picrorhiza kurroa* [22]. While the coding DNA sequences (CDS) of these genes are more than 95% identical (highly conserved), polymorphic variations have been found in the promoter. The promoter region sequence variability amongst the gene paralogues across various accessions, might be responsible for differential functioning in *Picrorhiza kurroa* accessions.

Transcription factors (TFs) are proteins that bind to the specific regulatory regions of the pathway genes to control the rate of transcription [23]. These proteins control gene expression both tissue-specifically and in response to a variety of environmental triggers such as humidity, temperature, soil conditions, biotic stress etc. These factors contribute to the regulation of secondary metabolic pathways by controlling biosynthetic pathway genes expression. Myb, WRKY, NAC, Zn finger and bZip are some of the families of TFs that have been linked to regulation of the biosynthesis of secondary metabolites in plants [24]. Expression of TFs requires *cis*-regulatory elements present in the promoter region of the targeted genes [25].

Correlation between TFs and gene paralogues might be contributing to variability in Picroside-I and Picroside-II contents among various tissues of *Picrorhiza kurroa*. Hence, TFs regulating one or more of these pathway gene paralogues needs to be studied in *Picrorhiza kurroa*.

1.6 Transporters involved in the transport of secondary metabolites

Picrosides biosynthesis occurs in shoots (Picroside-I) and roots (Picroside-II) of *Picrorhiza kurroa*; and finally both accumulate in the stolons/rhizomes through transporters [9], [26], [27]. Multiple transporters are required to transport secondary metabolites from organs of synthesis to organs of accumulation in plant species [28], [29]. Among numerous transporters reported, ABC transporters are known for transporting secondary metabolites such as terpenoids, flavonoids, alkaloids, lignins, and others through plasma membrane and tonoplast of plant species [30]. For instance, *NpABC1* was reported to transport a diterpenoid, Sclareol in *Nicotiana plumbaginifolia* [31]. There are no studies available on the role of transporters in the transport and accumulation of Picrosides in *Picrorhiza kurroa.*

1.7 Variation in *Picrorhiza* accessions for Picroside-I and Picroside-II

Variation in Picrosides content in *Picrorhiza kurroa* due to altitude or other environmental conditions have been reported in some studies. For instance, effect of altitude was indicated in a study, where Picroside-I and Picroside-II content in *Picrorhiza kurroa* collected from

Sonemarg was found to be highest and least in sample from Pulwama located in north-western region of Kashmir [32]. In another study, seven populations of *Picrorhiza kurroa* collected from different geographical locations of Kashmir showed variation Picrosides content ranging from 2.78% to 5.18% for Picroside-I and 2.53% to 5.39% for Picroside-II [33].

The metabolic diversity of *Picrorhiza kurroa* might be attributed to various genes encoding proteins involved in the regulation of Picrosides biosynthetic pathway. However, contents of secondary metabolites particularly in high altitude medicinal herbs, are sensitive to various factors such as altitude, temperature, humidity, soil factors, age, and developmental stage [34]–[36]. As of today, no information exists as to whether variation in different accessions of *Picrorhiza kurroa* for picrosides content is due to environment or genetic components. To address that we profiled a large collection of *Picrorhiza kurroa* accessions collected from geographically diverse locations of North-Western Himalayas and planted at one common location to identify accessions which vary for metabolites content due to inherent genetic differences rather environmental or developmental factors (Figure 1.2).

Figure 1.2 a) Germplasm stock centre of *Picrorhiza kurroa* accessions; b) *Picrorhiza kurroa* accessions collected from different location; c) *Picrorhiza kurroa* plants

1.8 Analysis of biosynthetic machinery components across accessions varying for Picrosides in *Picrorhiza kurroa*

In the current study, the accessions showed variations for Picrosides content that could be possibly due to differential expression of biosynthetic machinery components of which pathway genes are critical. The paralogues of pathway genes coupled with their regulatory genes (or TFs) are speculated to affect the differential accumulation of Picrosides content in various tissues/organs of this medicinal herb. Furthermore, the details of different transporters involved in the mobility of Picroside-I from shoots to the stolons and Picroside-II from roots to the stolons, as well as intracellular accumulation of Picroside-I and Picroside-II in various tissues, needs to be deciphered.

To address these questions, we have used natural variations in Picroside-I and Picroside-II content among various accessions of *Picrorhiza kurroa,* transcriptome data and expression analysis data to identify the role pathway gene paralogues, TFs and ABC transporters in Picrosides biosynthesis. In addition, co-expression network was constructed to identify additional components of biosynthetic machinery associated with the ABC transporters in different tissues. This is the first study to report ABC transporters in *Picrorhiza kurroa.*

Further, a robust co-expression network was generated to uncover the coordinated behaviour of TFs with pathway gene paralogues in association with Picroside-I and Picroside-II content in shoots and roots, respectively.

Thus, the current research is focused on identification, characterisation, and validation by expression analysis of pathway genes, and transcription factors to infer their coordinated role in biosynthesis of Picroside-I and Picroside-II in *Picrorhiza kurroa*. In addition, the putative ABC transporters possibly associated with Picroside-I and Picroside-II accumulation were identified through extensive transcriptome analysis followed by their molecular evaluation.

Therefore, the present study was initiated with a view to understand the molecular basis of Picrosides' biosynthetic machinery components in *Picrorhiza kurroa* accessions. To achieve the goals of the present study, following objectives were designed:

I. Characterization of *Picrorhiza kurroa* populations for contents of major compounds, Picroside-I and Picroside-II

II. Mining ABC transporters possibly associated with the transport of Picroside-I and Picroside-II specific to *Picrorhiza kurroa*

III. Dissecting the coordinated association of transcription factors and pathway gene paralogues in controlling differential contents of Picroside-I and Picroside-II across different tissues and populations of *Picrorhiza kurroa*

CHAPTER 2
REVIEW OF LITERATURE

Chronic liver problems are becoming prevalent globally, including India, regardless of gender, ethnicity, or age. Liver diseases are among top 10 leading cause of death in India. According to recent World Health Organization (WHO) data, 268,580 deaths in India are caused by liver diseases, accounting for 3.17% of all deaths[2]. Over the years, corticosteroids and interferons, two synthetic and recombinant drugs, have been used to treat a variety of liver disorders. Unfortunately, these medications have negative side effects, such as agitation, depression, retinal ischemia, anxiety, and nephrotic syndrome [37]. Consequently, plant-based treatments for liver disorders gained attention. Several medicinal plants with potent phytochemical constituents are available, for instance, *Berbris aristate, Crica papaya, Rubia cordifolia, Saussurea lappa, Cestrum nocturnum. Picrorhiza kurroa*-based herbal medicines have been found to be highly effective against a variety of liver disorders after comparative screening of various medicinal herbs [38]. This ancient discernment has been transformed through modern scientific research into excellent formulations for treating liver diseases introduced on by microorganisms, synthetic drugs, or food poisoning. Herbal preparations of *Picrorhiza kurroa* contain several essential phyto-chemicals that work as antioxidants and nutraceuticals to prevent and treat liver diseases [39], [40]. Therefore, in this section, we have summarised the existing knowledge on major iridoid glycosides from *Picrorhiza kurroa*, their metabolites, pharmacological activities, and status of biosynthetic machinery.

2.1 Introduction to *Picrorhiza kurroa*

Picrorhiza kurroa is an endangered, valuable medicinal herb native to North-Western Himalayan region (Figure 2.1). The plant is widely utilized in the formulation of various herbal drugs commercially used for the treatment of liver ailments.

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[2] Liver Disease in India (worldlifeexpectancy.com)

Figure 2.1: *Picrorhiza kurroa* plants

The plant is included in the family Plantaginaceae (formerly Scrophulariaceae). The taxonomical classification of the plant is mentioned in Table 2.1**.** *Picrorhiza kurroa* possesses hepatoprotective properties and thus benefits the liver and spleen. It has been used to treat all types of liver damage, cirrhosis, and inflammation. It defends the liver from Hepatitis C virus infection. Additionally, it has immunomodulatory, anti-inflammatory, and other medical benefits for upper respiratory conditions like bronchial asthma, dyspepsia, anorexia, periodic fever, and jaundice [2], [41]. It is frequently found in combination with herbs such as *Potentilla kashmirica, Aconitum violaceum, Sedum ewersii Lagotis cashmiriana,* and *Senecio jacquemontianus* [6].

Table 2.1: *Picrorhiza kurroa:* Taxonomic classification

Kingdom	**Plantae**
Class	Astride
Division	Dicotiledonae
Order	Scrophulariales
Family	Plantaginaceae
Genus	*Picrorhiza*
Species	*Kurroa*

Picrorhiza kurroa grows best in sandy loam/ clay soils which allows the rhizomes to spread horizontally underneath. Its cultivation requires organic content rich soil with a high moisture content. Additional areas with some shades are confronted to be ideal for maximising growth and productivity. It can be grown at elevations of 1800 to 2800 metres. The plant grows well

in cool and moist climate. The seed must fully mature in the plant after a year. September is the best month for harvesting the roots and rhizomes when the aerial parts (shoots) begin to wither and dry. The plantings can be raised through seeds or propagated through rhizomes, stolons or offsets. For growing a nursery, seeds can be collected in August and September. For a period of about six months, seeds are more than 60% viable. Compared to plants raised from seedlings, stem cutting-raised plants reach maturity almost a year sooner. Nevertheless, plants must be harvested prior to flowering in the months of September and October to obtain higher active contents [42].

2.2 *Picrorhiza kurroa* as a potential source of Picroside-I and Picroside-II

The pharmacological importance of this herb is ascribed to the exhibition of diverse iridoid glycosides fraction. *Picrorhiza kurroa* has been found to contain more than 50 secondary metabolites, including iridoid glycosides, cucurbitacins, and phenolic compounds. The pharmacological importance majorly hepatoprotective activity of *Picrorhiza kurroa* has been mainly demonstrated due to picrosides (Picroside-I and Picroside-II) and other iridoid category metabolites like Picroside-III, Picroside-IV, Picroside-V, kutkoside, etc. Other minor iridoids identified in this plant include vernicoside, minecoside, and 6-feruloyl catalpol, as well as the phenol glycosides picein and androsin. *Picrorhiza kurroa* also contains 23 distinct cucurbitacin glycosides, phenolic compounds such as apocynin/l androsin4 and vanillic acid, and flavonoids in addition to iridoid glycosides [6].

Picrorhiza kurroa extract exhibits an anti-inflammatory effect via -adrenergic blockade to alter the cell surface biology. Picroside-II provides hepatocyte protection by acting against apoptosis induced by D-galactosamine and lipopolysaccharide [43]. Experiments with Picroliv drug have demonstrated antioxidant and anti-inflammatory activity against ulcerative colitis induced in mice with dextran sulphate sodium [44]. Also, protective effects of Picroliv (Picroside-I and Picroside-II) observed in golden hamsters against Leishmania infection [45]. Picroliv and antilesihmanial drugs, paromomycin and miltefosine, increased phagocytosis and lymphocyte proliferation [46]. The hypolipidemic action of Picroliv was demonstrated by lower serum lipid levels and decreased cholesterol synthesis [47]. Picroside-II has shown to be beneficial in I/R-induced injury via improving animals neurological capabilities [16]. The medicinal value of Picroside-I and Picroside-II is detailed in Table 2.2.

Table 2.2: Pharmacological properties of Picroside-I and Picroside-II

S. No.	Treatments	Iridoid glycoside	Reference
1	Neuritogenesis	Picroside-I; Picroside-II	[48]
2	Anticancer	Picroside-I; Picroside-II	[10], [49]
3	Neuroprotective	Picroside-II	[12]
4	Hepatoprotective	Picroside-I; Picroside-II	[50], [51]
5	Treating Hypoxia/reoxygenation induced cardiomyocytes injury	Picroside-II	[52], [53]
6	Treating Myocardial ischemia reperfusion injury	Picroside-II	[54]
7	Treating Hind limb ischemia reperfusion injury	Picroside-II	[55]
8	Treating Renal ischemia reperfusion injury	Picroside-II	[56]
9	Anaphylaxis	Picroside-I (Picroliv)	[57]
10	Anti- allergic	Picroside-I (Picroliv)	[57]
11	Treating Allergic asthma	Picroside-II	[58]
12	Immunostimulatory/ Immunomodulator	Picroside-I, Picroside-II	[59]
13	antileishmanial	Picroside-I (Picroliv)	[45]
14	anti-inflammatory	Picroside-I, Picroside-II	[43], [57]
15	Hypoglycemic/ Antihyperglycemia	Picroside-I; Picroside-II	[60]
16	Treating Colitis	Picroside-I (Picroliv)	[44]
17	Treating cholestasis	Picroside-I (Picroliv)	[44], [59]
18	Used against NAFLD	Picroside-I; Picroside-II	[61]

19	Anti-acute Pancreatitis	Picroside-II	[62]
20	Treating Vitiligo	Picroside-I; Picroside-II	[63]
21	Treating Neuropathic Pain	Picroside-II	[64]
22	Treating Hepatic steatosis	Picroside-II	[65]
23	Nephroprotection	Picroside-I (Picroliv), Picroside-II	[66]
24	Protection against sepsis	Picroside-II	[67]
25	Inhibits Arthritis	Picroside-I; Picroside-II	[68]
26	Antioxidant	Picroside-II	[16]
27	Cardio-protective	Picroside-II	[16]
28	Antimicrobial	Picroside-I	[10]
29	Osteoclast genesis	Picroside-II	[15]
30	Hepatitis B	Picroside-I	[10], [11]

2.3 Variation in major phytochemical constituents in *Picrorhiza kurroa*

Increasing demand for *Picrorhiza kurroa* plant material has prompted many investigators to look for genotypes high in Picrosides, which do not exist in *Picrorhiza kurroa* as of today. The herbal drug industries preparing herbal formulations from medicinal plants are not only looking for higher contents of chemical constituents but also particular proportions of desired chemical constituents, which are uniquely present in elite chemotypes. It is important to have specific composition of active constituents in a herbal drug formulation for example, in Picroliv, a *Picrorhiza kurroa* based formulation contains Picroside-I and Picroside-II, in a definite ratio of 1:1.5 [59]. Therefore, screening of natural populations of *Picrorhiza kurroa* from various geographical locations is necessary to assess the extent of variation in picrosides content so as to identify superior accessions. Picroside-I and Picroside-II content in various plant parts of *Picrorhiza kurroa* sourced from different geographical locations with varying altitudes, viz. Sonemarg (2,740 m), Tangmarg (2,690 m), and Pulwama (1,630 m) in the North-Western Kashmir Himalayas was reported [32]. Picrosides content was found to be highest in populations collected from Sonemarg followed by Tangmarg and Puwama suggesting that picrosides accumulation is directly correlated with altitudinal change. In another study,

rhizomes of four accessions of *Picrorhiza kurroa* from different locations in Uttarakhand namely, Lohagang pass, Roopkund, Dewal and Ghat were quantified [69]. Picroside-I ranged from 2.74 – 4.91 % and Picroside-II from 3.12 – 5.39%, with Lohagang pass having highest amount of picrosides. Eighteen accessions of *Picrorhiza kurroa* were evaluated for picrosides accumulation pattern under field conditions at Chamba (Himachal Pradesh) located at an elevation of 2538 m and six accessions were identified with higher picrosides content [9]. Picrosides content ranged from 0.08- 8.05% in leaves and from 0.52- 9.02% in rhizomes. This study revealed that different accessions of Picrorhiza kurroa, which were grown at lower elevations and collected from the North-Western Himalayas, varied in their growth and content of picrosides (Picroside-I and Picroside-II). Similarly, rhizomes of seven accessions of *Picrorhiza kurroa* were analysed from Himachal Pradesh for variation in picrosides content which ranged from 2.21- 5.5% [70]. In another study, seven populations of *Picrorhiza kurroa* collected from different geographical locations of Kashmir showed variation Picrosides content ranging from 2.78% to 5.18% for Picroside-I and 2.53% to 5.39% for Picroside-II [33].

The variation of major phyto-chemicals across altitudinal ranges within the same species indicates that there is a significant connection between the quantity and quality of active compounds, habitat and environmental conditions found in various geographic locations. Concentration of active compound in a medicinal plant can influence its therapeutic potential. As a result, it is important to standardise factors like growth, environment, and storage conditions, among others for the cultivation of such medicinally important plants to achieve higher quality and thus improve therapeutic effects. Thus, the Indian pharmaceutical industry may use the assessment of roots and rhizomes of *Picrorhiza kurroa* populations collected from various locations in Himachal Pradesh and Uttarakhand regarding the Picrosides (Picroside-I and Picroside-Il) content for the formulation of high-quality herbal drugs.

2.4 Genome/transcriptome resources of *Picrorhiza kurroa*

Current 'omics' resources such as genomics, transcriptomics, proteomics and metabolomics could reveal the molecular components associated with the biosynthesis and accumulations of metabolites in medicinal plant species. *Picrorhiza kurroa* is a diploid plant with 2n=34 and genome size was estimated about 3452.34 Mbp using flow cytometry [71].

The first global analysis of *Picrorhiza kurroa* transcriptome under two temperature regimes (15°C and 25°C) by using strategy for *de novo* assembly of transcriptome using short-read sequence data was generated by Illumina sequencing technology [72]. Transcriptomes of 6

tissues of *Picrorhiza kurroa* have also been deciphered by our previous group differing in culture conditions, tissue and metabolite content using Illumina HiSeq 2000 platform [4].

Recently, first draft genome sequence of *Picrorhiza kurroa* has been generated using hybrid de novo assembly approach viz Illumina GAIIx and PacBio RSII SMRT platform [5]. *Picrorhiza kurroa* genome database is available at https://scbb.ihbt.res.in/picro-db/. The number of protein coding genes and non-coding genes were predicted 24,798 and 9,789 respectively in *Picrorhiza kurroa* [5]. The availability of genome and transcriptomes resources provides a robust dataset to mine for potential molecular components and correlate with Picrosides content.

2.5 Pathways involved in Picrosides biosynthesis

Picrosides biosynthesis is a combinative route involving mevalonate (MVA), mevalonate-independent/ methylerythritol phosphate (MEP), shikimate/ phenylpropanoid and iridoid pathway modules [27], [73]. Picrosides are iridoid glycosides with 10-carbon molecules, belonging to the group of monoterpenoids and are synthesized from a precursor with five carbon atoms, isopentenyl pyrophosphate (IPP) and its functional isomer, dimethylallyl pyrophosphate (DMAPP) that are biosynthesised from MVA and MEP pathways, respectively. MEP pathway occurs in plastids, while MVA pathway occurs in the cytosol [74]. IPP and DMAPP are then combined to create the 10-carbon compound geranyl pyrophosphate (GPP), which is regarded as a precursor to monoterpenoid molecules [75]. GPP undergoes sequences of oxidation and cyclization to form backbone of picrosides, catalpol. Cinnamic acid and vanillic acid from phenylpropanoid pathway undergo acylation with catalpol (iridoid moiety) to form Picroside-I and Picroside-II [20].

2.6 Regulation of secondary metabolism in plants

Plant secondary metabolites are essential bioactive compounds for plant defence as well as commercially beneficial for humanity. Therefore, its production needs to be up scaled by utilizing various metabolic engineering strategies. To accomplish this goal, details of different biosynthetic pathways and their components along with its spatio-temporal regulatory mechanisms needs to be elucidated. Furthermore, knowledge of cross-talk of these pathways with the broader metabolic networks would aid in developing techniques for enhancing the yield of these important metabolites [76]. However, biosynthesis and accumulation of secondary metabolites in plants is tightly controlled and mostly depends on tissue specificity, stage of plant development as well as various environmental factors [33]. The complexity of

all these factors needss to be kept in mind while designing strategies for its enhanced production and hence the role of developmental regulators should be considered in this regard. An important strategy could be to identify various tissues as well as the developmental stages involved in these pathways. In addition, identification of key rate limiting steps of these pathways as well as its upstream regulatory mechanisms would help to manipulate the specific enzymes/regulatory molecules for effective metabolic engineering of the biosynthetic pathways.

Recent advances in molecular techniques have offered numerous prospects regarding understanding of regulatory components associated with biosynthesis of secondary metabolites such as kinases, miRNAs, transcription factors, pathway genes and their paralogues, transporters, and other biochemical regulators. Moreover, genomic and transcriptome resources of *Picrorhiza kurroa* have made available pertinent datasets for the identification of these regulatory elements allied to Picrosides biosynthesis in *Picrorhiza kurroa*.

2.7 Transcription factors as regulators of secondary metabolites biosynthesis

Plant TFs are known to regulate cellular pathways involved in plant development, metabolic processes, environment adaptability as well as secondary metabolites in various stress responses including defence against pathogens [77], [78]. For instance, bHLH was reported to be important for shade-induced elongation [79], ZmDOF36, a Zn finger in maize has shown to positively control starch accumulation [80], AtMYB4 controls the synthesis of sinapate esters providing tolerance to UV-B irradiation [81].

Controlled transcription of biosynthetic genes is one key mechanism in the plant cells that governs the production of secondary metabolites. Various TF families such as WRKY, NAC, Zn fingers, SPL, MYB, Bzip and AP2/ERF have been reported to control various pathway genes for secondary metabolites biosynthesis in plants [82]. For instance, in eggplant, *SmWRKY44* showed correlated expression with *SmDFR, SmANS, SmF3H, SmF3′ 5′ H, SmMYB1*, and *SmTT8* suggesting that *SmWRKY44* might be involved in the biosynthesis of anthocyanin [83]. A Myb TF, *AaMYB15* was reported to regulate key pathway genes *ADS*, *CYP*, *DBR2*, and *ALDH1* for artemisinin biosynthesis in *Artemisia annua* [84]. Similarly, *ZmMYB111* and *ZmMYB148* were reported to regulate *PAL* gene of phenylpropanoid pathway in maize [85]. *BIS3*, a bHLH family TF was found to activate iridoid biosynthetic genes; *GES, G10H, 8HGO, IS, 7-DLGT* and *7DLH* for loganic acid in

Catharanthus roseus [86]. Recently, in *Camellia sinensis*, ten TFs were predicted to regulate saponin biosynthesis using comparative transcriptome analysis [87]. Various TFs involved in secondary metabolites biosynthesis in various plant species are detailed in Table 2.3.

Table 2.3: Transcription factors involved in secondary metabolite biosynthesis in plants

TFs	Secondary Metabolite	Plant	Reference
MYB3	Limonoids	*Citrus grandis*	[88]
DkbZIP5	Proanthocyanidin	*Diospyros kaki*	[89]
CrWRKY1	Terpenoid indole alkaloid	*Catharanthus roseus*	[90]
HlWRKY	Prenylflavonoid	*Humulus lupulus L.*	[91]
CbWRKY24	Saponin	*Conyza blinii* H. Lév	[92]
bHLH	Diterpenoids	*Oryza sativa*	[93]
MsYABBY5	Terpene biosynthesis	*Mentha spicata*	[94]
AP2/ERF	Terpenoid	*Medicago truncatula*	[95]
SmbHLH3	Phenolic acids and tanshinone	*Salvia miltiorrhiza*	[96]
MdWRKY11	Anthocyanin	*Malus domestica*	[97]
Myb4	Flavonoid	*Medicago sativa*	[98]
bHLH	Bisbibenzyls and flavonoids	*Plagiochasma appendiculatum*	[99]
HlMYB8	Flavonoid	*Humulus lupulus L.*	[100]
PaNAC03	Flavonol	*Norway spruce*	[101]

MYB	fLavonoid and phenylpropanoid	*Populus*	[102]
PAP2	Anthocyanin	*Arabidopsis thaliana*	[103]
SbMyb60	Lignin	*Sorghum bicolor*	[104]
SIMYB	Proanthocyanidin	*Camellia sinensis*	[105]
PtrMYB57	anthocyanin and proanthocyanidin	*Populus*	[106]
PdMYB118	Anthocyanin	*Populus*	[107]
VvMYB114	Anthocyanin and flavonol	*Vitis vinifera*	[108]
ERF115	phenolic acids	*Salvia miltiorrhiza*	[109]
HvWRKY23	Flavonoid glycoside and hydroxycinna mic acid amide	*Hordeum vulgare*	[110]
GmMYB29	Isoflavone	*Glycine max*	[111]
BrMYB28	Glucosinolates	*Brassica rapa*	[112]
MYB29	Glucosinolate	*Oleracea*	[113]
GbMYBFL	Flavonoid	*Ginkgo biloba*	[114]
GATA	Vindoline	*Catharanthus roseus*	[115]
ZCT1	Terpenoid Indole Alkaloid	*Catharanthus roseus*	[116]
TSAR1 and TSAR2	Triterpene Saponin	*Medicago truncatula*	[117]
R2R3-MYB	Flavonoid and terpenoid	*Vitis vinifera*	[118]

bHLH, *NbbHLH,* and *NbARF1*	Alkaloid	*Nicotiana benthamiana*	[119]
AaSPL2	Artemisinin	*Artemisia annua* L.	[120]
AP2/ERF	Vindoline and Serpentine	*Catharanthus roseus*	[121]
SmERF128	Diterpenoid	*Salvia miltiorrhiza*	[122]
OpWRKY3	Camptothecin	*Ophiorrhiza pumila*	[123]
CrBPF1	Terpenoid indole alkaloid	*Catharanthus roseus*	[124]
BIS1	Monoterpenoid indole alkaloid	*Catharanthus roseus*	[125]
TwDXR	Terpenoids	*Tripterygium wilfordii*	[126]
CitAP2.10	Valencene	*Citrus sinensis*	[127]
BIS1, ORCA3 and MYC2a	Monoterpenoid indole alkaloids	*Catharanthus roseus*	[128]
MYC2	Nicotine	*Nicotiana benthamiana*	[129]
SmbHLH92	Phenolic acids and tanshinones	*Salvia miltiorrhiza*	[130]
SmbHLH10	Tanshinones	*Salvia miltiorrhiza*	[131]
MYB and bHLH	Anthocyanin	*Nicotiana benthamiana*	[132]
*Hl*WRKY1 and *Hl*WDR1	Flavonoids	*Humulus lupulus* L.	[133]
CiMYB42	limonoids	*Citrus sinensis*	[134]
WRKY and MYC2	abietane diterpenes	*Salvia sclarea*	[135]
NtERF189 and NtERF199	Alkaloid nicotine	*Nicotiana tabacum*	[136]
R2R3-MYB	Anthocyanin	*Solanum lycopersicum*	[137]

MYC2 and GBFs	Terpenoid indole alkaloids	*Catharanthus roseus*	[138]
SmGRAS1 and SmGRAS2	Tanshinones and Phenolic Acids	*Salvia miltiorrhiza*	[139]
ATR1/MYB34	Glucosinolate	*Arabidopsis thaliana*	[140]
NIC2/ORCA3 ERF	Nicotine	*Nicotiana tabacum*	[136]
AaMYB2	Artemisinin	*Artemisia annua*	[141]
bHLH13	Anthocyanin and Glucosinolate	*Arabidopsis thaliana*	[142]
AaERF1 and AaERF2	Artemisinin	*Artemisia annua*	[143]

The expression of TFs is dependent on the induction of target genes, which requires identification of *cis*-regulatory elements in the promoter region of genes [25]. Many such elements have been reported in promoter regions of the secondary metabolite pathway genes in various plant species. For instance, promoter sequence of *G10H* was isolated and characterised for unique binding regions for various TFs in *C. roseus* [144]. In *Actinidia arguta* (kiwifruit), comparative promoter analysis identified putative NAC binding sites in *AcTPS1* promoter responsible for controlling monoterpene biosynthesis [145].

