UNRAVELING MOLECULAR BIOLOGY OF ACONITES BIOSYNTHESIS IN A HIGH VALUE MEDICINAL HERB *ACONITUM HETEROPHYLLUM* WALL

A THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENTS FOR

THE DEGREE OF DOCTOR OF PHILOSOPHY

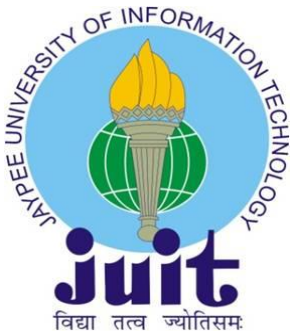
IN

BIOTECHNOLOGY

BY

NIKHIL MALHOTRA

Enrollment No. 106565



JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY

WAKNAGHAT

FEBRUARY, 2017

Dedicated to the scientific community

And

In fond memory of my grandmother

DECLARATION

I certify that:

a. The work contained in this thesis is original and has been done by me under the guidance of my supervisor.
b. The work has not been submitted to any other organisation for any degree or diploma.
c. Wherever, I have used materials (data, analysis, figures or text), I have given due credit by citing them in the text of the thesis.

Nikhil Malhotra

Enrollment No. 106565

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, India

Date: 18/2/2017

CERTIFICATE

This is to certify that the thesis entitled, **"Unraveling Molecular Biology of Aconites Biosynthesis in a High Value Medicinal Herb *Aconitum heterophyllum* Wall"** which is being submitted by **Nikhil Malhotra (Enrollment No. 106565)** in fulfillment for the award of degree of **Doctor of Philosophy** in **Biotechnology** at **Jaypee University of Information Technology, Waknaghat, India** is the record of candidate's own work carried out by him under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