2.8 Functional characterization of transcription factors

Functional characterization of TFs can be demonstrated using various gene-editing techniques such as CRISPR, TALEN, RNAi, VIGS and Zinc finger nucleases. Several studies have reported using different techniques for functional validation. For instance, overexpression of *CiMYB42* resulted in higher triterpenoid accumulation (limonin), whereas the nomilin accumulation was curtailed by downregulation through RNAi technique [134]. Similarly, in *C. roseus* hairy roots, overexpression of *G10H* or both *G10H* and *ORCA3* genes increased the quantity of catharanthine [146]. Various salvianolic acids, such as rosmarinic acid, lithospermic acid, salvianolic acid B were enhanced as a result of overexpression of *AtEDT1* which in turn increased the expression levels of salvianolic acid biosynthetic genes in *Salvia*

miltorhhiza [147]. Similarly, In *C. roseus,* gene expression in both the indole and iridoid branches of the TIA pathway was repressed resulting in the reduced alkaloid accumulation in the hairy roots when *CrMYC2 was* overexpressed or *CrGBF1* was suppressed through RNAi [138]. Virus-induced gene silencing was used to characterize transcription factors other than Myc that regulate nicotine biosynthesis in tobacco [136] and PatJAZ6 for patchouli accumulation in *P. cablin* [148]. Zhi et al used CRISPR/Cas9 to regulate anthocyanin biosynthesis in tomato plants by silencing *SIAN2*, a Myb transcription factor [137].In another study, a knockout mutant of AP2/ERF-family transcription factors, NtERF189 and NtERF199 using CRISPR/Cas9 reduced the level of alkaloid nicotine in tobacco [149].

Transient expression in transgenic tobacco indicated *HbWRKY1* as a negative regulator of *HbSRPP* involved in rubber biosynthesis in *Hevea brasiliensis* [150]. Similarly, transient expression of *AabHLH1* showed to regulate the biosynthesis of artemisinin by regulating genes involved in artemisinin biosynthesis; *ADS*, *CYP71AV1* and *HMGR* in *A. annua* [151]. In *Withania somnifera*, the genes related to secondary metabolism like *DXR, HMGR and CAS* had elevated expression levels following transient overexpression of *WsAP2* in plants, while *GGPPS* was maximum induced after *WsAP2* overexpression, indicating role of *WsAP2* in terpenoid metabolism [152]. Transient co-transfection assay has been used for the identification of various TFs in secondary metabolites. For instance, *CbWRKY 24* gene for saponin (terpenoid) content in *C. blini* [92], bHLH for monoterpenoid indole alkaloid biosynthesis in *Catharanthus roseus* [153].

Transient gene expression can be achieved using protoplast, biolistic bombardment, Agrobacterium-mediated (vacuum infiltration, cocultivation, agrobacterium infection). Some of the experiments are discussed. Transient transformation assays based on Agrobacterium are not equally successful in various plant species. For instance, lettuce and tobacco are much easier transformable than Arabidopsis [154].

Transient gene expression using protoplasts has been used since its first isolation from roots of tomato and is gaining importance as they are cost-effective and give high output. Protoplasts have been isolated from various parts of plants including leaves and roots [155]. Transient expression-based assay using mesophylls are also popularized and have been used to characterize the genes involved for secondary metabolites in various plants systems such as *Catharanthus* [156], tobacco [153], in barley for coumaryl glucosides [157], for benzoxazinoids in maize [158], for taxane production in *C. roseus* [159].

In recent years, chloroplast expression is emerging as a functional analysis tool for studying secondary metabolites biosynthesis. In tobacco, squalene biosynthesis genes farnesyl diphosphate synthase and squalene synthase were engineered via the *Nicotiana tabacum* chloroplasts to promote squalene biosynthesis [160]. In another study, for enhancing biosynthesis of artemisinin, engineering of two metabolic pathways were undertaken for various cellular structures like chloroplast, nucleus, and mitochondria) [161].

Further, identification of different parameters influencing the interactions between TFs and pathway genes can be achieved via electrophoretic mobility shift assays (EMSA), yeast one-hybrid system, chromatin immunoprecipitation experiments (ChIP-chip or ChIP-seq) and computational tools. Omics- based approaches such as transcriptome and targeted metabolic analysis are useful to identify novel TF genes in plants. In the last 15 years, the combination of co-expression analysis and metabolites profiling has been vital in the identification of genes involved in regulatory and biosynthetic pathways [162]. Alternatively, framework using statistical thermodynamics and the principles of physical chemistry can model the TF molecules binding of various DNA sections [163].

Galis et al used microarray, EMSA and time-course expression data to identify an R2R3 MYB-type transcription factor *(NtMYBJS1)* that was co-expressed in a close temporal pattern with the core phenylpropanoid genes phenylalanine ammonia-lyase (*PAL*) and 4-coumarate: CoA ligase (*4CL*) in tobacco. Further, overexpression of *NtMYBJS1* in tobacco cells caused the accumulation of specific phenylpropanoid conjugates in the cells [164]. Recently, association of *PbRVE1a*, *1b* and *7* with *PbDFR* and *PbANS* promoters was further confirmed using yeast one-hybrid assay in red pear [165]. In *Salvia miltiorrhiza*, promoter binding of *SmGRAS1/2* was confirmed by yeast one-hybrid, dual-luciferase, and EMSA. Yeast two-hybrid assays showed *SmGRAS1* interacted physically with *SmGRAS2* [139].

2.9 Pathway genes paralogues

Gene paralogues can be described as isoforms presenting in multiple copies in the genome of any organism. They may occur from splicing or duplication event [166]. Multiple copies of a gene belonging to categories such as kinases, transcription factors, pathway genes, and transporters have been found in organisms such as yeast, bacteria, insects, and humans.

Multiple copies of genes have been reported in tissues of different plant species as evident in various empirical studies. For instance, nine gene paralogues of *HMGR* have been reported in

Gossypium raimondii, nine in *Gossypium arboreum*, and eighteen paralogues in *Gossypium hirsutum* [167]. In *Arabidopsis thaliana*, differential functions of paralogues were observed; *HMGR1* functions in protein binding whereas *HMGR2* plays role in coenzyme binding [168]. In *Brassica napus,* two paralogues of *C4H* showed differential expression whereby, *BnC4H-2* was dominant in roots and old seeds over *BnC4H-1* which had higher expression in leaf and pericarp [169]. The paralogues have been reported to play role in secondary metabolite biosynthesis. For instance, *CgHMGR* gene in *Corylus avellana L Gasaway* is reported to play a role in Taxol biosynthesis and its expression was found to be elevated in roots while lower in leaves and stems, according to gene expression analyses [170]. Expression analysis identified two gene homologs of *IS* i.e., *SmIS1* and *SmIS2* displaying higher expression in leaves and stems, respectively implicated in enhancing Gentiopicroside production in *Swertia mussotii* [171]. Four isoforms (paralogues) of *4CL* in *Arabidopsis thaliana* showed role in lignin biosynthesis through functional validation by gene silencing [172]. Similarly, three isoforms of *PAL* had shown differential expression levels in different tissues (flower, fruit pericarp and different vegetative tissues) which were characterized through phylogenetic analysis [173]. Two gene paralogues of *DXPS* occurring in chloroplast have been reported in *Arabidopsis thaliana*; *DXPS1* that plays role in both chlorophyll and terpenoids biosynthetic process and *DXPS3* that is solely responsible for terpenoids biosynthesis [174], [175]. *DXPS2* was validated by gene silencing to regulate isoprenoid biosynthesis in *Solanum lycopersicum* whereby *DXPS2* played role in the biosynthesis of β-phellandrene, a monoterpene. Silencing of *DXPS2* by RNAi showed reduced monoterpene levels [175]. In *S. miltiorrhiza,* overexpression of *SmHMGR2* enhanced enzyme production resulting in the enhanced production of a protein that produced tanshinones and squalene in hairy roots [176].

In most of the studies, differential expression of pathway gene paralogues has been reported due to non-coding DNA regions having *cis*-regulating elements which bind sites to TFs in their promoter regions thus regulating the expression of these gene paralogues even though, the paralogues share more than 90% similarity with each other [177]. Further, binding region of TFs were reported in promoter region of pathway gene paralogues influencing secondary metabolite biosynthesis. For instance, six TFs (*PkWRKY 71, Pk WRKY12, PkMyb 46, PkERF18, PkNAC25* and *PkMyc2*) were shortlisted among which *PkNAC25, PkWRKY 71* and *PkERF18* were targeted as key TFs for picrosides biosynthesis [24]. MYB promoter site has been found to be present in *G10H* promoter in *C. roseus* [144]. *TSAR1* and *TSAR2*, a bHLH family members were reported to coregulate and transactivate *HMGR1* for triterpene saponin

biosynthesis in *Medicago truncatula* [117]. Recently, a *cis*-regulatory element (TGGTTA) known to bind *BmMYB35* transcription factor belonging to the MYB family member, was found to be present in the *BmG10H-1* promoter in *Bacopa monnieri*, thereby indicating its role in regulating *BmG10H-1* gene expression [178].

2.10 Transporters of secondary metabolites vis-à-vis Picroside-I and Picroside-II

ABC family constitutes the most common transporters found in cell membranes of plants, animals, bacteria, and fungi to translocate different substrates [179]. ABC family is one of the largest transporter proteins family, categorised into nine subfamilies ABCA–ABCI out of which ABCH is not found in plants and ABCG is found only in plants and fungi [180]. ABC transporters have nucleotide-binding domain (NBD) in addition to highly conserved motifs; Walker A (GXG-K-[ST]), Walker B ([RK]- X3-G-X3-L-[hydrophobic]3) motifs, and ABC signature motif ([LIVMFY]-S-[SG]-G-X3-[RKA]-[LIVMYA]-X- [LIVMF]-[AG]). They also have transmembrane domains (TMDs), in addition to NBDs which are composed of numerous hydrophobic α-helices. There are four key domains in a functional ABC transporter, viz., two NBDs and two TMDs (Figure 2.2). The two NBDs work together to bind and hydrolyse ATP, which act as a driving force for the transport of metabolites, while the TMDs participate in substrate recognition [31]. The classification of ABC transporter family is according to their sequences and TMD structures [181].

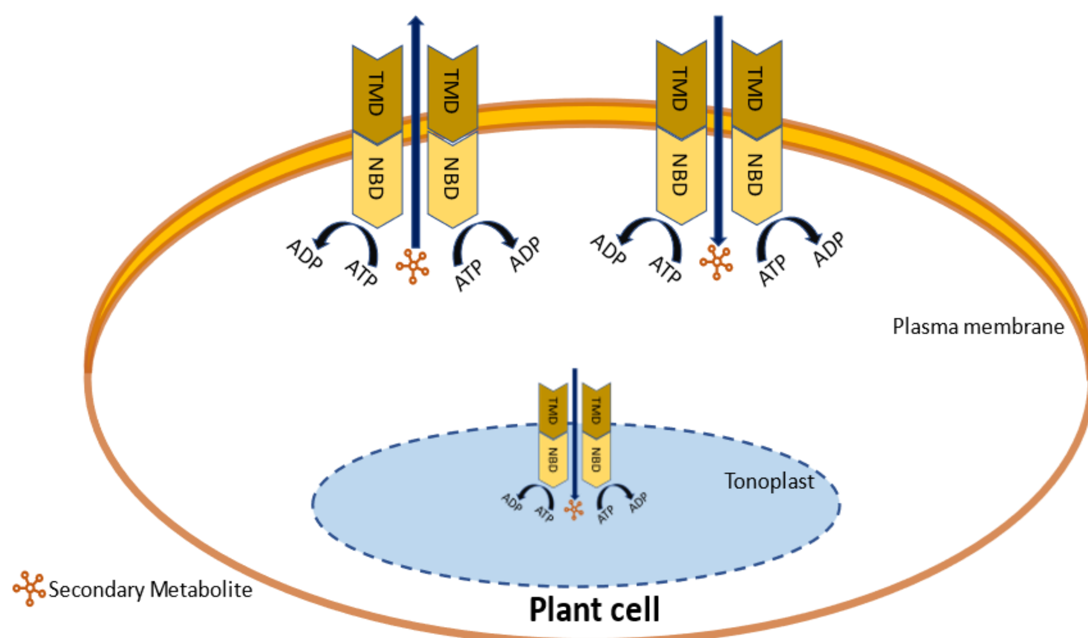


Figure 2.2: Schematic representation of ABC transporters in a plant cell. Adapted from Nogia et al. [28]

ABC transporters are involved in a variety of processes, including plant growth, nutrition, and development, as well as plant response to abiotic and biotic stresses and plant-environment interaction [182]. ATP-binding cassette (ABC) transporters are known to transport secondary metabolites between intercellular tissues, such as roots and shoots. In a plant cell, vacuole membrane possesses ABC transporters, channels, and pumps, facilitating metabolite transfer.

Secondary metabolites such as anthocyanins, terpenoids, alkaloids, flavonoids, and carotenoids are transported across the membranes via ABC transporters. For instance, in *C. japonica*, benzylisoquinoline alkaloid berberine is synthesized in roots and is transported to rhizomes by an ABCB-type transporter [183]. Similarly, *AaPDR3* is reported to transport a sesquiterpenoid named β-caryophyllene in trichomes of *A. annua* [184]. Transportation of diterpene compound, sclareol in *Nicotiana* species is mediated by a PDR member (*NpABC1*) [185]. ABC transporters also help to transport plant metabolites into the vacuoles of plant cells [186]. For instance, *ZmMRP3* belonging to ABCC family has been reported as vacuolar anthocyanin transporter in *Zea mays* [187], *CsABCC4* belonging to ABCC family has been reported using the transportomic approach to transport crocins in *Crocus sativus* (Saffron) [188], *VvABCC1* in *Vitis vinifera* functions as anthocyanidin 3-O-glucosides transporters [189]. ABC transporters associated with secondary metabolites transport and accumulation reported in various plant species is detailed in Table 2.4.

Table 2.4: ABC transporters involved in secondary metabolites in plants

Transporters	Plant Species	Subcellular localisation	Function	Reference
ABCG30/PDR2	*Arabidopsis thaliana*	Plasma membrane	Root exudation of phytochemicals	[190]
PDR1	*Nicotiana plumbaginifolia*	Plasma membrane	Terpene transport	[191]
ABCB4/PGP4	*Arabidopsis thaliana*	Plasma membrane	Polar auxin transport	[192]
ABCB14/PGP4	*Arabidopsis thaliana*	Plasma membrane	Polar auxin transport	[193]
ABCB15/PGP4	*Arabidopsis thaliana*	Plasma membrane	Polar auxin transport	[194]
AtDTX42	*Arabidopsis thaliana*	Plasma membrane	Citrate transport	[195]
ABCB19/PGP4	*Arabidopsis thaliana*	Plasma membrane	Polar auxin transport	[196]
CjMDR1	*Coptis japonica*	Vacuolar membrane	Berberine transport	[188]
ABCC1/MRP1	*Vitis vinifera*	Vacuolar membrane	Anthocyanidin 3-o-glucosides transport	[197]

MRP3	*Zea mays*	Vacuolar membrane	Anthocyanin accumulation	[198]
ABCG29	*Arabidopsis thaliana*	Plasma membrane	Monolignol transporter involved in lignin biosynthesis	[191]
CrTPT2	*Cathanranthus roseus*	Plasma membrane	Catharanthine export	[199]
VvABCC1	*Vitis vinifera*	Tonoplast	Anthocyanin transport	[189]
CjABCB2	*Catharanthus Japonica*	Plasma membrane	Berberine transport	[196]
CjABCB3	*Catharanthus Japonica*	Plasma membrane	Berberine transport	[196]
MtABCG10	*Medicago truncatula*	Plasma membrane	Transport of flavonoids	[200]
AtABCG37	*Arabidopsis thaliana*	Plasma membrane	Scopoletin transport	[201]
AtABCG40	*Arabidopsis thaliana*	Plasma membrane	Abscisic acid transport	[202]
SpTR2	*Sprirodela polyrrhiza*	Plasma membrane	Sclareol transport	[203]
AaPDR3	*Artemisia annua*	Plasma membrane	Sesquiterpene transport	[184]
CsABCC4	*Crocus sativus*	Tonoplast	Crocins transport	[188]
PhABCG1	*Petunia hybrida*	Plasma membrane	Volatile compounds emission	[204]

Thus, investigating the role of ABC transporters in migration of secondary metabolites is of immense interest for controlling secondary metabolites accumulation in specific tissues.

2.11 Functional validation of ABC transporters

Cloning and functional characterization of ABC transporters-encoding genes can be undertaken through various molecular techniques such as knockout/ knockdown through RNAi/CRISPR/VIGS; tissue-specific expression through transcriptional fusions with reporter genes; sub-cellular localization by in-situ hybridization, FISH, fluorescent fusions and expression of transporters in single cell systems of yeast mutants, *Xenopus* oocyte cells, plant cell cultures (BY2, Arabidopsis cell lines), etc. For instance, the suppression of *CrTPT2* expression in developing seedlings and mature plants by VIGS resulted in increased catharanthine–vindoline dimers within the leaves of *Catharanthus roseus* [199]. Also, overexpression of Petunia *PaPDR1* in *Medicago truncatula* demonstrated the role of *ABCG* in transport of orobanchol-type molecules [205]. In several studies, functions of transporters has been demonstrated in the intracellular environment by expression of transporters in different

host systems, for example mutant yeast strains and oocytes of *Xenopus* [206]. For example, *VvABCC1*, an ABC transporter was found to be localised to the tonoplast in the exocarp cells of *Vitis vinifera*, involved in the cotransport of anthocyanidin 3-*O*-glucosides and GSH when heterologously expressed in yeast [189]. Additionally, in *N. tabacum,* overexpression of *NtPDR1* in *N. tabacum* BY-2 cells, followed by its purification and reconstitution into liposomes, validated the role of *NtPDR1* in transport of diterpene and sesquiterpenes [28], [207].

The detailed literature review on *Picrorhiza kurroa*, has helped identify the following major gaps in our knowledge about this valuable medicinal herb:

1. No genetically defined accessions are available for commercial use
2. Lack of knowledge on pathway gene paralogues coupled with TFs regulating one or more of these pathway genes or paralogues in *Picrorhiza kurroa*
3. Lack of knowledge on ABC transporters possibly involved in the transport of Picroside-I and Picroside-II from different tissues and their tissue-specific accumulation in *Picrorhiza kurroa*

CHAPTER 3
MATERIALS AND METHODS

The present study was carried out in the Bennett University, Greater Noida, India. The materials used and the methodologies implemented to achieve objectives of the research are described here under:

3.1 Plant material

Eighty-five accessions of *Picrorhiza kurroa* were obtained from Himalayan Forest Research Institute (HFRI), Jagatsukh, Manali, Himachal Pradesh (1,900 m altitude, 200 35.6′–320 6.1′N and 780 57.8′–770 33.7′E) and coded as PK-01 to PK-85 (Table 3.1). These were sourced from different geographical locations in Himachal Pradesh and Uttarakhand and grown at Jagatsukh. After sample collection for analysis, the plants were separated into 3 tissues (shoots, roots, and stolons) followed by washing, drying, and were finally stored at -80ºC for further use.

Table 3.1: *Picrorhiza kurroa* accessions from different geographical locations of Himachal Pradesh and Uttarakhand used for quantification of picrosides

***Picrorhiza kurroa* accessions**	**Location/Site**	**District**	**State**	**Altitude (m)**
PK01	Pulag nath	Kullu	HP	3871
PK02	Malana jot	Kullu	HP	3747
PK03	Bhaggi	Kullu	HP	4259
PK04	Dalau Paddar	Kullu	HP	4189
PK05	Chitkul	Kinnaur	HP	3645
PK06	Rohtang	L &S	HP	4159
PK07	Dehl, GHNP	Kullu	HP	3460
PK08	Brighu	Kullu	HP	3817
PK09	Yangpa	Kinnaur	HP	3300
PK10	Shrigul Tung	Kullu	HP	4229
PK11	Chander Nahan	Shimla	HP	3600
PK12	Sari	Kullu	HP	4091
PK13	Katgaon	Kinnaur	HP	3046
PK14	Teita	Kullu	HP	4071
PK15	Hydan bhatori	Chamba	HP	3680
PK16	Existing stock 1 year	Kullu	HP	1902
PK17	Existing stock 2 year	Kullu	HP	1902
PK18	Existing stock 3 year	Kullu	HP	1902
PK19	Existing stock 4 year	Kullu	HP	1902
PK20	Tinnu gaon	L &S	HP	3707
PK21	Katgaon (kinnour)	Kinnaur	HP	3046

PK22	Bari (Nichar)	Kinnaur	HP	3727
PK23	Gue (spiti)	L &S	HP	4205
PK24	Moral Danda	Kinnaur	HP	3354
PK25	Tinnu gaon 2	L &S	HP	3635
PK26	Granfu	L &S	HP	3100
PK27	Dehl set2	Kullu	HP	3462
PK28	tinnu site 3	L &S	HP	3707
PK29	Chitkul Site 2	Kinnaur	HP	3645
PK30	kundaghat	Mandi	HP	3230
PK31	Pattal tissa	Chamba	HP	3784
PK32	Sari	Kullu	HP	4091
PK33	Chander Khani site2	Kullu	HP	3856
PK34	Banjar site 1	Kullu	HP	3250
PK35	Karguni	Chamba	HP	4218
PK36	Sural bhatori	Chamba	HP	3523
PK37	Salam Tith	Chamba	HP	3867
PK38	Udipur	L &S	HP	3772
PK39	Patalsu top	Kullu	HP	3993
PK40	Chatru	L &S	HP	3566
PK41	Hanuman tibba	Kullu	HP	4410
PK42	Bansheru	Kullu	HP	3616
PK43	Chander Khani site 3	Kullu	HP	3952
PK44	Brighu	Kullu	HP	3817
PK45	Singh kothi farm	Kullu	HP	2237
PK46	Ropa nursery	Kullu	HP	1536
PK47	Shangar Top	Kullu	HP	3897
PK48	Barshangar Top	Kullu	HP	4067
PK49	Nasogi top site 2	Kullu	HP	4050
PK50	Nanda tith	Kullu	HP	3560
PK51	Diudi top	Shimla	HP	3715
PK52	Litham thach	Shimla	HP	3511
PK53	Jamari Dhar	Kullu	HP	4204
PK54	Nasogi top	Kullu	HP	3835
PK55	Auli	Chamoli	UK	2805
PK56	Kalpur	Chamoli	UK	3230
PK57	Badrinath	Chamoli	UK	3110
PK58	Lalmati	Chamoli	UK	3345
PK59	Kedarnath	Rudraprayag	UK	3500
PK60	Sngoode gangotri	Uttarkashi	UK	3546
PK61	Bansi narayan	Chamoli	UK	3605
PK62	Tung nath	Rudraprayag	UK	3600
PK63	Mangdeswar	Uttarkashi	UK	3597
PK64	Mayer	L&S	HP	3491
PK65	Pulga top	Kullu	HP	4214
PK66	Hamta top	Kullu	HP	3345
PK67	Seri	Kullu	HP	4091
PK68	kundaghat site 2	Mandi	HP	3230

PK69	Badrinath2	Chamoli	UK	3200
PK70	Bara	Kullu	HP	3867
PK71	Battal	L&S	HP	4392
PK72	Deosoor	Kullu	HP	4014
PK73	Kali Mithidhar	Chamba	HP	3672
PK74	Mandal forest	Rudraprayag	UK	3689
PK75	Pandu ropa	Kullu	HP	3891
PK76	Patesu top	Kullu	HP	4116
PK77	Rondhar	Chamba	HP	3901
PK78	Sachar	Kullu	HP	3718
PK79	Sari dhar	Chamba	HP	3857
PK80	Semandhar	Kullu	HP	3623
PK81	Stogi forest	Rudraprayag	UK	3560
PK82	Soil	Kullu	HP	3906
PK83	Vedani pugthal	Rudraprayag	UK	3548
PK84	Yeche top	L&S	HP	3952
PK85	Tunda (Tandhelidhar)	Solan	HP	1680

3.2 Extraction of samples for Picroside-I and Picroside-II analysis

For the analysis, the three tissues (shoots, stolons, and roots) of eighty-five *Picrorhiza kurroa* accessions were ground in liquid nitrogen using a pestle to fine powder (Figure 3.1). Extraction from powdered samples (100.0 mg) was done using 10.0 mL of 80% methanol (HPLC grade). The samples were then run through a vortex mixer and were kept overnight at room temperature (25°C) for extraction to complete. Samples were centrifuged at 10,000 rpm for 15 minutes and the supernatant was filtered through 0.22µm filter (Millipore) and used for HPLC analysis [9].

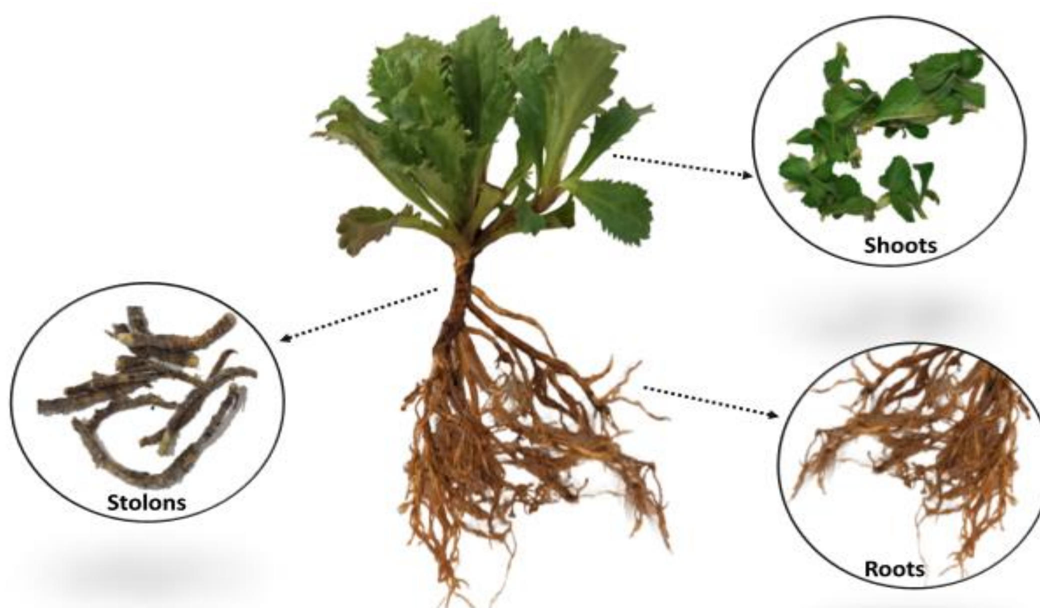


Figure 3.1: Different parts of *Picrorhiza kurroa* plant used for HPLC quantification

3.3 Quantification of Picroside-I and Picroside-II content through HPLC analysis

Reverse phase HPLC system containing[3] was used for the separation of compounds. The mobile phase was made up of water (ultrapure), methanol (Merck, USA) and acetonitrile (Merck, USA) in the ratio of 70:15:15, respectively. To the mobile phase, 0.03% trifluoroacetic acid (TFA) was added and used for the isocratic elution from column at a flow rate of 1.0 mL per min. The injection volume was 20 µL and cycle time of HPLC analysis was 30 minutes at 30°C [208]. The picrosides were detected at a wavelength of 280 nm and identified by comparing their retention time and UV spectra with reference Picroside-I and Picroside-II standards (Sigma). The experiment was performed in biological and technical triplicates (Figure 3.2).

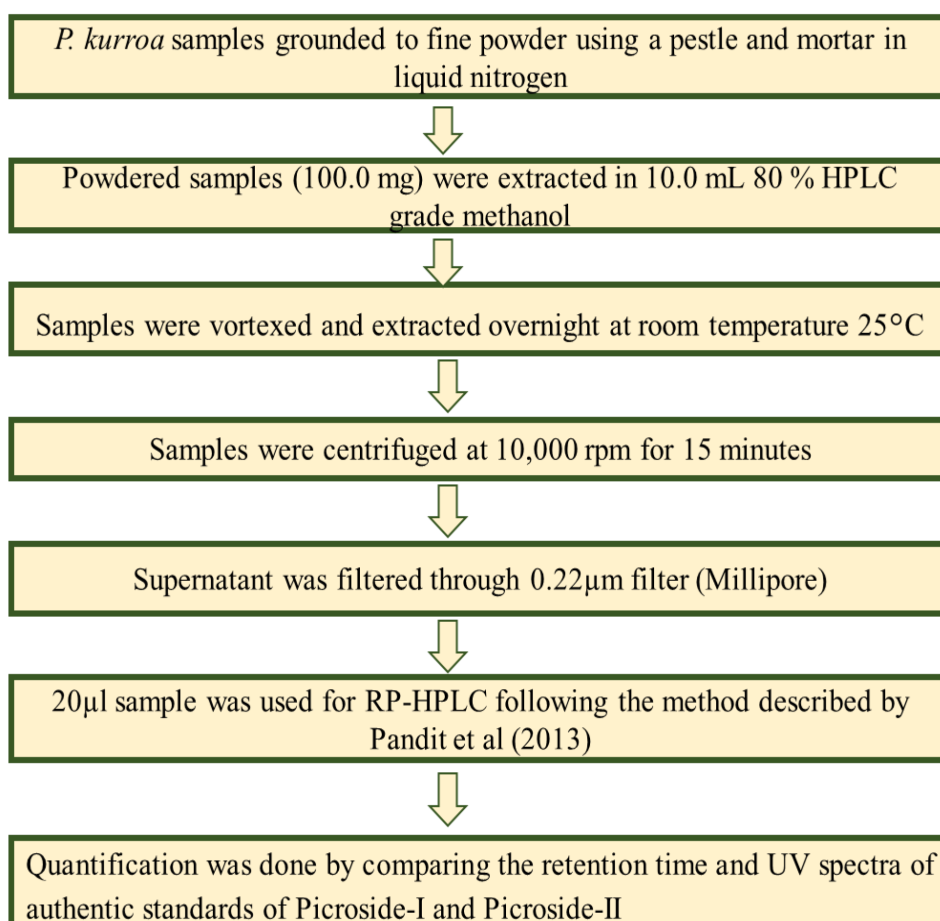
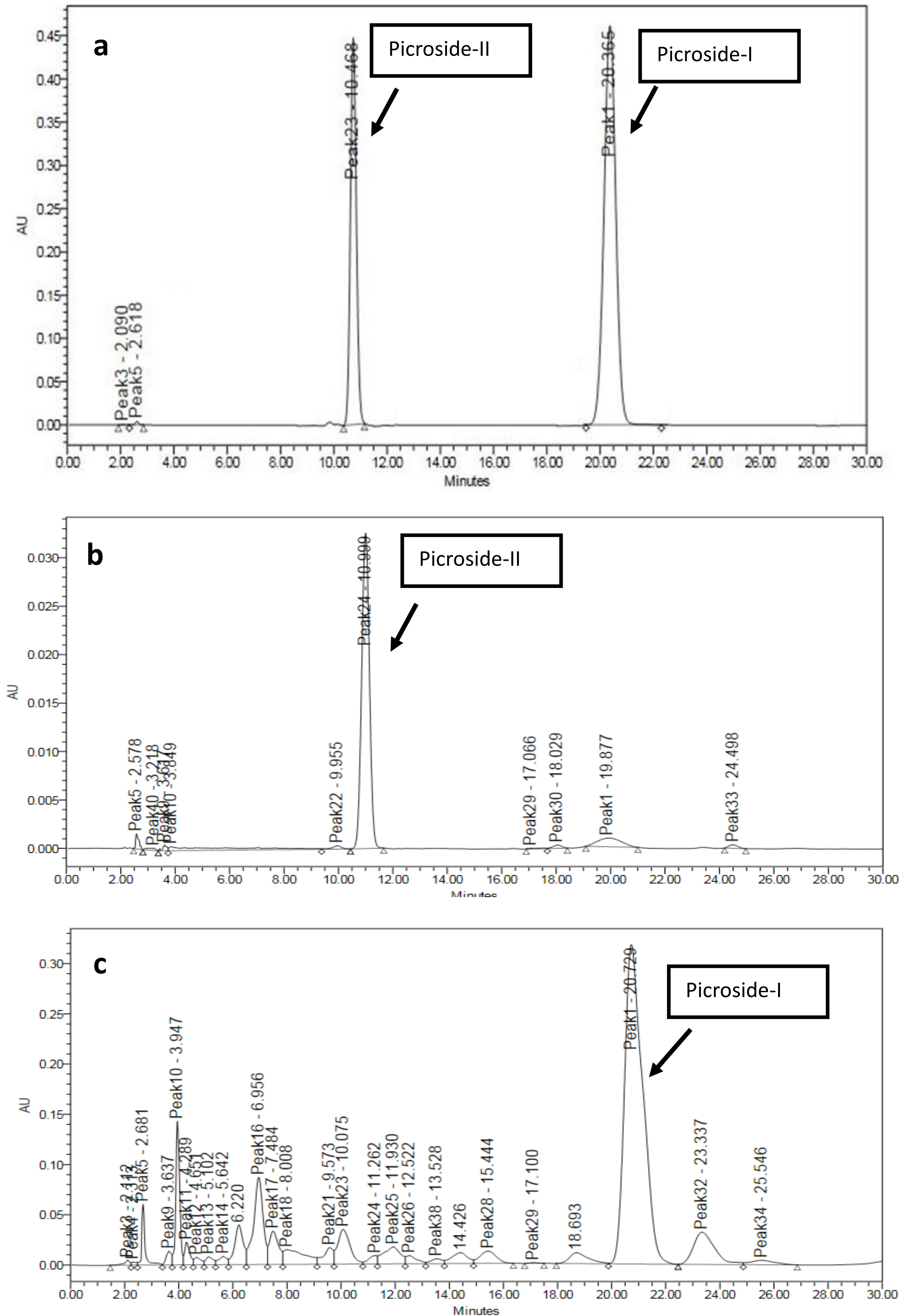


Figure 3.2: Experimental workflow utilized for HPLC analysis.

The HPLC chromatograms of Picroside-I and Picroside-II in shoots, roots, and stolons

[3] C18 column (4.6×250 mm, 5 µm) and photodiode array detector (PDA; Waters 2996)

samples along with Picroside -I and Picroside-II standards are depicted in Figure 3.3.



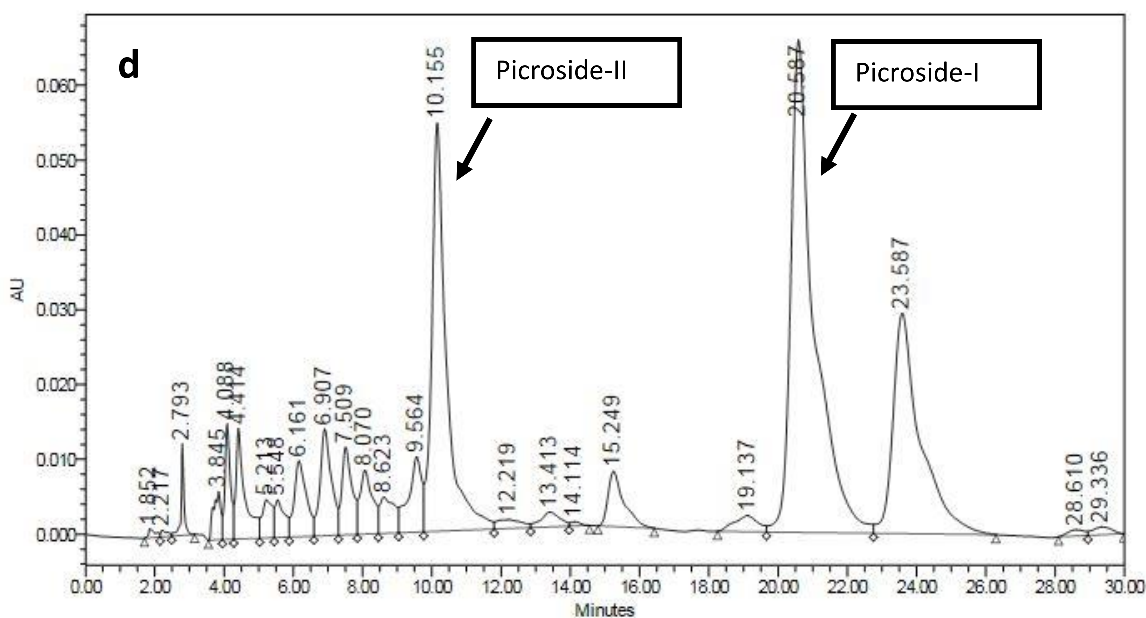


Figure 3.3: Chromatogram: a) Standard of Picroside-I and Picroside-II, b) Root tissue, c) Shoot tissue, d) Stolon tissue

3.4 Selection of differential metabolite content accessions of *Picrorhiza kurroa*

Based on quantitative analysis of Picroside-I and Picroside-II through RP-HPLC, *Picrorhiza kurroa* accessions with high and low content were identified and selected for further experiments to characterize components of biosynthetic machinery involved in the synthesis of Picroside-I and Picroside-II.

3.5 Generation of NGS transcriptomes for *Picrorhiza kurroa*

Transcriptomes of three tissues of *Picrorhiza kurroa* varying for tissue type and metabolite content were utilized for mining components of biosynthetic machinery associated with picrosides biosynthesis. NGS transcriptomes were generated and assembled for tissues having differential Picroside-I (shoots-PKSS) and Picroside-II (roots-PKSR) and Picroside-I + Picroside-II (stolons- PKSTS) contents (Table 3.2) [9]. Total RNA from these tissues was extracted and the quality was analysed on 1% denaturing agarose gel. Whole transcriptomes were generated de novo using paired end (PE) sequencing method on Illumina HiSeq 2000 platform. TruSeq RNA library preparation kit was used for preparation of pair-end cDNA sequencing libraries for each sample. Cluster generation was carried out on cBOT using TruSeq

PE Cluster kit v3-cBot-HS. Each cluster was sequenced on a flow cell using TruSeq SBS v3-HS kit using sequencing by synthesis method [9].

Table 3.2: Content of picrosides varying in shoots, roots & stolons of *Picrorhiza kurroa* [9]

Transcriptome datasets	**Description**	**Picroside-I content (% fresh w/w basis)**	**Picroside-II content (% fresh w/w basis)**
PKSS	*Picrorhiza kurroa* shoots	2.70	-
PKSTS	*Picrorhiza kurroa* stolons	1.70	0.99
PKSR	*Picrorhiza kurroa* roots	-	0.40

3.6 Quantification of transcript abundance

The transcript abundance of the transcripts encoding transcription factors, and ABC transporters in transcriptomes of *Picrorhiza kurroa* was calculated based on Transcripts per million (TPM) parameter using Salmon tool. For extraction of transcripts with significant expression, transcript identifiers with TPM values >0 were recovered using MS Excel. These IDs were used as a query in Samtools faidx to extract the FASTA sequences that matched them. Transcripts from each sample were examined.

3.7 Identification of transcription factors in transcriptomes of *Picrorhiza kurroa*

The transcriptomes of *Picrorhiza kurroa* shoots (PKSS), stolons (PKSTS) and roots (PKSR) were analysed for identification of TFs possibly involved in regulation of picrosides biosynthesis. Out of several TF families obtained, ten TF classes (WRKY, NAC, MYB, bHLH, bZIP, AP2/ERF, TCP, MYC, SPL and Zinc finger TFs) were selected for experimental analysis. Forty-five TFs belonging to the selected families were mined from transcriptomes data of PKSS, PKST and PKSR. Transcript abundance of transcription factors genes was

calculated by TPM in the *Picrorhiza kurroa* transcriptomes. Redundant TFs encoding sequences with 100% sequence identity were removed. Further, *in silico* comparative analysis in PKSS, PKST and PKSR followed by qRT-PCR was done to pull out candidate TFs associated with Picroside-I and Picroside-II biosynthesis. Methodology used for identification and shortlisting of TFs has been provided in Figure 3.4

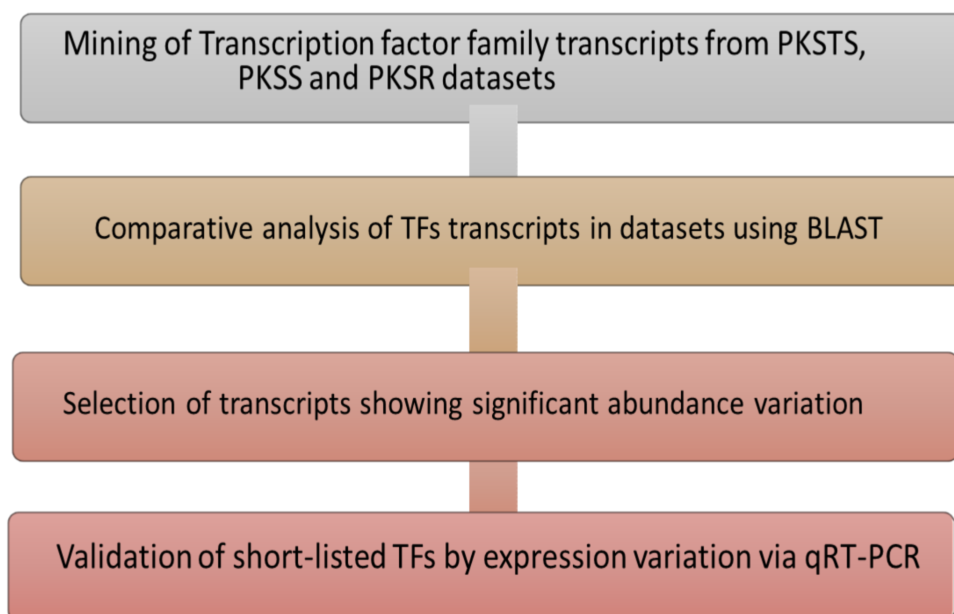


Figure 3.4: Experimental strategy for identification and shortlisting of transcription factors in transcriptomes of *Picrorhiza kurroa*

3.8 Identification of 5′ upstream regions of Picrosides biosynthetic pathway genes

Picrorhiza kurroa genome sequence contigs were retrieved from *Picrorhiza kurroa* genome database (https://scbb.ihbt.res.in/picro-db/) and 2 kb sequence upstream of translational start codon ATG of key pathway genes were annotated using GenScan (http://hollywood.mit.edu/GENSCAN.html). Pathway gene paralogues (*PKHMGR, PKPAL, PKDXPS, PK4CL, PKG10H* and *PKIS*) reported to positively influence Picroside-I and Picroside-II content in *Picrorhiza kurroa* were taken for the analysis [22]. The *cis*-regulatory elements were mined in promoter regions of candidate pathway gene paralogues to explain the basis of the differential expression of these paralogues using PlantCARE [209] and PLACE [210] The strategy involved in identification of *cis*-regulatory elements is demonstrated in Figure 3.5.

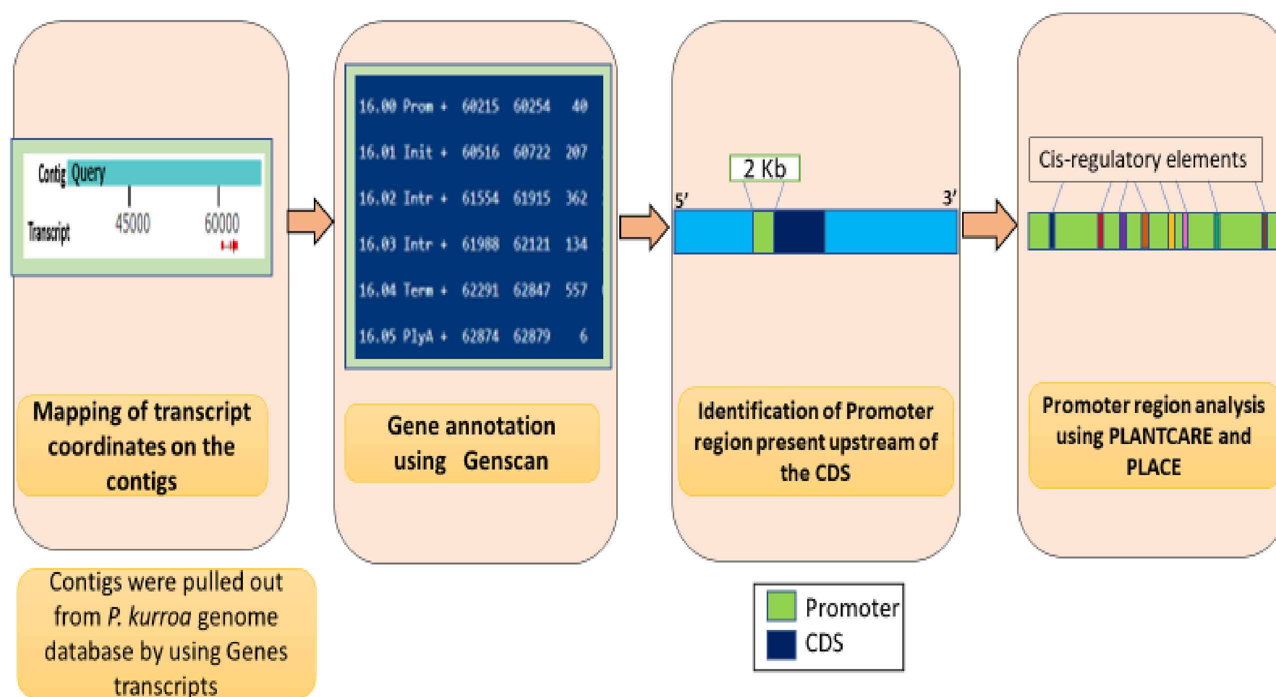


Figure 3.5: Experimental scheme to identify *cis*-regulatory elements in promoter region of pathway gene paralogues in *Picrorhiza kurroa*

3.9 Computational mining of ABC transporters in transcriptomes of *Picrorhiza kurroa*

For identification of putative ABC transporters that are possibly associated with the transport and accumulation of Picroside-I and Picroside-II in *Picrorhiza kurroa*, three transcriptome datasets viz. PKSS, PKSTS, and PKSR differing for Picrosides contents were mined computationally for sequences that have putative ABC transporter function. The transcripts were further shortlisted based on their TPM values. The detailed experimental strategy has been described in Figure 3.6.

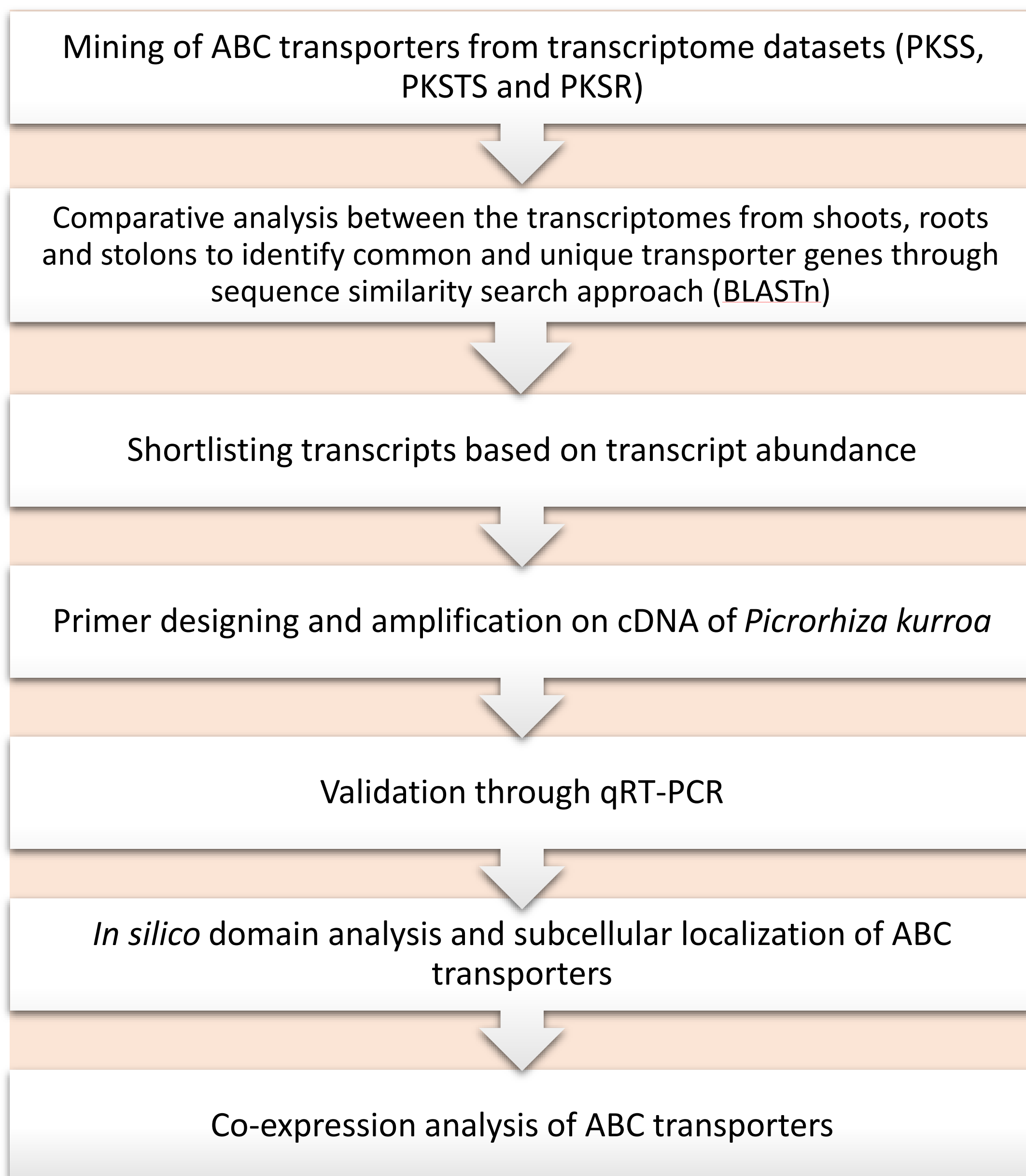


Figure 3.6: Experimental scheme for identification and shortlisting of ABC transporters in transcriptomes of *Picrorhiza kurroa*

3.9.1 Comparative Analysis of putative ABC transporters from NGS transcriptome datasets

Comparative analysis was performed using three transcriptome datasets to shortlist ABC transporters relating to Picroside-I and Picroside-II transport and accumulation. BLASTn tool was used to pull out sequences from different combinations of three transcriptome datasets for comparative analysis of mined ABC-encoding transcripts. ABC transporters unique in shoots were shortlisted for Picroside-I transport and those unique in roots were shortlisted for

Picroside-II. Similarly, transporters unique to stolons were shortlisted for accumulation of both Picroside-I and Picroside-II. The candidate transcripts with higher TPM values were shortlisted for qRT-PCR verification.

3.9.2 *In silico* domain analysis and subcellular localization of ABC transporters

The shortlisted ABC transporters were checked for domain/motif similarity with functionally characterized transporters in different plant species. The validation was accomplished by aligning transcripts with the draft genome of *Picrorhiza kurroa* [5] resulting in identification of genome sequence contigs. The sequence contigs were further annotated using GenScan (http://hollywood.mit.edu/GENSC AN.html) for predicting genes and proteins. The predicted proteins encoding ABC transporters were further analysed for domain analysis using Pfam 35.0 [211]. Additionally, Cell Ploc2 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2/) was used to verify cellular location of the candidate protein sequences.

3.9.3 Co-expression network analysis

The strategy defined in our previous study was applied to build gene co-expression networks for three different transcriptomes of *Picrorhiza kurroa* [212]. GENIE3 was used to generate individual co-expression network for every transcriptome [213] with parameter of "ensemble" with K value 7, and number of ensembles 50. Every relationship between transcripts in the form of a link list had a connectivity threshold of>0.005. Three separate linked lists representing three transcriptome samples were created. The gene co-expression linked list was used to extract linked pairs of putative function related to ABC transporters, which were then displayed using Cytoscape Network Visualization software [214]. The Cytoscape software's network analyser was used to determine the degree of each node in the network. For comparison of transcript abundance amongst co-expressed organ-specific sub-networks, TPM values of nodes were included as an effective addition to the generated network graphs. The size in the network represents the degree of freedom, and the colour represents expression in the form of TPM value.

3.10 Isolation of RNA and cDNA synthesis

RNA was extracted from three tissues of *Picrorhiza kurroa*: shoots, roots, and stolons using total RNA isolation kit (Takara) using the manufacturer's protocols. Quality of RNA was analysed on 1% (w/v) ethidium bromide-stained agarose gel and was quantified in NanoDrop spectrophotometer (Thermo Scientific) by measuring absorbance at 260 nm wavelength and

purity at 260/280 nm ratio. First-strand complementary DNA (cDNA) was synthesized from 1µg of RNA using cDNA synthesis kit (Takara) as per the manufacturer's instructions. cDNA concentration for every sample was adjusted to equal volume for qRT-PCR analysis.

3.11 Expression analysis using qRT-PCR

The qRT-PCR analysis of paralogues of pathway genes, transcription factors and ABC transporters was performed on different tissues of *Picrorhiza kurroa* varying for Picroside-I and Picroside-II contents to evaluate the association of a particular genes with picrosides content. Transcript sequences from the *Picrorhiza kurroa* transcriptomes were extracted for designing gene specific primers using Primer3 online tool. Gene specific primers were checked initially on cDNA for amplification of single product using standard PCR. qRT-PCR reaction was performed in a CFX96 system (Bio-Rad Laboratories) with the Hi-SYBR master mix including Taq Polymerase (Hi-media) using gene-specific primers (Table 3.3, Table 3.4, Table 3.5). The PCR protocol was as follows: denaturation for 3 min at 95°C, followed by 35 cycles each of denaturation for 30 s at 95°C, annealing for 45 s at 50–65°C, and elongation for 30 s at 72°C. Housekeeping genes, 26s rRNA was used as internal controls for normalization. The comparative Ct value approach was used to determine the relative expression of each gene. The statistical significance of the discrepancies between the transcripts was assessed using the standard deviation.

Table 3.3: Primer details for Transcription factors and annealing temperatures used in qRT-PCR analysis

Sequence ID	TFs	Forward Primer	Reverse Primer	Tm (°C)
TF-01	BHLH53	CCGCCTGAACTTTATTGGAA	TTTCCCCAACATGGATCAGT	51
TF-02	Zinc finger	CCGTGTCTCAGCCTTTGTTT	GGAGCCTTGTTTGCGATTAT	53
TF-03	NAC32	AGGGCATCAAGACAGATTGG	TGTGGTCGTAACTCCAGCAA	53
TF-04	NAC 75	GGATTTGCCAGGTGATGC	TTCCTTTTGGAGGCTTTCCT	50

TF-05	NAC 94	CTTCTACGAGGGCAGAGCAC	CGGTGATAGCGGAGTCTTGA	51
TF-06	WRKY40	CCTCCTCAGAACGGTAAACG	TTCATCATCGTCCCCATCAT	52
TF-07	COL 7	TCCTTTATTACTCCCAACGA	CTGCGTTCAGTATCACAAAA	48
TF-08	ERF6	GAAGAAACACTACAGAGGCGTA	GAAGTTGAGTTTGGCTTTGG	54
TF-09	CPRF 2	AGCCAAAAATACAACGAAGC	AGCTGAGGTGTCTGGACTTC	52
TF-10	MYB6	CTCCGAAACTCAAGGATTGTG	GATAAAGCAAATGGCACGCT	53
TF-11	BLHL80	TGCTGAGATAGACCGAATTT	CCTATCCATATTGGGAACAA	51
TF-12	AP2	ATCAAACATTTCGTCACCAT	GCCCCATTAATCATCTCATA	51
TF-13	Bzip60	GTCTGCTGTGCTCTTGTTGG	GCCACTTTTCCCCACTCTTC	53
TF-14	BHLH50	GAAAAACTTAATGGCGGAGA	GCTGTTCTGTCCATCTTGCT	52
TF-15	MYB4	AAGAAGACCGAAAGCAACGA	CGACCAGAAACTCCCATCC	55
TF-16	MYB3	ACCATCGCTAACACTTG	GCAATAGACAGCTCCAAGTC	51
TF-17	MYB5	TTAGGGTTTCGTCGGTGTTC	CCACTCCAAAGTTCGCATCT	53
TF-18	NAC14	TCCCAGATTTGGCTTTGTAT	AAGGCCTTCTTGATCCCTAC	52

TF-19	NAC72	GCTTTTGGTGCAGTACCTCT	ATTTTCGGTCTCTTGGACTG	52
TF-20	NAP	TCTTTAGCCCTCGTGACAGAA	GTTTTGATTCCCTTTGGTGGT	56
TF-21	WRKY40	ATTCGTCCAAGAAACAGAGG	TGAAGTATGCTCTCGGACAA	52
TF-22	pkdoubleWRKY	GGGTGGTCTTAGGTGAAGTG	TGTAGTTGTGCCAGCTCATT	49
TF-23	WRKY34	AAGTTAACCCGAAGATTTCC	CTTCTTTTTGACTGGACAGC	50
TF-24	WRKY40	ACAGCAGCTCAAGTGATGAA	TTACTGGACAAGTTGGAGCA	53
TF-25	WRKY20	GTCATCGACCACCATTAACT	CACTTGGAATTCGGTAAATC	51
TF-26	ERF7	GAAGAATTACAGGGGTGTGA	GAAAATTAAGCTTCGCTCTG	48
TF-27	MYB108	GAACAAGAAACATCCTCTGC	ACCATATTCTTGCGTAGCAT	52
TF-28	SNAC1	CTGTCTCGGGATATTGGAAG	CTTGGATTCGCTTAGTCGAT	48
TF-29	WRKY17	AAACCGCAATCTCTGTTCTGA	GGAGAAGTTTTCGGAATGGTC	52
TF-30	WRKY41	AGAGAAAAATGCAGCACACA	AAATATTGTCGGGTCGTCAT	50
TF-31	WRKY4	GGAGAAAGTATGGTCAGAAGG	TTATGCTTCCCCTCGTATGT	52
TF-32	WRKY27	GAATTCGTCTTCAACGACCT	AGTTTTCGGAATGGTCATGT	52

TF-33	GBF3	TTATGGAATGCATCTCCTGCT	CTCCGAGCAGACTCCCTATTT	51
TF-34	BZIP5	GATTAAAGGGGATGCAGCTA	TCTTCAGCCTGTTGAGATGA	50
TF-35	COL10	CACTTTTTGGCGAATCTCTTG	GATTGTGCTGGTCTTGGAGAG	53
TF-36	ERF32	GGAAACGAATTTTGCTGCTG	ATACTCTCGCGCCGTTCTTA	53
TF-37	ERF118	AATGGTGAGAGAGCCCCTAAA	TTCAAATTCCAAGCGTTTCAG	50
TF-38	NUTCRACKER	CTTCAATCAAGGTGGTTTCCA	GTTAGGGTTTGGGTTGTGGTT	52
TF-39	MYC2	TCTCCTTCACATCTGGCTTA	TTCTCCCTCCTTTGTCTCTC	53
TF-40	Zinc finger	CGAGCACCTATCCAAGTTAC	TTGTCGCAGACCTTACACTT	52
TF-41	bHLH6	TCTGAGGGAAGAAGCGGTAA	TTGACATGTGGAGGTGGAGA	54
TF-42	bHLH11	CAAATTCAAGCGGGTCAT	CGATTCTTTCGCGTTTTC	53
TF-43	Bzip1	GCCGTTTGAAGCTGACTC	ATCCACGGTAGTGCGAAG	53
TF-44	MYB	GCCGATGAATCAGTCGAT	GGCAACGAAAGTGGGATA	52
TF-45	bHLH14	CCGTTGTGTCCAGTTCCT	TGATCAGGTGCAACTCCA	52

Table 3.4: Primer details for pathway genes paralogues and annealing temperatures used in qRT-PCR analysis

Pathway Genes	Forward Primer	Reverse Primer	Tm (°C)

26s	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	51
DXPS1	TGCTGCGAGTCTTGAAGAGA	CAATTCATCGGCCAATCTTT	53
DXPS2	GGACGGATTACAAGAAAGCTCAAC	CGACACTACGTAAACAATCTCTTCC	53
IS1	TAATCGGGTTGTCCTCGTTC	CTCGAGCGTGGCCTTAATAG	53
IS2	CTCGAGCGTGGCCTTAATAG	TAATCGGGTTGTCCTCGTTC	54
IS3	ACTGGGAAGATAGAAAGTCATGATCCT	CCACCTCTTCAAACAGAATATCCTCC	52
IS4	ATTCGGCATAATCAAGCCCCACGAGA	TGGAACGTTCTCGTGTTTGCAAATTG	51
PAL1	GATCCACCACCCTAAGCAAA	GCGATTGATTTGAGGCATTT	58
PAL2	TCCTGAACTCCTCCACCATC	GGAAAATTCACACGCACCTT	60
PAL3	CGAATCGGATGCCAGAGTAT	GACCGACAGCTATGGGGTTA	54
4CL1	GATCCAGAGTCGACGGAGAG	ACCACAAAGGCAACTGGAAC	55
4CL2	CTAATGGGAAGATCGCCAAA	GTGCCCATGAAGGATGAAGT	54
4CL3	CCTGGTCGGCAATACCGGAA	TTCTGGGTGATTCCCAGATTGGCC	55
G10H1	GGAGTCGGAAAGTGCAAGAG	CCGAAAAAGGGTCAGTCAAA	53
G10H2	GATCAACGAAAGGACCGAAA	GCCATTGCCCATTCTAGTGT	53
HMGR1	CATCGATTTCGTCCAGTCCT	CTCGCCAGAAACAACAGACA	52
HMGR3	GGTCCTTCACTCAGGCCATGGGAA	ACGTGACGAGTGCAGCGATCT	52
HMGR4	CCTTCTCCGATCAAACCCCAATTCTC	GATGAGGCAGAGGATGGCTG	53

Table 3.5: Primer details for ABC transporters and annealing temperatures used in qRT-PCR analysis

Name	Forward Primer	Reverse Primer	Tm (^{0}C)
PKABCB1	CTTCCACTCTCTCCGACCAG	GCGGCTGCTTCTATATTTGC	53
PKABCB2	GGAGATTAGAGCAGCGGTTG	CTCGTAGCCTCGTCCAAAAG	53
PKABCB3	ACGAAGCAACCAGTGCTCTT	TTGACACCAACCCATTAGCA	52
PKABCF1	CAAGGTTTCCCGTCATCCTA	AGCTCACGGCAACCTAAAGA	52
PKABCE1	TGCTGAAACCAGGGGTAATC	AAGGGACCCAACAAATTTCC	52
PKABCC1	GAGGTCTCGTTTTGGGATCA	TTGTCACCGCTGTCAAGAAC	53
PKABCF2	CACGCAAAAATAACCCGACT	TTCGGCGTCACAATCATTTA	53
PKABCF3	AGTCGTTCTCCACCACCATC	GCTTCCTATTCCGGATCACA	52
PKABCB4	ATGTTGGCTCGGATTGTTTC	TTGTTGGAGAGAGCGGAAGT	52
PKABCB5	ATCCCTCACGGCACAACTAC	CCTTTCCGTATGCGATGTTT	53
PKABCG1	GACTAGAGCGGTGCCAAGAC	CGGCCTCAGCTATTTCATGT	54
PKABCF5	GACTTTGGGGTTGACCTTGA	TTTCCATGTCCAGCTTTTCC	52
PKABCB6	GTCGATTTAACTCCCGGACA	TCCACAATTTTCCCACCATT	54
PKABCA1	GCTTTCGCCTTTATGCTGTC	ATTTGGGGGAAAGAATGACC	53
PKABCC2	TGAAGACAACGGTGGAAACA	ATTAACGTTCCTTGCCATGC	54

3.12 Correlation network analysis

Expression values of transcription factors and gene paralogues calculated through qRT-PCR in different accessions of *Picrorhiza kurroa* were considered for calculating correlation analysis with Picroside-I and Picroside-II contents. The expression value of each transcription factor and pathway gene paralogue were represented in the form of heatmap using pheatmap package of R[4]. The matrix consisting of ΔΔCt value and Picroside-I/ Picroside-II content in different

[4] (https://www.rdocumentation.org/packages/pheatmap/versions/0.2/topics/pheatmap).

accessions were evaluated through Pearson’s correlation coefficient formula resulting in a correlation matrix showing a pair-wise correlation coefficient of all gene paralogues, transcription factors and picrosides contents.

$$r = \frac{\sum(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum(x_i - \bar{x})^2 \sum(y_i - \bar{y})^2}}$$

r = Pearson’s Correlation coefficient

x_i = Values of the x variables in the sample

$\bar{x}$ = Mean of the values of x variables

y_i = Values of the y variables in the sample

$\bar{y}$ = Mean of the x variables in the sample

The pairs of correlation greater than 0.5 and less than -0.5 were considered positive and negative correlations, respectively. These pairs were further visualized using Cytoscape [214] with an additional style feature to represent the type of correlation through colors of edges and network analyzer to calculate the degree of freedom for each edge.

CHAPTER 4

RESULTS

4.1 Picroside-I and Picroside-II contents in different tissues of *Picrorhiza kurroa*

In this section, the results obtained from quantitative analysis of Picroside-I and Picroside-II content in three tissues (shoots, roots and, stolons) across 85 accessions of *Picrorhiza kurroa* are described.

4.1.1 Differential Picroside-I contents in shoots of *Picrorhiza kurroa* accessions

Total Picroside-I content ranged from 0.23% to 7.31% in shoots of 85 accessions (Figure 4.1). Out of the five accessions exhibiting the maximum Picroside-I content, PKS 54 accession showed maximum level of Picroside-I of 7.31% followed by PKS 78 (5.46%), PKS 22 (5.39%), PKS 52 (4.46%) and PKS 85 (4.16%), respectively. Low level of Picroside-I was observed in PKS 30 (0.23%), PKS 42 (0.23%), PKS 8 (0.25%), PKS 25 (0.15%) and PKS 29 (0.27%). Picroside-II was not detected in shoots of any of *Picrorhiza kurroa* accessions. The accessions having high Picroside-I contents in shoots are listed in Table 4.1.

Table 4.1: *Picrorhiza kurroa* chemotypes for high Picroside-I contents in shoots

Sample Name	Location (Source)	PICROSIDE-I%
PK Shoot 54	Nasogi Top	7.31
PK Shoot 78	Sachar	5.46
PK Shoot 22	Bhuri (Nichar)	5.39
PK Shoot 52	Litham Tach	4.46
PK Shoot 45	Singh Kothi Farm	4.37
PK Shoot 6	Rohtang	3.92
PK Shoot 68	Kundaghat site 2	3.70
PK Shoot 46	Ropa Nursery	3.56
PK Shoot 17	Existing stock 2 year	3.43

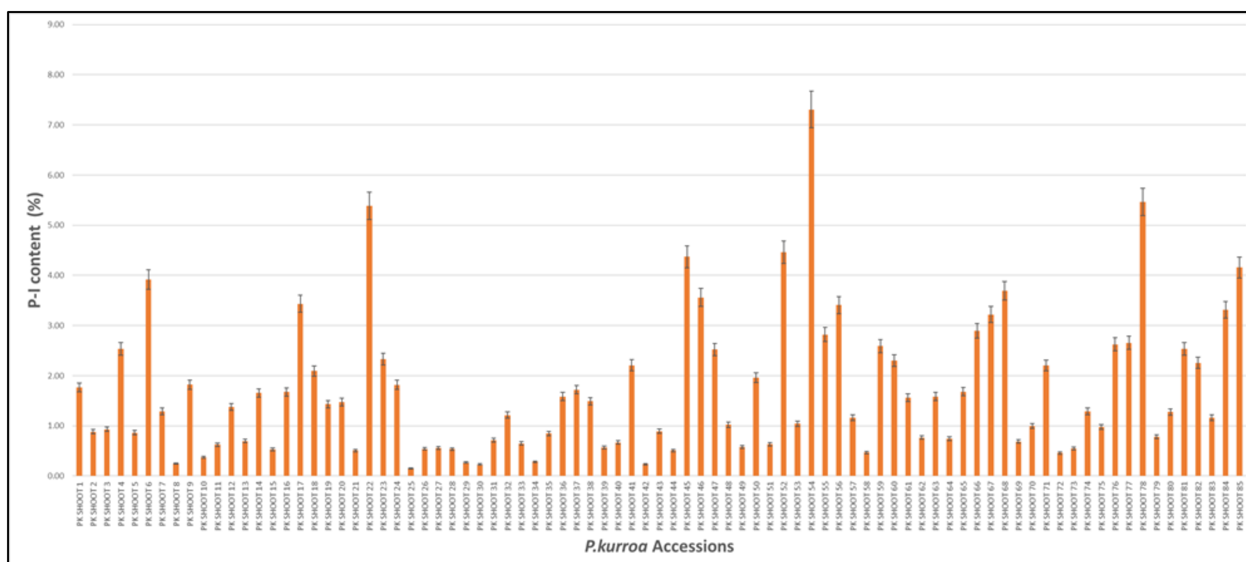


Figure 4.1: Variation for picroside-I content in shoots of *Picrorhiza kurroa* accessions. The data presented as means ± SD (n = 3).

4.1.2 Differential Picroside-II contents in roots of *Picrorhiza kurroa* accessions

The analysis of root tissues of 85 different *Picrorhiza kurroa* accessions revealed significant variations in Picroside-II content (Figure 4.2). Total Picroside-II content ranged from 0.02% to 2.68% with accession PKR 84 showing the highest amount of Picroside-II of 2.68%, followed by PKR 82 (2.37%), PKR 74 (1.69%), PKR 83 (0.91%) and PKR 71 (0.84%), respectively. Minimum amount of Picroside-II was observed in PKR 13 (0.02%), PKR 62 (0.02%), PKR 27 (0.03%), PKR 31 (0.03%) and PKR 11 (0.04%). Picroside-I was also detected in roots of some accessions of *Picrorhiza kurroa* with maximum of 0.29% in PKR 71, followed by PKR 36 (0.38%), PKR (0.15%) and PKR 01 (0.13%) as given in Table 4.2.

Table 4.2: *Picrorhiza kurroa* accessions for high Picroside-I and Picroside-II content in roots

Sample Name	Location (Source)	Picroside-I%	Picroside-II%
PK Root 71	Patesu top	0.29	0.84
PK Root 36	Sural bhatori	0.28	0.35
PK Root 81	Stogi forest	0.15	0.53
PK Root 01	Pulag nath	0.13	0.26
PK Root 56	Kalpur	0.11	0.33
PK Root 59	Kedarnath	0.10	0.18

The accessions having high Picroside-II contents in roots are listed in Table 4.3.

Table 4.3. *Picrorhiza kurroa* accessions for high Picroside-II content in roots

Sample Name	Location (Source)	Picroside-II%
PK Root 84	Yeche Top	2.68
PK Root 82	Soil	2.37
PK Root 74	Mandal Forest	1.69
PK Root 83	Vedani pugthal	0.91
PK Root 71	Battal	0.84
PK Root 41	Hanuman Tibba	0.82
PK Root 73	Kali Mithidhar	0.79
PK Root 46	Ropa Nursery	0.78
PK Root 66	Hamta top	0.59
PK Root 75	Pandu Ropa	0.58

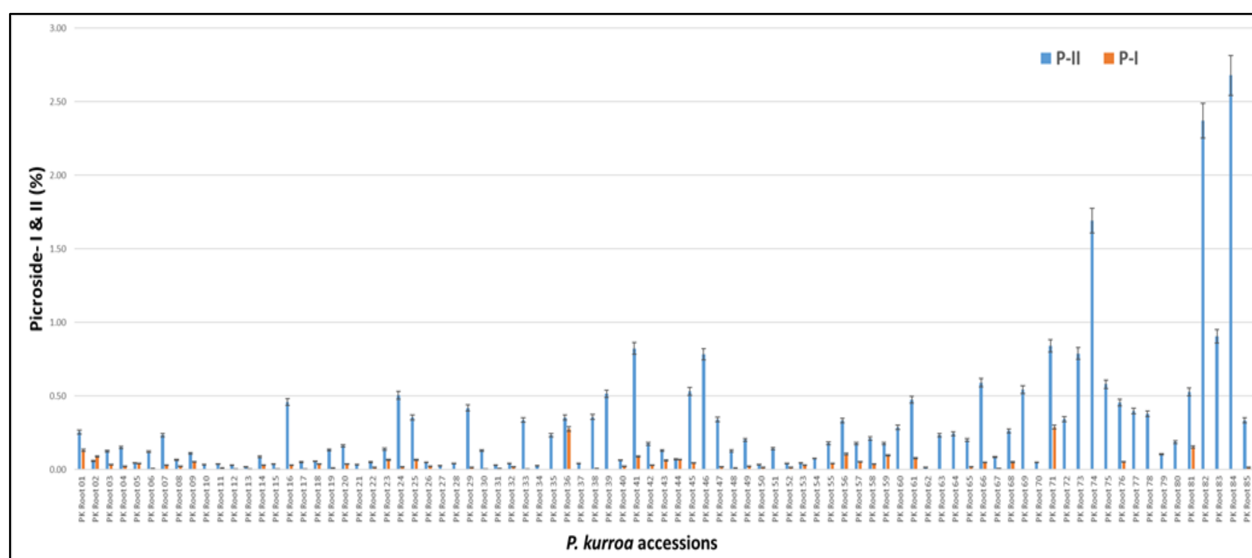


Figure 4.2: Variation for Picroside-II content in roots of *Picrorhiza kurroa* accessions. The data presented as means ± SD (n = 3).

4.1.3 Differential Picroside-I and Picroside-II contents in stolons of *Picrorhiza kurroa* accessions

Stolons (which eventually mature into rhizomes) of eighty-five accessions were quantified for Picroside-I and Picroside-II contents as illustrated in Figure 4.3. Overall Picroside-I and Picroside-II content in stolons of *Picrorhiza kurroa* ranged from 0.10% to 7.36%. The accessions PKST 47, PKST 57, PKST 61, PKST 62 and PKST 67 showed higher amount of

Picroside-I ranging from 4.73% - 7.36%. The maximum amount of Picroside-I and Picroside-II was observed in PKST 67 (7.36%), followed by PKST 47 (7.14%), PKST 57 (5.12%), PKST 62 (4.87%) and PKST 61 (4.73%). In contrast, minimum amount of Picroside-I and Picroside-II was detected in PKST 3 (0.10%), PKST 43 (0.12%), PKST 58 (0.20%), PKST 21 (0.24%) and PKST 25 (0.26%). The accessions having high Picroside-I and Picroside-II contents in stolons are listed in Table 4.4.

Table 4.4: *Picrorhiza kurroa* accessions for high Picroside-I and Picroside-II content in stolons

Sample Name	Location (Source)	Picroside-I%	Picroside-II%	Picroside-I+Picroside-II %
PK Stolon 67	Seri	5.05	2.31	7.36
PK Stolon 47	Shangar nursery	4.64	2.50	7.14
PK Stolon 57	Badrinath	4.21	0.91	5.12
PK Stolon 62	Tungnath	1.32	3.55	4.87
PK Stolon 61	Bansi narayan	3.68	1.06	4.73

The accessions having ratio of 1:2 between Picroside-I: Picroside-II in stolons is listed in Table 4.5 as this composition is used in most of the herbal drug formulations.

Table 4.5: Stolons of *Picrorhiza kurroa* accessions varying for Picroside-I and Picroside-II contents in 1:2 ratio

Sample Name	Location (Source)	Picroside-I%	Picroside-II%
PK Stolon 80	Semandhar	1.34	2.73
PK Stolon 70	Bara	1.00	1.94
PK Stolon 48	Barshangar nursery	0.91	1.87
PK Stolon 7	Dhel, GHNP	0.75	1.61
PK Stolon 38	Udaipur	0.70	1.41
PK Stolon 52	Litham thach	0.48	1.00
PK Stolon 77	Ron dhar	0.49	0.83
PK Stolon 36	Sural bhatori	0.43	0.83
PK Stolon 22	Bhuri (Nichar)	0.25	0.51

PK Stolon 10	Shrigul Tung	0.22	0.38
PK Stolon 9	Yangpa	0.16	0.26

Table 4.6: *Picrorhiza kurroa* accessions varying for high and low Picroside-II contents in stolons

Sample Name	Location (Source)	Picroside-II%	Picroside-I%
PK Stolon 62	Tung nath	3.55	1.32
PK Stolon 80	Semandhar	2.73	1.34
PK Stolon 84	Yeche top	2.50	1.79
PK Stolon 47	Shangar Top	2.50	4.64
PK Stolon 67	Seri	2.31	5.05
PK Stolon 58	Lalmati	0.10	0.10
PK Stolon 42	Bansheru	0.09	0.25
PK Stolon 43	Chander Khani site 3	0.09	0.03
PK Stolon 37	Salam Tith	0.08	0.58
PK Stolon 03	Bheegi	0.08	0.02

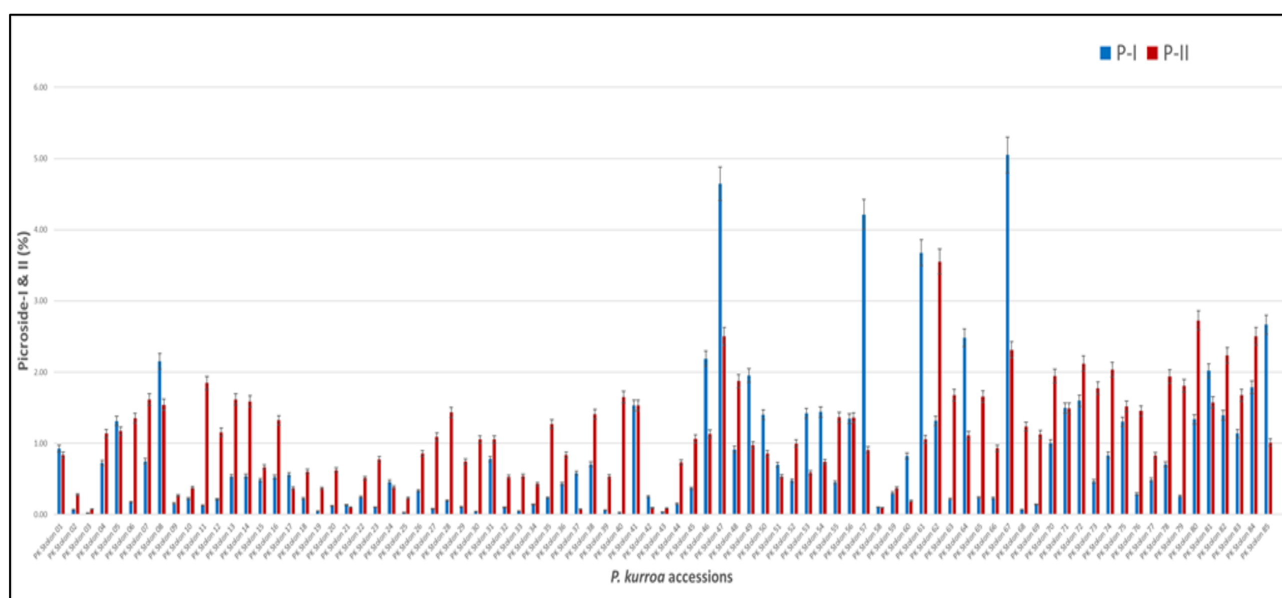


Figure 4.3: Variation for picrosides (Picroside-I vs Picroside-II) content in stolons of *Picrorhiza kurroa* accessions. The data presented as means ± SD (n = 3).

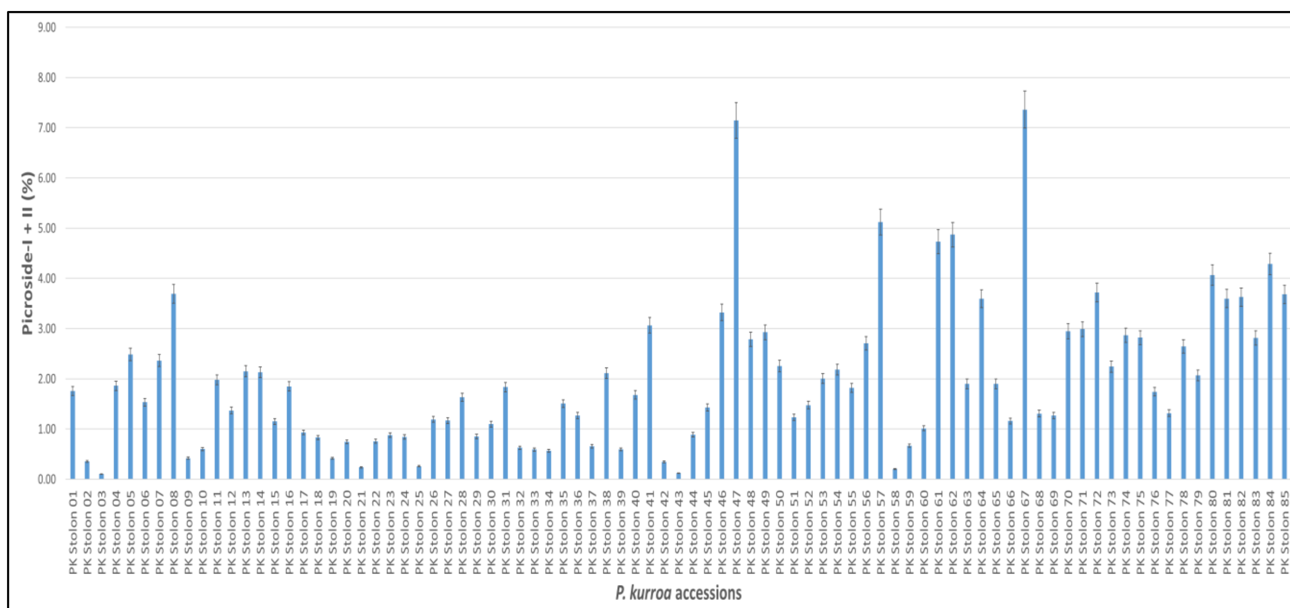
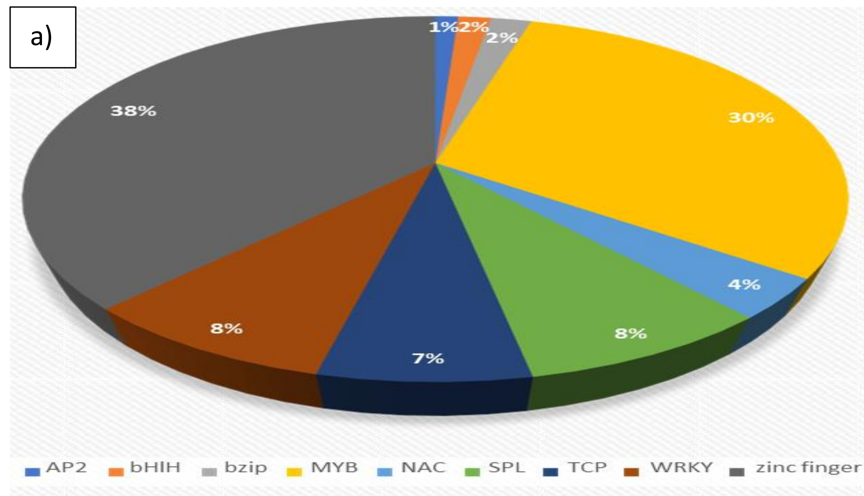


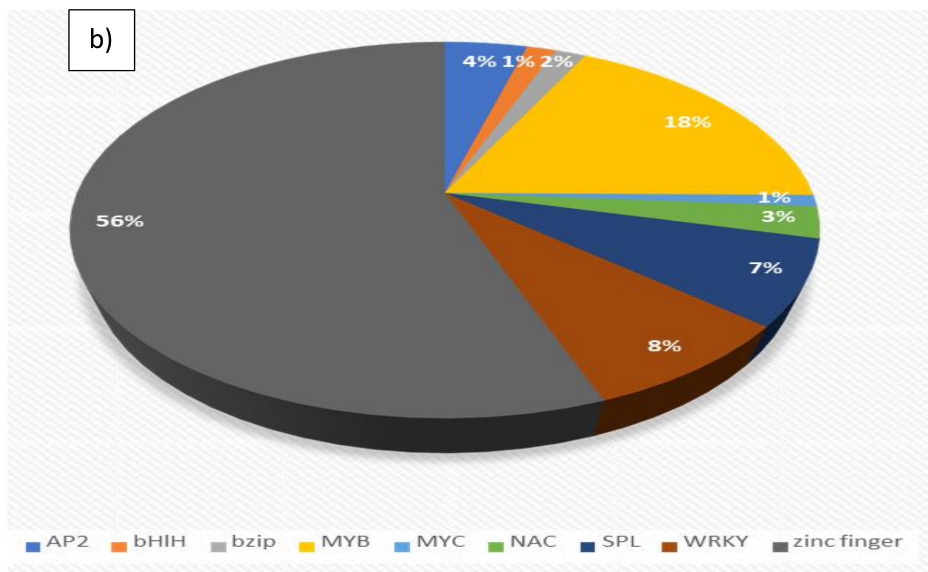
Figure 4.4: Determination of total picrosides (Picroside-I+Picroside-II) content in stolons of *Picrorhiza kurroa* accessions. The data presented as means ± SD (n = 3).

4.2 Identification of candidate transcription factors (TFs) in *Picrorhiza kurroa* transcriptomes

The mining of *Picrorhiza kurroa* transcriptomes derived from shoots (PKSS), roots (PKSR) and stolons (PKST) resulted in the identification of 1108, 922 and 1334 transcripts encoding TFs belonging to 86 families. The identified transcripts encoding for TFs were associated with various biological processes such as seed germination, flower development, drought resistance, photosynthesis, disease resistance, biosynthesis of secondary metabolites, etc. We analysed further TFs involved in regulation of picrosides biosynthesis in *Picrorhiza kurroa*. Out of several TF families obtained, ten TF classes (WRKY, NAC, Myb, bHLH, bZip, AP2/ERF, TCP, Myc, SPL and Zinc finger) were selected for further experimental analysis.

In the shoot and root transcriptome datasets, bZip family was the most abundantly present i.e, 38%, and 56%, respectively whereas in stolons, Myb family was dominantly present with 26%. Myb was the second most abundant TFs class in shoot and stolon transcriptomes. TCP were found in shoot and stolon transcriptome indicating their unique presence in Picroside-I containing tissues whereas Myc TFs were uniquely present in root transcriptome (Figure 4.5).





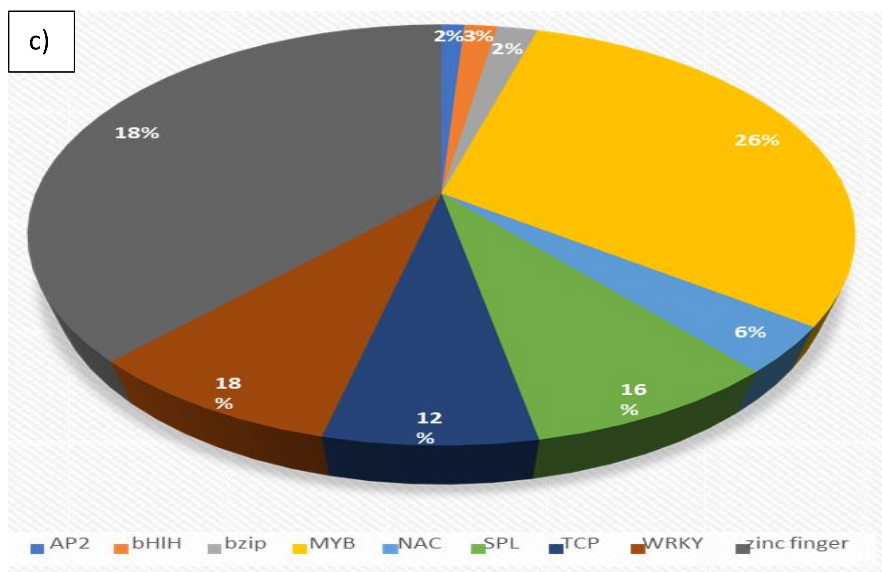


Figure 4.5: Distribution of TFs families in *Picrorhiza kurroa;* a) shoots, b) roots and c) stolons transcriptomes

All transcripts of selected TF families obtained from the transcriptomes (PKSS, PKSR and PKSTS) were annotated using BLASTX and EGGNOG and were compared with NR database of NCBI. Unreliable annotations like probable, predicted and hypothetical were removed. TFs classified by NR annotation were used as the reliable dataset for further analysis. A total of 106, 122, and 83 transcripts encoding different TFs families were identified in PKSS, PKST and PKSR transcriptomes, respectively.

4.3 Transcript abundance of TFs

Transcript abundance values for the unique and common TFs were analyzed in different tissues: shoots, roots and stolons transcriptomes of *Picrorhiza kurroa* in the form of TPM value. TPM values corresponding to respective TFs ranged from 1.27- 216.76 in shoots, 2.09-174.87 in roots and 0.08- 272 in stolons of *Picrorhiza kurroa.* The transcriptome analysis revealed that transcripts encoding for NAC-25 have the highest TPM value of 272 in stolons. In case of shoots, transcript encoding for WRKY 71 showed the highest TPM value of 216.76. Similarly, in roots, transcript encoding for bHLH 81 showed the highest TPM value of 174.87. On analyzing the common TFs in shoots and stolons, the transcript abundance ranged from 4.46-210.43 whereas TPM value of common TFs among roots and stolons ranged from 2.83- 123.98. Further, based on transcript abundance (TPM) variation, 45 TFs were selected for expression analysis via qRT-PCR.

4.4 Expression analysis of TFs using qRT-PCR

To identify candidate TFs possibly associated with Picroside-I and Picroside-II biosynthesis, the transcripts fold changes were compared independently between transcriptomes of three tissues, i.e., roots, shoots, and stolons. Further, through qRT-PCR, *Myb 46* (~8 folds), *ERF 118* (~17 folds), *NAC 25* (~4 folds) and *NAC 94* (~4 folds) showed maximum expression in shoots and stolons compared to other TFs whereas *bHLH 93* (~12 folds), *WRKY 71* (~10 folds), *NAC 32* (~10 folds), *WRKY 12* (~9 folds), and *Myc2* (~11 folds), showed maximum expression in roots and stolons (Figure 4.6). The relative expression status of transcription factor genes between different tissues is thus expected to provide us a realistic association with the biosynthesis of Picroside-I and Picroside-II.

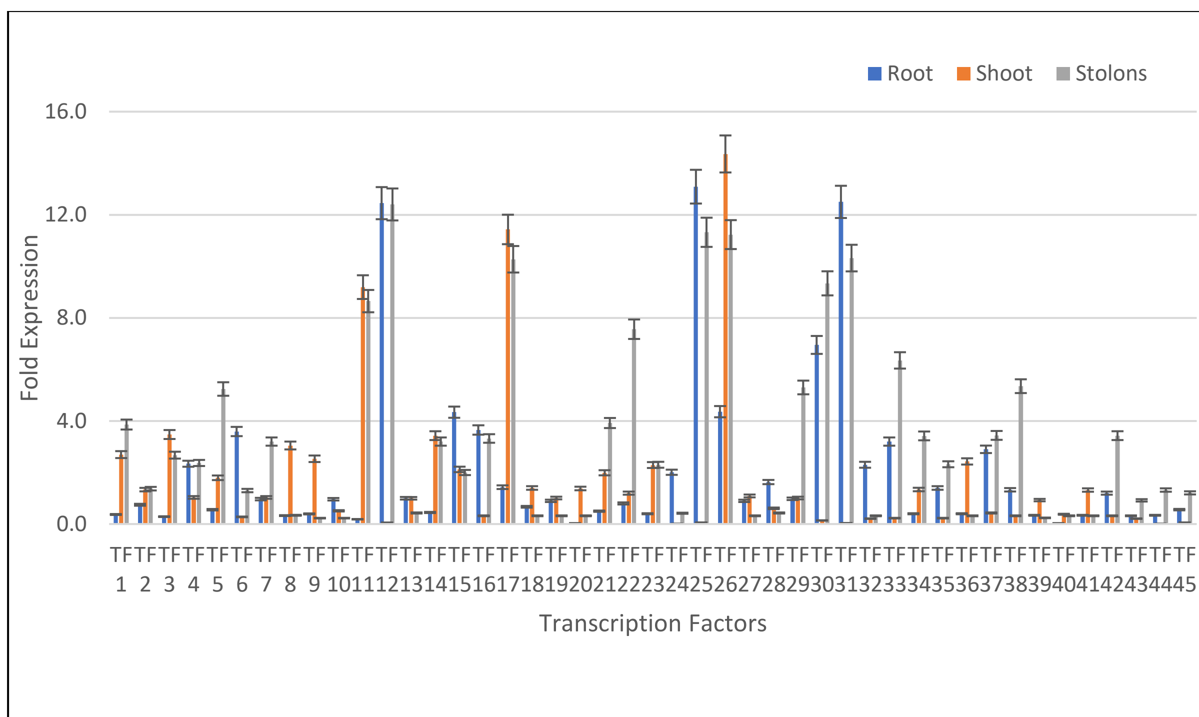


Figure 4.6: Expression profiling of transcription factors in different tissues (Roots, shoots, and stolons) varying for Picroside-I and Picroside-II contents. Error bars indicate percentage error (5%)

4.5 Mining *cis*-regulatory elements in the promoter regions of pathway gene paralogues

Expression of gene paralogues is under the spatio-temporal control of its upstream regulatory elements as well as transcription factors. Thus, to identify the regulatory mechanisms of the biosynthetic machinery of picrosides, we carried out the mining of genome sequences which in turn helped us to extract the promoter region of the pathway genes paralogues. These promoter regions were then used to identify different binding sites of TFs.

The numbers of *cis*-regulatory elements varied in the promoter regions of paralogues of the same genes. Six transcription factors binding sites were searched in promoters of the paralogues as detailed in (Table 4.7). The binding motifs of TF 24 was same in all the paralogues of *4CL*, although TF 35 binding sites were highest in promoter of *4CL2* (13) as compared to *4CL1* (7) and *4CL3* (3). In case of *DXPS2*, two binding motifs for TF 24, three binding motifs for TF 23 were present, however seven TF 35 binding sites were detected in promoter of *DXPS1* paralogue. Promoter of *PAL1* had comparatively higher binding motifs of candidate TFs in comparison to paralogues, *PAL2* and *PAL3*. In promoters of paralogues of

HMGR, binding sites of TF 10 and TF 24 were uniformly present, in contrast, eleven TF35 were present in *HMGR2*. Also, four and seven binding motifs of TF 14 and TF 35, respectively were predicted in the promoter of *G10H1* paralogue as compared to one and three in *G10H2* paralogue. In case of *IS*, three binding motifs of TF 35 were predicted in promoter regions of *IS1* and *IS2* paralogues, which might explain their differential expression as compared to *IS3*.

Table 4.7 Distribution frequency of the transcription factor binding motifs present in promoters of pathway gene paralogues of *Picrorhiza kurroa*

	HMGR			PAL			IS				DXPS		4CL			G10H	
Transcription factor	HMGR 1019	HMGR 3054	HMGR 2216	PAL 2040	PAL 4114	PAL 3124	IS 9637	IS 11679	IS 12400	IS 13192	DXPS 2543	DXPS 4421	4CL 4225	4CL 9400	4CL 33467	G10H 94082	G10H 11577 1
WRKY 12	1	2	3	4	2	1	1	2	1	1	1	3	3	2	3	3	3
MYC 2	2	2	1	2	2	2	2	2	1	-	2	1	4	2	2	1	2
MYB 46	3	1	4	4	1	4	-	1	1	1	2	2	3	1	1	2	4
WRKY 71	3	11	5	8	3	2	7	13	-	-	7	3	7	13	3	3	7
NAC 25	2	2	3	2	2	2	3	3	-	1	-	2	2	2	2	1	4
ERF18	2	1	1	1	0	1	1	1	1	1	-	1	3	2	1	2	1

4.6 Expression analysis of transcription factors and pathway gene paralogues in different *Picrorhiza kurroa* accessions

The expression analysis of different TFs and pathway gene paralogues was performed through qRT-PCR in shoots and roots of accessions with contrasting variations for contents of Picroside-I and Picroside-II. Shortlisted 6 TFs and 17 gene paralogues of six pathway genes were selected for the analysis. Paralogue *DXPS2* showed lower expression in accessions PKS 78 (5.46 % fresh weight Picroside-I) and similar expression in all other accessions whereas *DXPS1* showed no expression. Paralogues, *HMGR1* and *HMGR3* showed higher fold change in PKS 56 (3.41 % fresh weight Picroside-I) and lower expression in PKS 78 (5.46 % fresh weight Picroside-I) which is a high Picroside-I containing accessions. In contrast, paralogue *HMGR4* showed analogous expression in all the accessions. Out of 4 paralogues of *IS, IS2* showed lower expression in low Picroside-I containing accessions, PKS 42 (0.23 % fresh weight Picroside-I) and PKS 08 (0.25 % fresh weight Picroside-I). In case of *PAL* paralogues, *PAL3* showed less expression in low Picroside-I containing shoots of PKS 42 (0.23 % fresh

weight Picroside-I), PKS 08 (0.25 % fresh weight Picroside-I) and PKS 64 (0.74 % fresh weight Picroside-I). Expression status of TF 04, TF 14, TF 23, and TF 24 was similar throughout shoots of different accessions. TF 10 and TF 35 showed higher expression in PKS 78 (5.46 % fresh weight Picroside-I) and PKS 54 (7.31 % fresh weight Picroside-I) as shown in Figure 4.7.

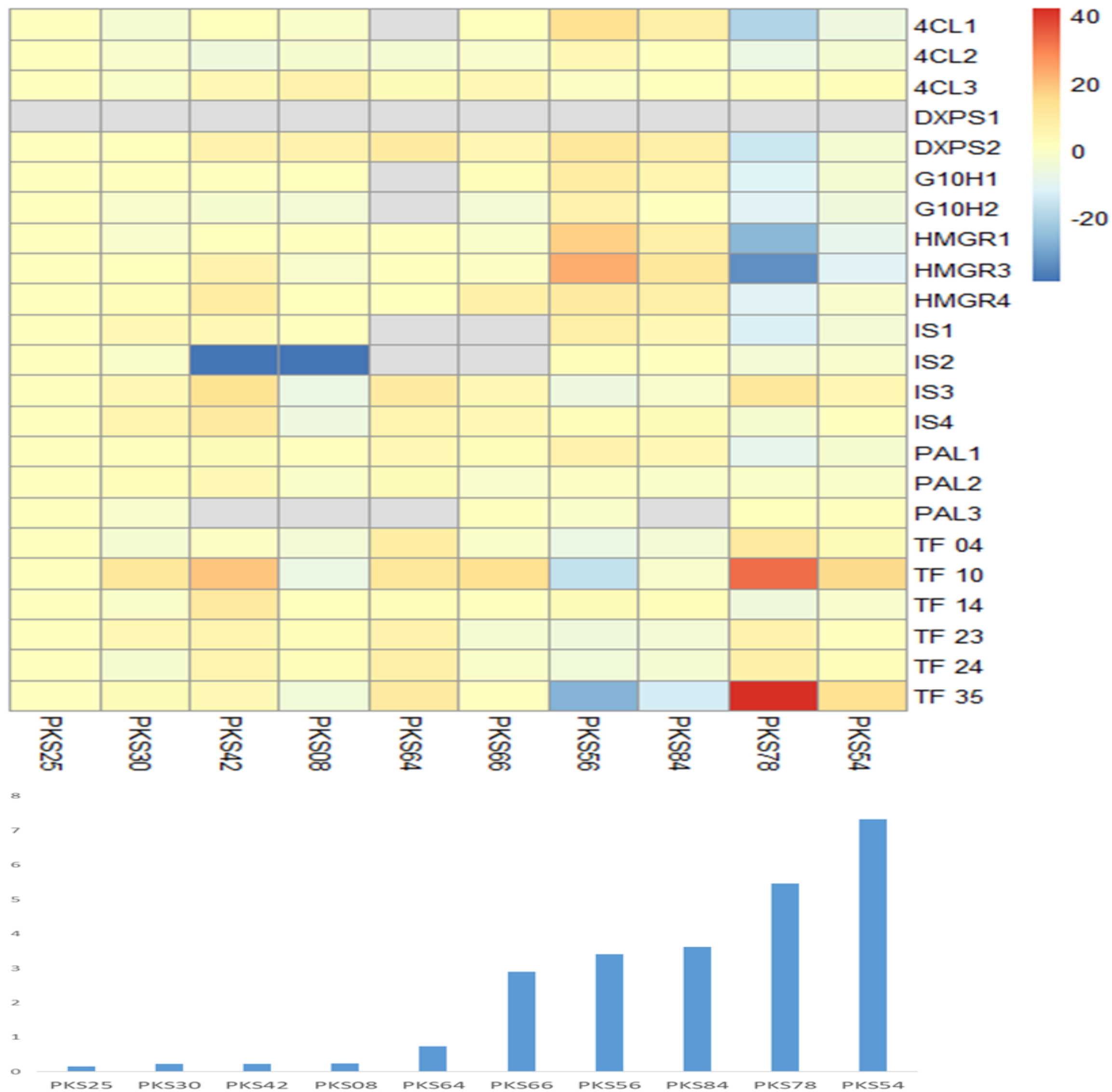


Figure 4.7: Expression profiles of transcription factors and pathway gene paralogues with the least (in blue) and most (in red) in shoots of *Picrorhiza kurroa*

In roots, *PAL* paralogues, *PAL2* showed less expression in high Picroside-II containing roots in contrast to *PAL3* which showed no expression in all the accessions. In *HMGR* paralogues, *HMGR4* showed negative expression throughout the accessions whereas *HMGR3* showed comparatively higher expression in all the accessions. *DXPS2* showed lower expression in high

Picroside-II containing roots of PKR 71 (0.84 % fresh weight Picroside-II), PKR 74 (1.69 % fresh weight Picroside-II) and PKR 84 (2.68 % fresh weight Picroside-II). Paralogues of *G10H, G10H1* showed lower expression in PKR 31 (0.03 % fresh weight Picroside-II), and PKR 28 (0.04 % fresh weight Picroside-II), whereas *G10H2* showed no expression in high Picroside-II containing accessions but showed low expression in PKR 31 (0.03 % fresh weight Picroside-II). Out of 3 paralogues of *4CL, 4CL3* showed negligible expression in all Picroside-II containing roots, whereas *4CL1* and *4CL2* showed low expression in PKR 31 (0.03 % fresh weight Picroside-II), PKR 81 (0.53 % fresh weight Picroside-II) and PKR 69 (0.54 % fresh weight Picroside-II). In case of TFs, TF 35 showed higher expression in PKR 81 (0.53 % fresh weight Picroside-II) and PKR 84 (2.68 % fresh weight Picroside-II) which are high Picroside-II containing accessions. TF 14 also showed higher expression in high Picroside-II accession; PKR 71 (0.84 % fresh weight Picroside-II) and PKR 74 (1.69 % fresh weight Picroside-II). In contrast, TF 24 showed lower expression in high Picroside-II accessions (Figure 4.8).

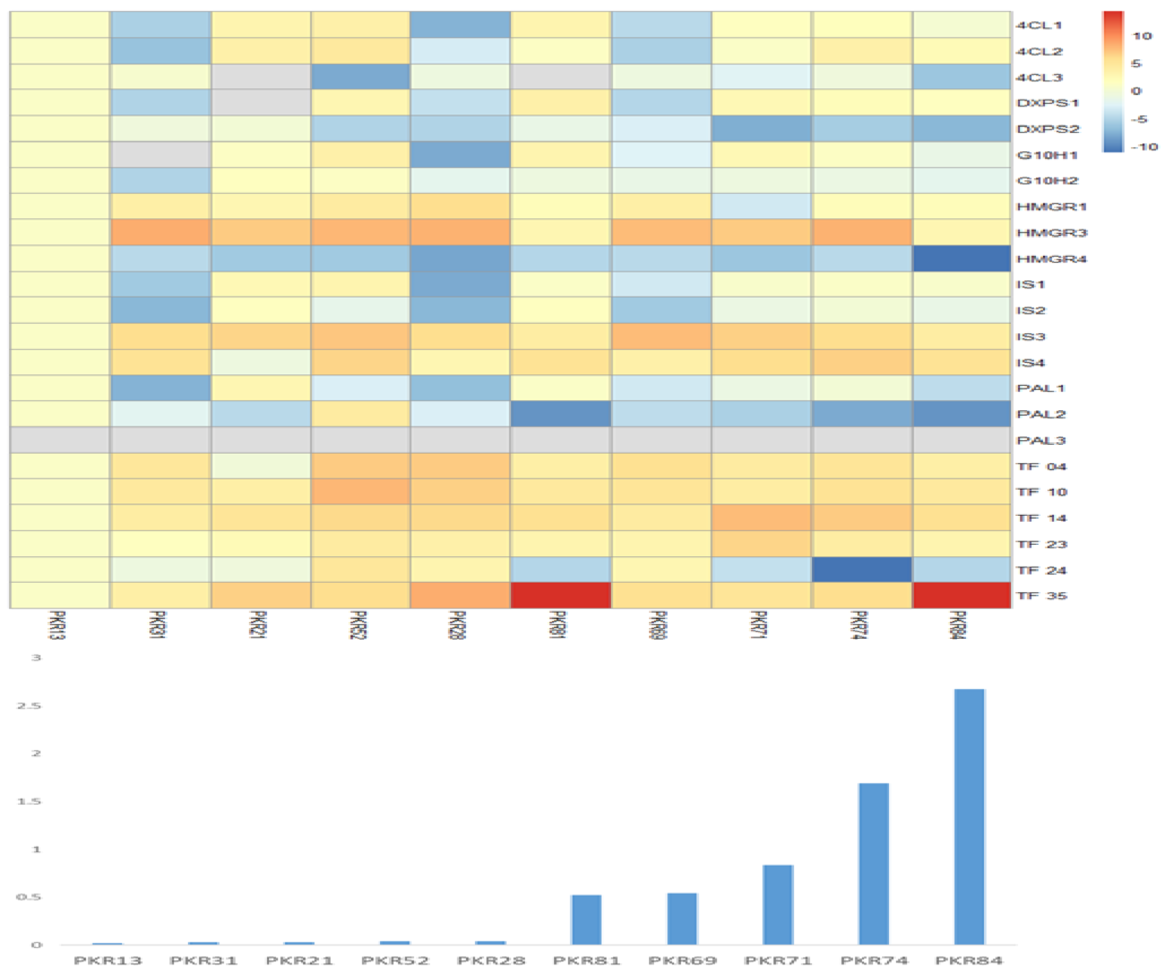


Figure 4.8: Expression profiles of transcription factors and gene paralogues with the least (in blue) and most (in red) in roots of *Picrorhiza kurroa*

4.7 Correlation network analysis of candidate gene paralogues with transcription factors

Co-expression network based on pairwise correlation (co-expression) was generated to understand relationship between pathway gene paralogues and selected transcription factors associated with Picroside-I and Picroside-II contents in different tissues of accessions. A gene regulatory network was used to visualize the flow of gene association within the network (i.e., how one gene is interacting with other network genes within and between the pathways).

In *Picrorhiza kurroa* shoots, based on degree of connectivity, TF 35 had highest degree with 10 interactions showing positive correlation with *DXPS2*, *IS3*, TF 23, TF 24 and TF 4 whereas negative correlation with *PAL1*, *IS1*, *IS2*, *G10H1* and *G10H2*. TF 24 showed positive interaction with *IS3*, TF 4, TF 23, TF 35 but negative interaction with *G10H1*, *G10H2*, *IS1* and *IS2*. Comparably, *DXPS2* showed positive interaction with *4CL3*, *HMGR4*, *IS3*, *IS4*, TF 10, and TF 35 whereas negative interaction with *PAL3*. Nevertheless, Picroside-I showed positive association with paralogues, *DXPS2*, and *HMGR4* whereas, negative association with *G10H1*, *HMGR1*, *HMGR3*, *PAL1*, and *PAL2* paralogues. On the contrary, TF 35, TF 24 and TF 23 showed negative interaction with both paralogues, *G10H1* and *G10H2*. *HMGR3* and *PAL2* co-expressed together to regulate Picroside-I. Interestingly possibly negatively, *4CL2*, *HMGR3*, *PAL2, IS1* and *IS4* had least degree of interactions with other genes, and *PAL2* and *HMGR3* were negatively correlated with Picroside-I in shoot network implying their possible downregulation might enhance the Picroside-I content in shoots. Picroside-I biosynthesis was positively associated with *DXPS2* and *HMGR4*. TF 35 and TF 10 both interacted positively with *DXPS2*, while TF 10 positively interacted with *HMGR4* (Figure 4.9 a-b).

In root network, *PAL2* had highest degree of interaction with other genes. *DXPS2, HMGR4*, and TF 24 correlated negatively with *PAL2* whereas, *IS2*, TF 14 and TF 35 correlated positively. Similar pattern was observed in case of TFs where, TF 24 showed highest interaction with 7 degrees; positively with *DXPS2* and *PAL2* and negatively with *4CL1, 4CL2, IS2* and TF 14. Least degree of interaction in the network was with *G10H1, IS3* and *PAL1*. Picroside-II was possibly downregulated by *PAL2*, TF 14 and TF 24 and upregulated by TF35. Small amount of Picroside-I is also found in root tissues of *Picrorhiza kurroa* which was possibly due to negative regulation by *4CL2, G10H2, HMGR1, IS1* and *IS2* whereas positively regulation by TF4 and TF 23. In contrast, *DXPS1* is possibly upregulated by TF 4 which further upregulates Picroside-I in roots. This analysis deciphers regulatory networks interplaying

between the candidate TFs and the gene paralogues which tend to exhibit similar expression patterns (Figure 4.9 c-d).

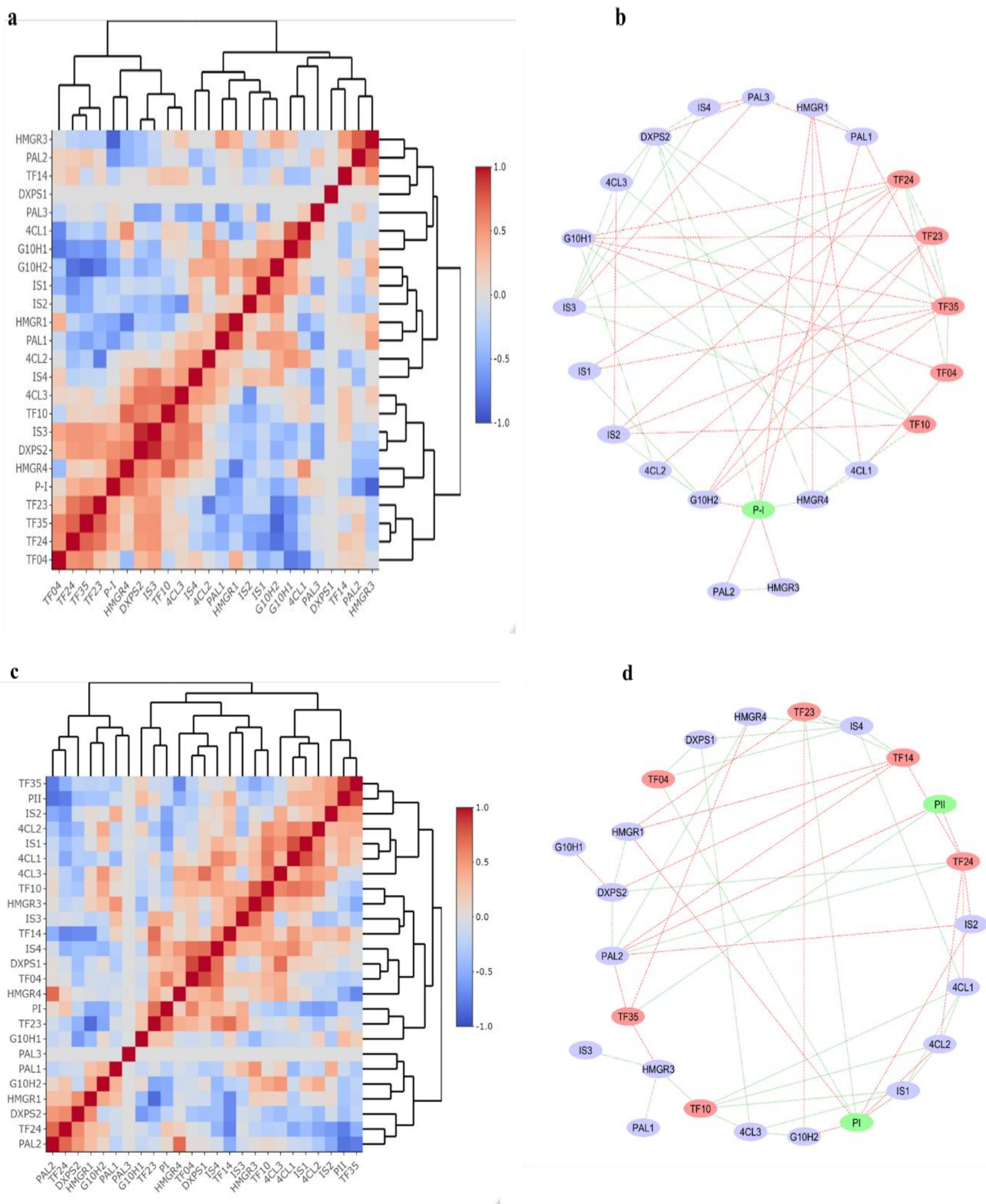


Figure 4.9: Correlation analysis of transcription factors (TFs) and pathway gene paralogues of (a) shoots and (c) roots of *Picrorhiza kurroa.* Correlation network representing interaction of transcription factors (pink), gene paralogues (purple) and picroside(s) (green) in (b) shoots and (d) roots

4.8 Transcript abundance of ABC transporters possibly associated with the transport of Picroside-I and Picroside-II in *Picrorhiza kurroa*

ABC transporters were mined from PKSS, PKSR and PKSTS transcriptome datasets of *Picrorhiza kurroa*. In total 99 transcripts encoding ABC transporters were identified that witnessed tissues/organ-specific expression, out of which 9 were ubiquitously expressed in all three tissues, 39 were expressed in PKSS, 23 in PKSR and 37 in PKSTS. Distribution of ABC transporters was computed to be 10% in PKSTS as compared to PKSS and PKSR with 9% and 6%, respectively (Figure 4.10).

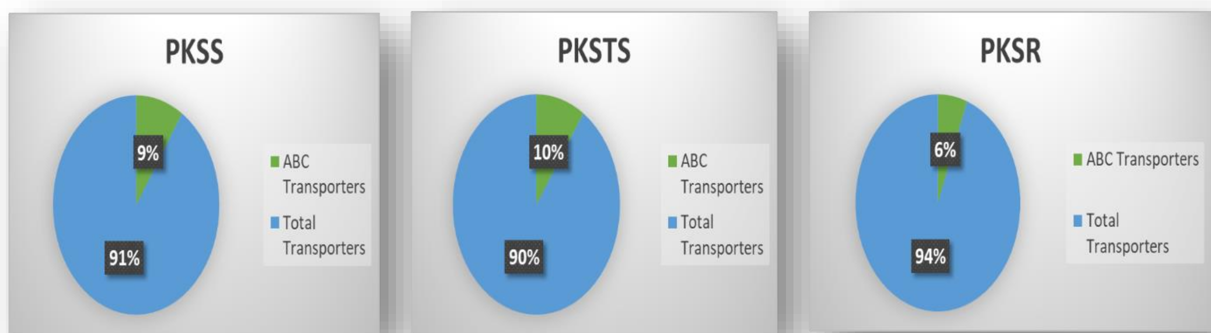


Figure 4.10: Proportion of ABC transporters among PKSS, PKSR and PKST transcriptomes of *Picrorhiza kurroa*

The ABC transporters were differentially distributed in shoots, roots, and stolons of *Picrorhiza kurroa.* In shoot, a total of 14 ABC B family members were observed in contrast to 6 for ABC F family. Similarly, in stolons, only three ABC F family members were discovered, compared to 15 ABC B family (Figure 4.11).

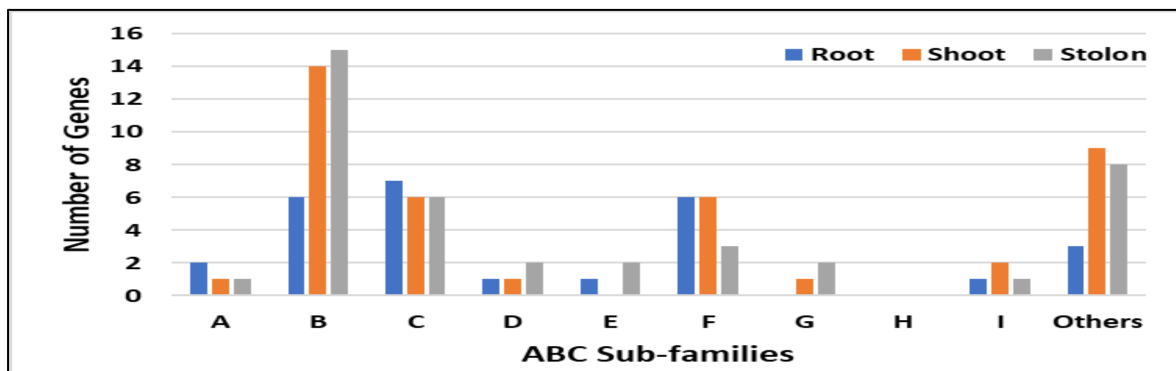


Figure 4.11: Distribution of ABC transporters among different subfamilies in transcriptomes of *Picrorhiza kurroa*

Abundance of common transcripts encoding ABC transporters was calculated in the form of TPM values. Transcripts having high TPM value were selected for comparative analysis for the identification of candidate transporters associated with Picroside-I and Picroside-II contents. TPM values were determined for 99 ABC transporters using Salmon tool. Transcript abundance for commonly present ABC transporters ranged from 0.16 to 44.20 in PKSS, 0.44 to 25.43 in PKSR and 0.68 to 60.18 in PKSTS datasets. Transcripts pknode_807 showed highest expression in PKSS (44.20 TPM value), pknode_74 in PKSR (25.43 TPM value) and pknode_738 in PKSTS (60.18 TPM value). The ABC transporter transcripts with higher transcript abundance were further shortlisted for validation through qRT-PCR to assess their association with Picroside-I and Picroside-II accumulation.

4.9 Identification of common and unique ABC transporters

In this study, a detailed comparative analysis of ABC transporters in the transcriptomes of three tissues of *Picrorhiza kurroa*, i.e., PKSS, PKSR and PKSTS was carried out to identify candidate ABC transporters possibly involved in transport of Picroside-I and Picroside-II. 33 ABC transporters were found to be commonly present in PKSS and PKSTS transcriptomes while 6 and 7 transporters were uniquely present in PKSS and PKSTS, respectively. Similarly, 16 ABC transporters genes were commonly present in PKSR and PKSTS transcriptomes while 8 and 21 transporters genes were uniquely present in PKSS and PKSTS, respectively. The unique ABC transporters identified in shoots transcriptomes of *Picrorhiza kurroa* were considered possibly associated with the transport of Picroside-I from shoots to stolons/rhizomes. Similarly, unique ABC transporters genes in roots transcriptome may be responsible for the transport of Picroside-II from roots to stolons/rhizomes. The common transcripts among tissues might suggest their involvement in the transport of some common primary or secondary metabolites.

4.10 Expression analysis of candidate ABC transporter genes among *Picrorhiza kurroa* accessions varying for picrosides contents

The relative expression of 15 shortlisted ABC transporter genes was analysed using qRT-PCR in shoots, roots, and stolons tissues of *Picrorhiza kurroa* varying for Picroside-I and Picroside-II contents. *Picrorhiza kurroa* accessions PKS 54 (7.31 % fresh weight Picroside-I) and PKS 78 (5.46 % fresh weight Picroside-I) showed higher accumulation of Picroside-I whereas PKS 08 (0.25 % fresh weight Picroside-I) and PKS 25 (0.15 % fresh weight Picroside-I) showed less accumulation of Picroside-I content in shoots. For Picroside-II, accessions PKR 84 (2.68 % fresh

weight Picroside-II) and PKR 82 (2.37 % fresh weight Picroside-II) showed higher accumulation of Picroside-II whereas PKR 57 (0.18 % fresh weight Picroside-II) and PKR 08 (0.07 % fresh weight Picroside-II) showed lesser amount. Similarly, in stolons, PKST 67 (7.36 % fresh weight Picroside-I+Picroside-II) and PKST47 (7.15 % fresh weight Picroside-I+Picroside-II) showed higher accumulation of both the compounds (Picroside-I + Picroside-II) whereas PKST 43 (0.12 % fresh weight Picroside-I+Picroside-II) and PKST03 (0.10 % fresh weight Picroside-I+Picroside-II) accumulated in less amount for both the compounds. Overall, these accessions provided contrasting content phenotypes of Picroside-I and Picroside-II, therefore, were used to pinpoint association of 15 transporters responsible in transport of Picrosides.

According to gene expression patterns, 4 transcripts; *PkABCA1, PkABCG1, PkABCB5* and *PkABCB4* were highly expressed in high Picroside-I content accessions (PKS 54 and PKS 78) with relative expression ranging from 1.11 to 8.98 in comparison to shoots of low-content accessions, PKS 08 and PKS 25 (Figure 4.12 a). Similarly, for high Picroside-II content in roots (PKSR 84 and PKSR 82), 3 genes; *PkABCB2, PkABCF3* and *PkABCC2* exhibited higher relative expression ranging from 4.21 to 10.98 (Figure 4.12 b). In stolon tissues of PKST 64 and PKST 47 having high Picroside-I and Picroside-II content, 3 genes; *PkABCB3, PkABCC1* and *PkABCF1* exhibited higher relative expression ranging from 2.32 to 5.34 in comparison to accession having low Picroside-I and Picroside-II in stolons (PKST 43 and PKST 03) (Figure 4.12 c). By contrast, 2 genes; *PkABCF5* and *PkABCB6* showed low expression levels in high Picroside-I content shoots (PKS 54 and PKS 78). For Picroside-II in roots, *PkABCB1* and *PkABCF2* exhibited low expression level PKR 84 and PKR 82. In stolon tissues, *PkABCE1* exhibited low relative expression in PKST 67 and PKST 47.

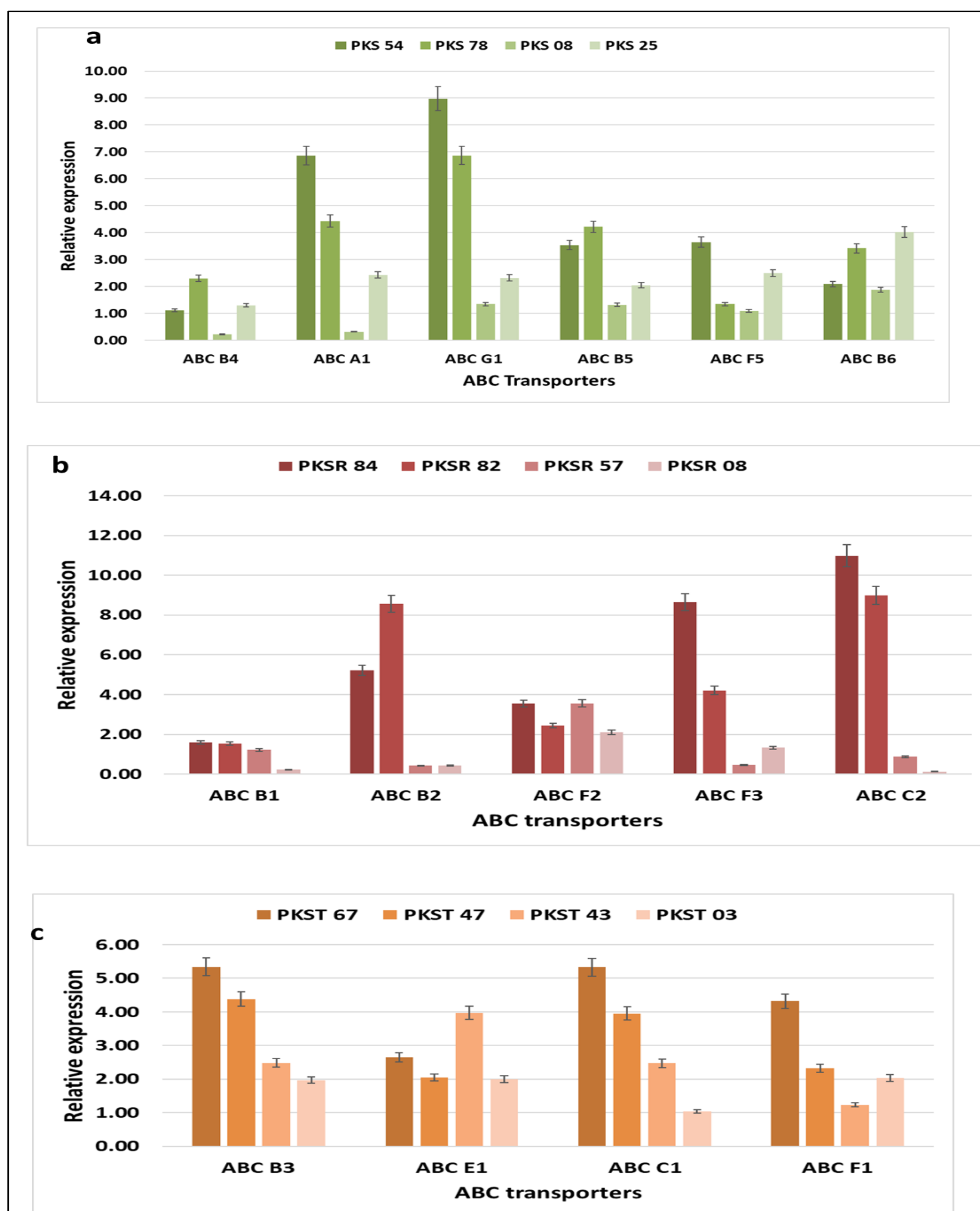


Figure 4.12: Relative expression of ABC transporters in *Picrorhiza kurroa* accessions **(a)** PKS 54 and PKS 78 vs PKS 08 and PKS 25 varying for Picroside-I content (PKS 54- 7.3%, PKS 78- 5.46%, PKS 08-0.25% and PKS 25-0.15%) in shoots **(b)** PKR 84 and PKR 82 vs PKR 08 and PKR 57 varying for Picroside-II content (PKR 84- 2.68%, PKR 82- 2.37%, PKR 08-0.07% and PKR57-0.18%) in roots **(c)** PKST 67 and PKST 47 vs PKST 43 and PKST 03

varying for Picroside-I+ Picroside-II contents (PKST 67- 7.36%, PKST 43- 0.12%, PKST 43- 0.07% and PKST 03-0.10%) in stolons

Furthermore, expression level of 15 genes was checked in all tissues of *Picrorhiza kurroa.* Accession PK 82 exhibiting relatively high content in all the 3 tissues (Picroside-I:2.26%; Picroside-II: 2.37% and Picroside-I and Picroside-II: 3.36% in shoots, roots, and stolons, respectively). The 6 genes; *PkABCB4, PkABCA1, PkABCG1, PkABCB5, PkABCF5* and *PkABCB6* expressed high in the shoots while *PkABCB2, PkABCF3* and *PkABCC2* expressed in roots of PK 82. Moreover, 5 genes, *PkABCB3, PkABCE1, PkABCF2, PkABCC1* and *PkABCF1* showed high expression in stolons rather than in other tissues of PK 82 (Fig 4.13).

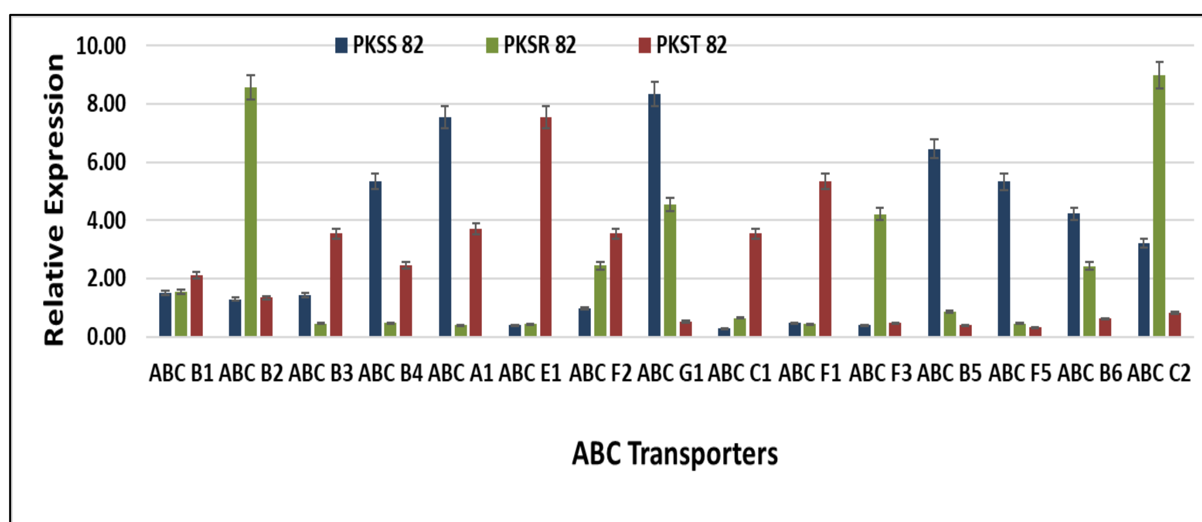


Figure 4.13: Relative expression of ABC transporters in shoots (PKSS), stolons (PKSTS), and roots (PKSR) of *Picrorhiza kurroa*

4.11 *In silico* domain/motif analysis and sub-cellular localization of candidate ABC transporters

The predicted proteins from genome sequence contigs corresponding to shortlisted ABC transporters-encoding transcripts were tested for presence of signature domains and motifs. The proteins shared significant similarity with transmembrane domain and nucleotide binding domain of ABC transporters in Pfam analysis. Also, the functionally characterised ABC transporters; *VvABCC1* in *Vitis vinifera*, *NtPDR1* in *Nicotiana tabacum* and *CjABCB2* in *Coptis japonica* shared domain/motif similarity with the shortlisted ABC transporter transcripts. Additionally, *in silico* analysis for the sub-cellular localisation of the putative ABC transporter

proteins using Cell Ploc2 suggested that these proteins are membrane localised as detailed in Table 4.8.

Table 4.8: *In silico* sub-cellular location of candidate ABC transporter proteins using Cell Ploc2

Transporter	Sub-cellular location using CELL PLOC 2.0
ABCB2	Cell membrane
ABCB5	Cell membrane
ABCC1	Chloroplast
ABCG1	Cell membrane, chloroplast
ABCB3	Cell membrane
ABCB4	Cell membrane

4.12 Co-expression network analysis to capture other components of Picrosides biosynthetic machinery

4.12.1 Sub-network module specific to Picroside-I in shoots

In shoots (PKSS), network module had 107 nodes and 213 edges. Among all the identified ABC transporters, *ABCB* and *ABCC* genes were characterized as major hubs with degree of freedom 50 and 26, respectively showing strong interconnections with co-expressed genes in the network (Figure 4.14). ABCB and ABCC are the main hubs connected with kinases, transcription factors; *MYB4* and *WRKY71* showing strong influence with the transporters. Some important metabolic enzymes such as NADH dehydrogenase, Glutathione-S-transferase, serine threonine-protein phosphatase, fructose-bisphosphate aldolase, 3-phosphoshikimate 1-carboxyvinyltransferase and Ketol-acid reductor-isomerase showed co-expressed interaction with ABCB family of transporters. Also, ABCB subfamily members were interlinked with other members suggesting that these transporters play an important role in transport of metabolites. *STK* has a role in maintaining the metabolic balance between different terpenoids [212]. Moreover, ABCG transporter gene co-expression network might provide additional clue to Picroside-I accumulation and transport via gene-gene co-expressed interactions.

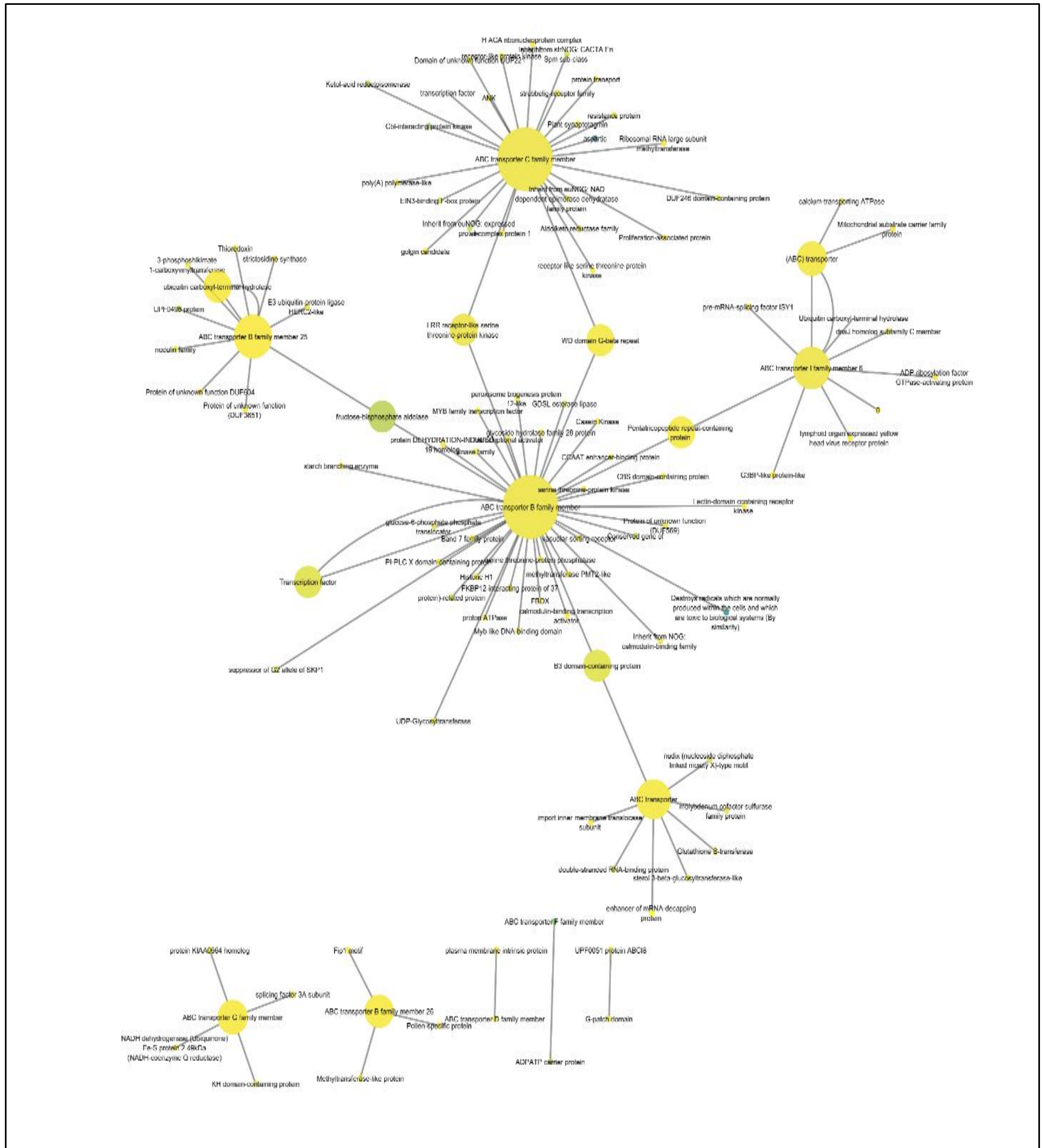


Figure 4.14: ABC family sub-network module constructed from *Picrorhiza kurroa* shoot-derived transcriptome (PKSS)

4.12.2 Sub-network module specific to Picroside-II in roots

In roots (PKSR), network module had 75 nodes and 141 edges. Among all identified ABC transporters, ABCB and ABCC genes were characterized as major hubs with degree of freedom 26 and 25, respectively showings co-expression with genes according to the network analysis

(Figure 4.15). The ABC hub was found to be associated with kinases, transcription factors, and key metabolic enzymes such as hexokinase, glucose-6-phosphate 1-dehydrogenase and an important biosynthetic enzyme, i.e., serine acetyltransferase which catalyses the last step of Picroside biosynthesis [215]. This enzyme was co-expressed with ABCB suggesting its role in Picroside-II biosynthesis in roots of *Picrorhiza kurroa*. ABCC family members had co-expressed linkage with Zn fingers which regulate secondary metabolites biosynthesis in plants [24] that might influence Picroside-II biosynthesis by regulating the enzymes of their biosynthetic pathway.

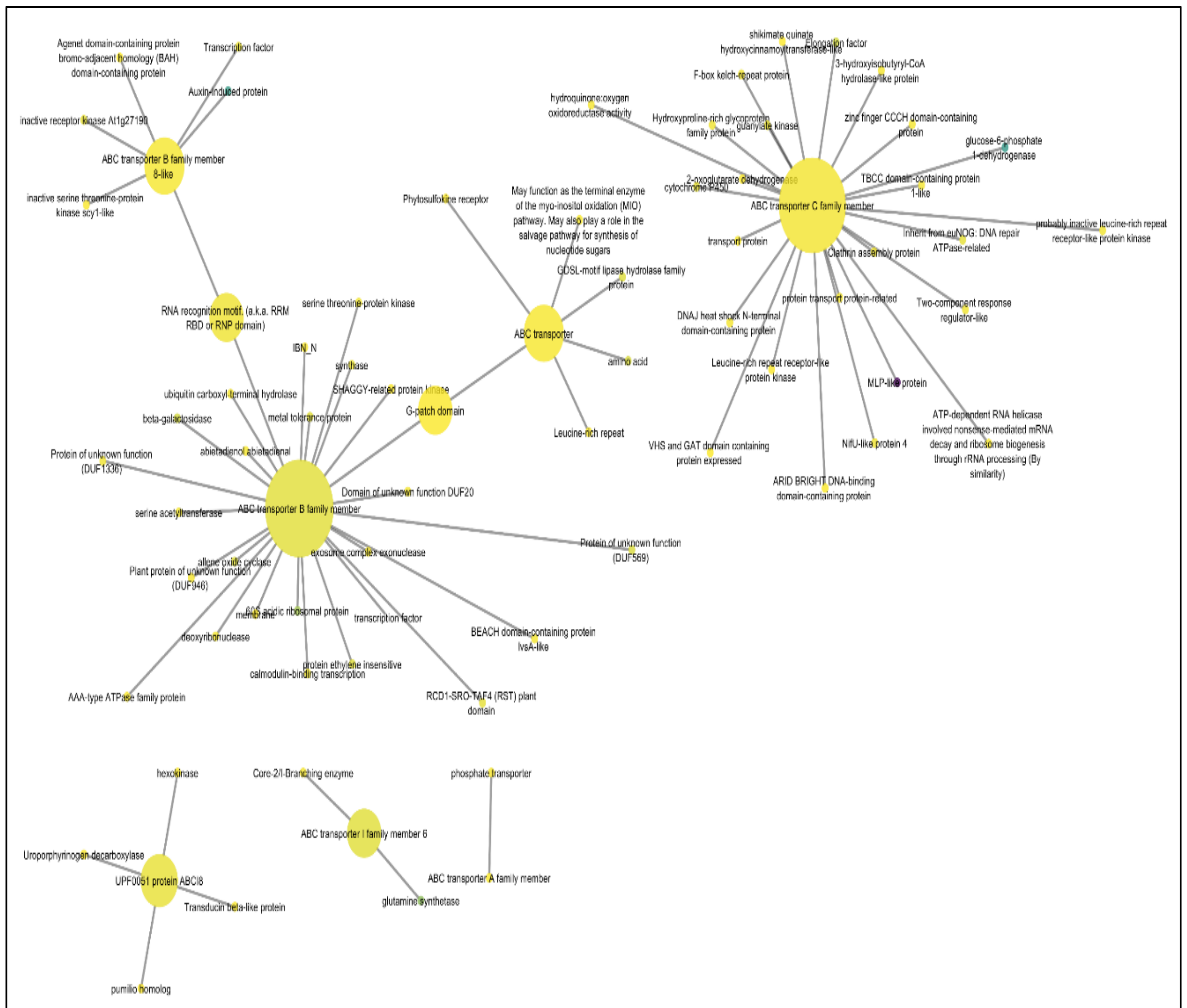


Figure 4.15: ABC family sub-network module constructed from in *Picrorhiza kurroa* root-derived transcriptome (PKSR)

4.12.3Sub-network module specific to combined Picroside-I and Picroside-II in stolons

In stolons (PKST), network module had 94 nodes and 190 edges. Among all the identified ABC genes, ABCB and ABCC genes were characterized as hub genes with degree of freedom 54 and 23 showing strong interconnections with co-expressed genes according to the co-expressed network analysis (Figure 4.16). ABCB, ABCG and ABCC were in the centre or main hub connected with serine threonine-protein kinases, sulphate transporter, magnesium transporter and some ion channels for transport of metabolites. Also, certain metabolic enzymes such as Arogenate dehydratase prephenate dehydratase, aminotransferase, E3 SUMO-protein ligase, cellulose synthase, acyl-coenzyme A oxidase and *4-coumarate-CoA ligase* that catalyses 4-coumarate to 4-coumaroyl-CoA showed connections in this network module.

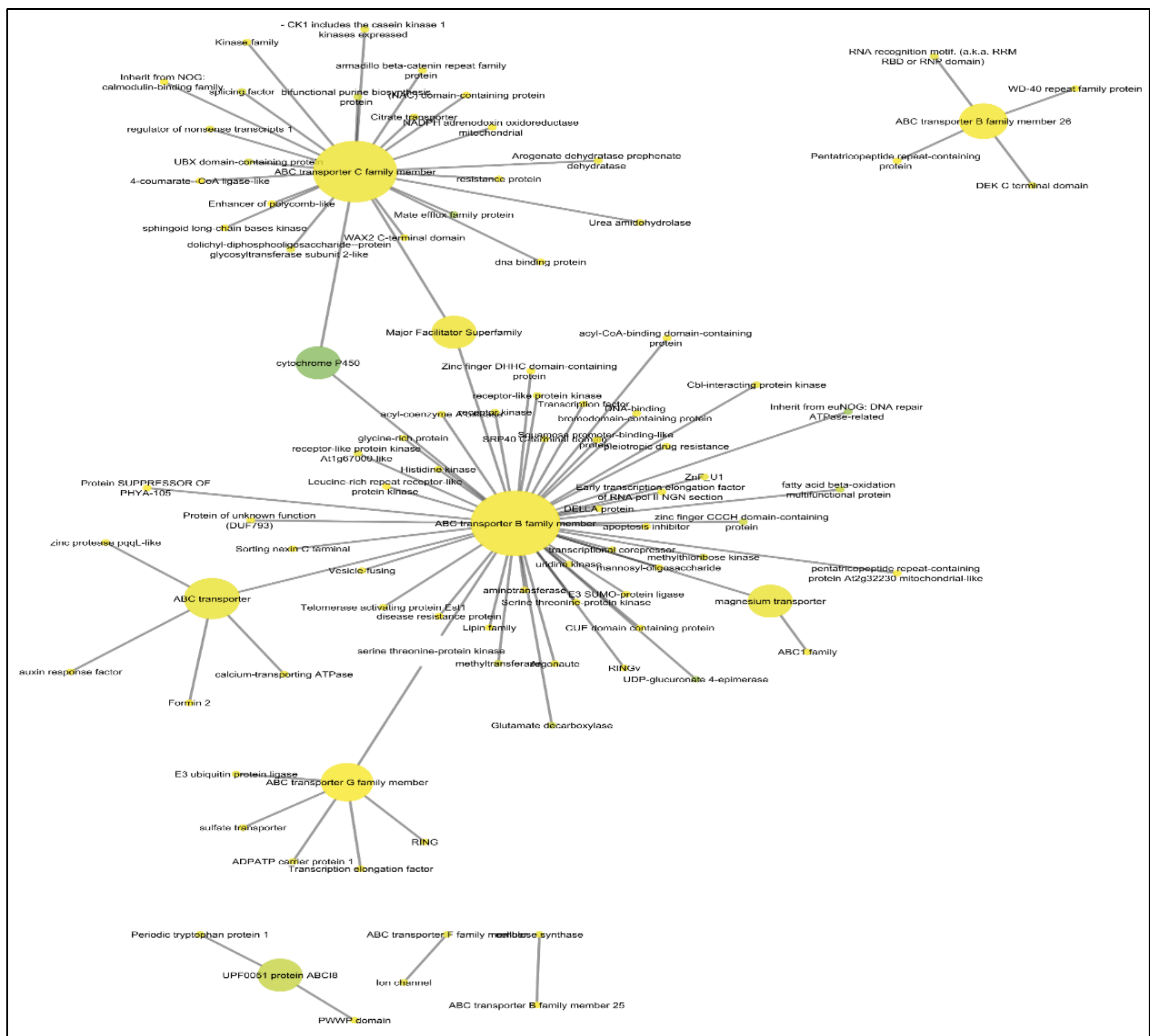


Figure 4.16: ABC family sub-network module constructed from in *Picrorhiza kurroa* stolon-derived transcriptome (PKST)

CHAPTER 5
DISCUSSION

Picrorhiza kurroa is a significant source of a wide range of therapeutic properties which make it extremely valuable to the herbal drug industries. Picroside-I and Picroside-II are the marker compounds in *Picrorhiza kurroa* which have been the subject of extensive pharmacological and biochemical research due to their medicinal properties mainly hepatoprotective. Several *in vitro* methods have been developed to meet the growing demand since plant material is the only source of these significant metabolites, but yield is still very low when compared to natural habitat. Constrained knowledge regarding Picrosides biosynthesis and regulation is a significant barrier to develop methods for increasing its production. Despite the fact that recent research has expanded our understanding of the Picrosides biosynthetic pathways, the regulatory control of their biosynthesis and transport is yet to be investigated. The discovery of these pathways' regulatory mechanisms and their interactions with larger metabolic networks may provide several opportunities to achieve optimal yield through molecular genetic interventions.

To gain insight into the role of molecular machineries in mediating the Picrosides production in *Picrorhiza kurroa*, the present work was designed with the goal of identifying the regulatory factors linked to control Picrosides biosynthesis. First, eighty-five *Picrorhiza kurroa* accessions from various North-Western Himalayan regions were examined for their Picroside-I and Picroside-II contents. The result showed that *Picrorhiza kurroa* accessions varied widely in their Picrosides content. Since multiple pathways interact during Picrosides biosynthesis, regulatory factors such as transcription factors that can regulate different facets of Picrosides biosynthesis were investigated in various tissues of *Picrorhiza kurroa*. Additionally, TFs regulating pathway gene paralogues were studied to better understand their role in Picrosides variation in *Picrorhiza kurroa*. Further, ABC transporters were identified to dissect their role in Picrosides biosynthesis, particularly transport in *Picrorhiza kurroa.* The present research produced some significant findings, which are discussed below:

5.1 Picroside-I and Picroside-II content analysis in *Picrorhiza kurroa*

Picroside-I and Picroside-II are major pharmacologically important metabolites in *Picrorhiza kurroa* as they are used in preparation of numerous herbal drug formulations, therefore, accessions with the desired concentrations of Picroside-I and Picroside-II must be identified. As of today, there are inadequate reports on variation in Picrosides content in limited number of accessions of *Picrorhiza kurroa* from the North-Western Himalayan states [9], [216]. Therefore, Picroside-I and Picroside-II contents in different tissues: shoots, roots and stolons in eighty-five accessions of *Picrorhiza kurroa* from various geographical locations in Uttarakhand and Himachal Pradesh were quantified. Our analysis showed significant variation in Picrosides content in shoots (0.23% to 7.31% Picroside-I), roots (0.02% to 2.68% Picroside-II) and stolons (0.10% to 7.36% Picroside-I and Picroside-II). Additionally, variations in Picroside-I or Picroside-II contents were seen, with some accessions having higher Picroside-I concentrations while others had higher Picroside-II concentrations. To ascertain whether variations in Picroside-I and Picroside-II contents are caused by environmental factors at different sites of collection or due to genetic variation, all accessions were planted at the same location at the Regional Research Station of the Himalayan Forest Research Institute (H.F.R.I), Jagatsukh, H.P., India. This analysis would result in identifying genetically superior accessions for their commercial production. Furthermore, discerning differential Picrosides containing *Picrorhiza kurroa* accessions will aid in deciphering and comprehending the biosynthetic machinery components of Picrosides in *Picrorhiza kurroa*.

5.2 Correlation analysis of pathway gene paralogues and transcription factors vis-à-vis Picrosides content

Transcription factors belonging to families comprising MYB, bHLH, WRKY, NAC, ERF, and bZIP represent as predominant families, which regulate secondary metabolites biosynthesis in plants [82]. Also, it is evident that a specific TF can regulate multiple genes in secondary metabolites pathways in plants [217]. For instance, in *Oryza sativa*, a WRKY TF, *OsWRKY45* has been reported to regulate the biosynthesis of diterpenoid phytoalexins; phytocassane, momilactone, and oryzalexin [218]. Also, in *Hyoscyamus albus,* role of *HaWRKY1* in regulating the expression of *hahpo1* gene for biosynthesis of solavetivone was reported [219]. In *C. roseus*, *BIS3*, a bHLH family member was reported to activate promoter of iridoid pathway genes, *GES, G10H, 8HGO, IS, 7-DLGT* and *7DLH*. Overexpression of *BIS3* in flower petals of *C. roseus* substantially upregulated the expression of iridoid pathway genes and

increased accumulation of loganic acid [86]. In *A. thaliana*, *MYB46/ MYB83* act as master regulators of nine monolignol biosynthetic genes (*PAL*, *C4H*, 4*CL*, *HCT*, *C3'H*, *CCoAOMT*, *F5H*, *CCR*, and *CAD*) involved in lignin biosynthesis [220]. *AaMYC2* was found to directly regulating the expression of *CYP71AV1* and *DBR2* genes, and other terpene synthase genes regulating artemisinin biosynthesis in *A. annua* [155]. In *Taxus* sp, MYC TF has been characterised to play a regulatory role in taxol biosynthesis [221]. In *Picrorhiza kurroa*, two WRKY genes, *Pkd*WRKY and *PksWRKY* were identified in the promoter regions of *Pkhmgr* and *Pkdxs*, the key genes in isoprenoid pathway [222]. Consequently, this study gives a comprehensive insight on identification of putative TFs possibly acting as *master switches* to regulate picrosides biosynthesis through their differential interactions with pathway genes in different *Picrorhiza kurroa* accessions.

The present work enumerates comparative transcriptomic analysis of *Picrorhiza kurroa* leaf and root tissues, further identifying and annotating various transcripts encoding Picroside-I and Picroside-II pathway gene paralogues and associated TFs, along with the underplaying active correlation networks. Moreover, promoter regions of pathway gene paralogues were captured, followed by the identification of candidate *cis*-regulatory elements in their promoter regions. For the expression analysis, 10 differential content accessions varying for Picroside-I content in shoots and similarly, 10 differential content accessions having variable Picroside-II contents in roots were selected to ensure that the differences in contents of major metabolites are primarily due to genetic differences but not due to origin, growth and development of tissue/organ or environmental factors.

Through comparative transcriptome and expression analysis via qRT-PCR, six TFs (*PkWRKY 71, Pk WRKY12, PkMyb 46, PkERF18, PkNAC25* and *PkMyc2*) were shortlisted among which *PkNAC25, PkWRKY 71* and *PkERF18* were targeted as key TFs for picrosides biosynthesis [24]. *In silico* promoter region analysis showed presence of their binding sites in the promoter regions of the pathway gene paralogues. For instance, two binding motifs of TF 24 were present in *DXPS2* promoter which might explain its differential role as compared to *DXPS1.* Also, four binding motifs of TF 14 were predicted in promoter of *G10H1* paralogue as compared to only one in *G10H2* paralogue which might justify the regulatory role of TF 14 in *G10H1* as speculated in correlation network outcome. In case of *IS*, three binding motifs in promoter regions of *IS1* and *IS2* paralogues might explain their differential expression as compared to *IS3.* In accordance with our study, MYB promoter site has been found to be present in *G10H* promoter in *C. roseus* [144]. *TSAR1* and *TSAR2*, bHLH family members were reported to

coregulate and transactivate *HMGR1* for triterpene saponin biosynthesis in *Medicago truncatula* [117]. Recently, a *cis*-regulatory element (TGGTTA) known to bind *BmMYB35* transcription factor belonging to the MYB family member, was found to be present in the *BmG10H-1* promoter in *Bacopa monnieri*, thereby indicating its role in regulating *BmG10H-1* gene expression [178].

The network analysis directed us to infer correlation between 'TF-paralogue partners' and probable TF family possibly regulating various steps in Picroside-I and Picroside-II pathways across accessions. In paralogues of *HMGR*, *HMGR1* was found to negatively correlate with Picroside-I in shoots and Picroside-II in roots conversely *HMGR4* was positively correlating with Picroside-I in shoots and Picroside-II in roots. *HMGR3* correlated negatively with Picroside-I in shoots whereas no role was found for Picroside-II in roots. Similarly, *DXPS2* correlated negatively with Picroside-II in roots but positively with Picroside-I in shoots. Paralogues of *G10H*, *G10H2* interacted negatively with Picroside-I and Picroside-II in shoots and roots respectively whereas *G10H1* positively with Picroside-II in roots but negatively with Picroside-I in shoots. This was also the case with paralogues of IS, where *IS1* and *IS3* positively correlated with Picroside-II in roots and Picroside-I in shoots, respectively. Our studies also showed that a particular TF can differentially regulate paralogues of a gene. For instance, TF 35 and TF 24 positively correlated with *IS3* paralogue whereas negatively with paralogues, *IS1* and *IS2* in shoots. Similarly in roots, TF 24 showed positive correlation with *PAL1*, conversely negatively with *PAL2*. Conclusively, TF 35 and TF 24 showed causal association with key pathway enzymes *DXPS, IS PAL, HMGR, G10H* and *4CL*.

In several studies, it has been reported that TFs can act as positive or negative regulators of pathway genes. For instance, *CiMYB42* positively regulates a triterpenoid, limonoid biosynthesis by regulating the expression of *CiOSC* by binding to the TTGTTG sequence of its promoter as overexpression of *CiMYB42* resulted in significant accumulation of limonin, whereas the downregulation by RNAi resulted less nomilin accumulation [134]. Transient expression in transgenic tobacco indicated *HbWRKY1* as a negative regulator of *HbSRPP* involved in rubber biosynthesis in *Hevea brasiliensis* [150]. In another study, transient expression of a bHLH TF, *AabHLH1* showed to positively regulate the biosynthesis of artemisinin by regulating genes involved in artemisinin biosynthesis; *ADS*, *CYP71AV1* and *HMGR* in *A. annua* [151]. Similarly, in *C. roseus* hairy roots, overexpression of *G10H* or both *G10H* and *ORCA3* genes increased the quantity of catharanthine [146]. In *Withania somnifera*, the expression levels of secondary metabolism-related genes like *CAS, HMGR, DXS*, and *DXR*

were elevated following transient overexpression of *WsAP2* in plants, while GGPPS was maximum induced after *WsAP2* overexpression, indicating role of *WsAP2* in terpenoid metabolism [152].

This study might help in understanding the differential functioning of secondary metabolites pathway gene paralogues in various plants. For instance, two gene paralogues of *DXPS* occurring in chloroplast have been reported in *Arabidopsis thaliana*; *DXPS1* that plays role in both chlorophyll and terpenoids biosynthetic process and *DXPS3* is solely responsible for terpenoids biosynthesis [174], [175]. *DXPS2* was validated by gene silencing to regulate isoprenoid biosynthesis in *Solanum lycopersicum* whereby *DXPS2* played role in the biosynthesis of β-phellandrene, a monoterpene. Silencing of *DXPS2* by RNAi showed reduced monoterpene levels [175]. In *S. miltiorrhiza,* overexpression of *SmHMGR2* enhanced enzyme activity which subsequently enhanced the production of a protein that produced tanshinones and squalene in hairy roots [176]. In *Arabidopsis thaliana*, differential functions of paralogues were observed; *HMGR1* functions in protein binding whereas *HMGR2* plays role in coenzyme binding [168]. Similarly, *CgHMGR* gene in *Corylus avellana L Gasaway* is reported to play a role in Taxol biosynthesis and its expression was found to be high in roots and low in leaves and stems, according to gene expression analyses [170].

Several studies have reported TFs controlling pathway genes of plant secondary metabolites biosynthesis; however, very limited information is available on how paralogues of pathway genes are differentially regulated by same or different TFs, either or/and in different tissues/ genetic backgrounds, which our study has provided preliminary outcomes. The study has provided significant leads that accessions of a medicinal herb, adapt to varying environments through variations in the biosynthesis of secondary metabolites, possibly through evolution of different genetic machineries. Further corroborations to these outcomes can be authenticated through functional analysis, either by gene silencing or over-expression or demonstrating interactions of promoter sequence elements with identified TFs.

5.3 Experimental validation and expression profiling of ABC transporters

Transporters play an important role in a variety of physiological and biochemical processes in a plant system. ABC transporters are the largest transporter family in plants, that play a major role in the transport of several substrates across membranes of the cell [180]. Numerous ABC proteins have been linked to the transport of secondary metabolites in various plant species. ABC transporters have a broad range of substrate specificity. ABC family is divided into nine

subfamilies ABCA-ABCI, of which ABCH is not present in plants and ABCG is only present in plants and fungi [180]. Further, ABCB/MDR (multi drug resistance), ABCC/MRP (multidrug and resistance protein), and ABCG/PDR (pleiotropic drug resistance) were the most identified ABC family transporters in different plants [185], [223]. There is no report on the discovery of ABC transporter genes and the analysis of their function in the accumulation of secondary metabolites in *Picrorhiza kurroa*. Development of RNAseq technology in the post-genomic era has made it possible to identify candidate transporters in non-model plants based on expression analyses in pertinent tissues [224] and through co-expression analyses with known process related genes (e.g., biosynthetic pathway genes) [225]. Hence, *Picrorhiza kurroa* tissue-specific transcriptome data was mined by shortlisting ABC transporters-encoding transcripts.

Comparative analysis among transcriptomes was done using BLASTn and TPM values were measured to correlate the expression of ABC transporters with the contents of Picrosides. A total of 99 ABC transporters identified in various organs, shoots, roots, and stolons of *Picrorhiza kurroa* were further reduced to six ABC transporter genes by utilizing a combinatorial approach of expressional analysis and co-expression network analysis. In the study, ABCB family members were more abundant; in contrast, no member of ABCH family was identified. Moreover, we shortlisted *PkABCB4, PkABCB5* and *PkABCG1* showing higher expression in shoots whereas *PkABCB2, PkABCF3* and *PkABCC2* showed relatively higher expression in roots suggesting their key role in Picroside-I and Picroside-II transport and accumulation. *PkABCC1, PkABCB3* and *PkABCF1* showed higher expression in stolons suggesting their role in transport and accumulation for both Picrosides, Picroside-I and Picroside-II. Furthermore, the putative ABC transporters showed similarity with the functionally characterised ABC transporters; *VvABCC1* in *Vitis vinifera*, *NtPDR1* in *Nicotiana tabacum* and *CjABCB2* in *Coptis japonica*, which are reported to play role in transport of anthocyanin, diterpene and berberine, respectively [28]. These transporters had TMD and NBD domains which were also identified in our shortlisted ABC transporters. In addition to identification and shortlisting of potential transporters, we also utilized co-expression network analysis and captured components such as serine-threonine kinases, *Myb* and *WRKY* transcription factors, enzymes and other transporters linked to the hubs giving more clarity on the source and sink correlation between organ/tissue in *Picrorhiza kurroa*.

ABC transporters family members perform diverse functions such as providing resistance through exporting various hydrophobic metabolites across the plasma membrane of the cell [226]. Among all ABCs, ABCG subfamily of proteins have lipophilic compounds and terpenoids functioning as their substrates. Also several evidences report that ABCG transporters are localized in the plant plasma membrane [227]. In *N. plumbaginifolia,* a plasma membrane-localized ABCG transporter *NpPDR1* is induced by diterpenoids sclareol and sclareolide [185]. In *S. polyrrhiza*, *SpTUR2*, a PDR5-like ABC transporter was reported to transport a diterpene sclareol [228]. ABCG members are also reported to transport different plant metabolites. For instance, *AtABCG29* has been characterized to transport p-coumaryl alcohol, a monolignol involved in lignin biosynthesis [191] and *AtABCG25* transports abscisic acid (ABA) in *A. thaliana* [229]. Similarly*, CrTPT2* mediates efflux of catharanthine to the surface of leaves in *Catharanthus roseus* [199]. Therefore, identification of *PkABCG1* in shoots might imply its involvement in the transport of Picroside-I from shoots to other parts of the plant.

ABCC subfamily members are mostly located on tonoplast of vacuoles in a cell. In *Crocus sativus*, *CsABCC4* functions as a vacuolar transporter for crocins [188]. *AtABCC2/ AtMRP2*, another ABCC member localised on vacuolar membrane has been reported to play role in xenobiotic detoxification, Cd/Hg/As resistance and chlorophyll catabolite transport [230], [231]. There is possibility that *PkABCC1* might be responsible for short distance vacuolar transport in stolon tissues of *Picrorhiza kurroa.*

ABCB (*MDR*) subfamily members are reported to be present in plasma membranes of cells. In *C. japonica*, *CjABCB1/ CjMDR1* and *CjABCB2* are reported to transport an alkaloid, berberine from roots to rhizomes [196]. Thus, *PkABCB2, PkABCB4* and *PkABCB5* might play a role in transport of Picroside-I and Picroside-II from one tissue to another in *Picrorhiza kurroa.*

5.4 Sub-cellular location prediction of ABC transporters

It is apparent that understanding of both intracellular and intercellular movement of intermediates, precursors, and final plant metabolites is a pre-requisite for effective metabolic engineering. The molecular basis of biosynthesis and inter/ intra-cellular transport of secologanin- an iridoid glycoside and an important precursor to several monoterpenoid indole alkaloids (MIAs) has been studied in *Catharanthus roseus* [232]. This medicinal herb is rich in several monoterpenoid indole alkaloids (MIAs) and the pathway for MIA is localized in

many subcellular compartments of at least four different types of cells. Before being exported to the cytosol, geraniol is synthesised in the plastids of internal phloem-associated parenchyma and transformed to loganic acid, which is then converted to secologanin, an iridoid glycoside in the cytoplasm of the epidermis cells. Secologanin is exported from apoplast to cytosol via *CrNPF2.4, CrNPF2.5* and *CrNPF2.6* transporters localised in the plasma membrane. *Strictosidine synthase*, which catalyses the formation of strictosidine from tryptamine and secologanin, is localised in the lumen of vacuole of epidermal cells in leaves. Strictosidine is then transported from the vacuolar lumen to the cytosol by *CrNPF2.9* acting as potential iridoid glucoside importer [186], [232]. Thus, as an analogy can be speculated between the transport of secologanin and the picrosides as both are iridoid glycosides as well as biosynthesized and transported to different cell types. Our previous studies have reported that Picroside-I [9], [26] is biosynthesized in leaf cells whereas Picroside-II in root cells [8], [20] and both finally accumulate in stolons/rhizomes [8], [21]. Picrosides biosynthesis is a combinative route involving mevalonate (MVA), mevalonate-independent/ methylerythritol phosphate (MEP), shikimate/ phenylpropanoid and iridoid pathway modules [29], [38]. Picrosides belonging to the class of monoterpenoids are synthesized from a 5-carbon precursor, isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP). These two precursors are biosynthesised from MVA and MEP pathways, respectively [74]. Subsequently, IPP and DMAPP form a 10-carbon product geranyl pyrophosphate (GPP), which is a precursor of monoterpenoid [75]. GPP undergoes sequences of oxidation and cyclization to form backbone of picrosides, catalpol. Cinnamic acid and vanillic acid from phenylpropanoid pathway undergo acylation with catalpol (iridoid moiety) to form Picroside-I and Picroside-II [20]. However, which of the shortlisted ABC transporters are involved in inter/ intra-cellular transport of pathway intermediates or even the picrosides remain to be investigated.

5.5 Functional analysis of ABC transporters

It is notoriously difficult to assign a functional role to a specific transporter protein or to identify a transporter with a precise substrate specificity. Significant progress has recently been made in functional characterisation, phenotypic characterisation, and *in silico*-based approaches. Several novel tools for transporter identification have emerged in recent years [233]. These include receptor-based expression cloning of phytohormone transporters in yeast [234], fluorescing metabolite sensor-based expression cloning [235], [236], synthetic biology-based identification of specialized metabolite exporters [237], expression cloning in Xenopus oocytes

[237], [238] and *Arabidopsis* mutant collections for targeted reverse genetics screens [202], [239]. For instance, accumulation of β-caryophyllene in *Artemisia annua* was found to be increased when *AaPDR3* was overexpressed, whereas it was reduced when the transporter was downregulated by RNAi [184]. Similarly, RNAi-mediated silencing of the *MtABCG10* gene resulted in reduced translocation of isoflavone medicarpin precursors *Medicago truncatula* [240]. When infected with *Phytophthora infestans*, the leaves of *Solanum tuberosum* showed *StPDR2* to be involved in the secretion of diterpene sclareol, implying a possible role in biotic stress responses [241]. In another study, *C. japonica CjABCB1* expression studies in the Xenopus oocyte system showed that the activity of the transporter was significantly inhibited when exposed to certain chemical agents [183]. *PDR2* is considered to play a significant role in controlling Petuniasterone levels in *Petunia* leaves and trichomes by analysing trichome contents from *PDR2* plants (knockdown by RNAi) [242]. *ABCG25* was characterised using Sf9 insect cells as the expression host. The import of radiolabelled ABA in inside-out membrane vesicles isolated from *AtABCG25*-expressing Sf9 insect cells was used to measure *ABCG25* export activity [229]. Interestingly, *ABCG25* has also been demonstrated to mediate ABA export when expressed in Xenopus oocytes [243].

The identified ABC transporter genes are believed to contribute to accumulation of Picrosides, due to a correlation between transcript abundance, expression analysis and Picrosides content. This study has thus not only identified putative ABC transporter genes that might play role in the transport and accumulation of Picrosides but has also identified other components of biosynthetic machinery such as transcription factors, enzymes, kinases, and other transporter genes associated with these metabolites. The ABC transporters were further shortlisted and validated by qRT-PCR not only among different tissues varying for Picroside-I and Picroside-II contents but also among *Picrorhiza kurroa* accessions to rule out possibility of differential expression of transporters due to developmental stage of different tissues. Thus, the knowledge of ABC transporters has been further advanced through molecular and functional characterization. This can immensely help in understanding the molecular basis of Picrosides transport and accumulation so that a defined genetic strategy can be implemented to modulate Picrosides content in tissue-specific manner in *Picrorhiza kurroa*.

CONCLUSION AND FUTURE PROSPECTS

The current study was undertaken to investigate the molecular basis of higher picrosides content in *Picrorhiza kurroa* and to use this information for future genetic improvement. Different accessions of *Picrorhiza kurroa* were collected from various geographical locations in North-Western Himalayas to ascertain differential picrosides content. The estimation of Picroside-I and Picroside-II contents in shoots, roots and stolons of *Picrorhiza kurroa* revealed a distinct pattern of biosynthesis and storage of Picroside-I and Picroside-II such that the Picroside-I was detected in the shoots, Picroside-II in the roots with traces of Picroside-I in some accessions and both Picroside -I and Picroside-II in the stolons.

Further, the outcome of the present research work shed light on identification and validation of crucial molecular components (pathway gene paralogues, TFs and ABC transporters) contributing to Picrosides biosynthesis machinery in *Picrorhiza kurroa*. A detailed comparative and expression analysis of TFs and ABC transporters through two different platforms: *in silico* TPM values and qRT-PCR analysis in different tissues of *Picrorhiza kurroa* accessions was presented in this study. Expression patterns of TFs revealed valuable insights possibly underlying the regulation of Picroside-I and Picroside-II biosynthesis. Additionally, the combination of metabolites content data and the corresponding expression analysis data would facilitate to identify the candidate TFs regulating particular gene paralogue vis-à-vis Picroside-I and Picroside-II biosynthesis in *Picrorhiza kurroa.*

Moreover, the work on ABC transporters would lay a basis for understanding the transport of Picrosides by revealing the candidate transporters genes ABCB, ABCC and ABCG family that showed positive correlation with Picrosides content. The identified TFs, pathway gene paralogues and ABC transporters might be key targets for designing a suitable genetic intervention strategy through metabolic engineering to enhance the Picroside-I and Picroside-II content in *Picrorhiza kurroa*. Overall, this work provides a foundational base for future studies aimed at deciphering the regulatory networks of Picrosides biosynthesis in *Picrorhiza kurroa*. Furthermore, functional analysis through overexpression or silencing of the shortlisted genes might provide a platform for enhancing Picroside-I and Picroside-II content in *Picrorhiza kurroa.*

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LIST OF PUBLICATIONS/ AWARDS

Ph.D. Research Publications

- **Roma Pandey,** Ashish Sharma, Hemant Sood, Rajinder Singh Chauhan. 2022. "ABC Transporters Mined Through Comparative Transcriptomics Associate with Organ-Specific Accumulation of Picrosides in a Medicinal Herb, *Picrorhiza kurroa*", **Protoplasma**. DOI :10.1007/s00709-022-01786-7

- **Roma Pandey,** Anjali Kharb, Ashish Sharma, Hemant Sood, Rajinder Singh Chauhan "Pathway gene paralogues and transcription factors differentially associate with contents of Picrosides in tissues and populations of a medicinal herb, *Picrorhiza kurroa*", **Molecular Genetics and Genomics**. (Under review)

International Conference

- **Pandey R**., Chauhan RS., "Identification of key transcription factors associated with Picrosides biosynthesis in *Picrorhiza kurroa*", Poster presented at: International Conference on Emerging Areas in Biosciences and Biomedical Technologies at IIT Indore, Feb 7-9, 2020.

Awards/ Fellowship

- Awarded FITM (Ministry of Ayush) Doctoral Research Fellowship (2021) for the project entitled *"Developing standardized guidelines and procedures for post-harvest processing and value addition in herbal raw material of high value medicinal plants for small scale farmers and industries"* under the supervision of Dr. R.S. Chauhan, (Professor and Head, Department of Biotechnology), Bennett University.

Other Research Publications

- Anjali Kharb, Shilpa Sharma, Ashish Sharma, Neeti Nirwal, **Roma Pandey**, Dipto Bhattacharyya, Rajinder Singh Chauhan. "Capturing Acyltransferase(s) Transforming Final Step in the Biosynthesis of a Major Iridoid Glycoside, (Picroside-II) in a Himalayan Medicinal Herb, *Picrorhiza kurroa.*" **Molecular Biology Reports** (2022).

doi.org/10.1007/s11033-022-07489-9

- Ashish Sharma, Shilpa Sharma, **Roma Pandey**, Hemant Sood, Rajinder Singh Chauhan. "Pathway gene paralogues and transcription factors differentially associate with contents of Picrosides in tissues and populations of a medicinal herb, *Picrorhiza kurroa."*. (In Process)

MOLECULAR CHARACTERIZATION OF PICROSIDES BIOSYNTHETIC MACHINERY COMPONENTS IN POPULATIONS OF A MEDICINAL HERB, *Picrorhiza kurroa Royle ex Benth*

Thesis submitted in partial fulfilment of the requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

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CONCLUSION AND FUTURE PROSPECTS

The current study was undertaken to investigate the molecular basis of higher picrosides content in *Picrorhiza kurroa* and to use this information for future genetic improvement. Different accessions of *Picrorhiza kurroa* were collected from various geographical locations in North-Western Himalayas to ascertain differential picrosides content. The estimation of Picroside-I and Picroside-II contents in shoots, roots and stolons of *Picrorhiza kurroa* revealed a distinct pattern of biosynthesis and storage of Picroside-I and Picroside-II such that the Picroside-I was detected in the shoots, Picroside-II in the roots with traces of Picroside-I in some accessions and both Picroside -I and Picroside-II in the stolons.

Further, the outcome of the present research work shed light on identification and validation of crucial molecular components (pathway gene paralogues, TFs and ABC transporters) contributing to Picrosides biosynthesis machinery in *Picrorhiza kurroa*. A detailed comparative and expression analysis of TFs and ABC transporters through two different platforms: *in silico* TPM values and qRT-PCR analysis in different tissues of *Picrorhiza kurroa* accessions was presented in this study. Expression patterns of TFs revealed valuable insights possibly underlying the regulation of Picroside-I and Picroside-II biosynthesis. Additionally, the combination of metabolites content data and the corresponding expression analysis data would facilitate to identify the candidate TFs regulating particular gene paralogue vis-à-vis Picroside-I and Picroside-II biosynthesis in *Picrorhiza kurroa.*

Moreover, the work on ABC transporters would lay a basis for understanding the transport of Picrosides by revealing the candidate transporters genes ABCB, ABCC and ABCG family that showed positive correlation with Picrosides content. The identified TFs, pathway gene paralogues and ABC transporters might be key targets for designing a suitable genetic intervention strategy through metabolic engineering to enhance the Picroside-I and Picroside-II content in *Picrorhiza kurroa*. Overall, this work provides a foundational base for future studies aimed at deciphering the regulatory networks of Picrosides biosynthesis in *Picrorhiza kurroa*. Furthermore, functional analysis through overexpression or silencing of the shortlisted genes might provide a platform for enhancing Picroside-I and Picroside-II content in *Picrorhiza kurroa.*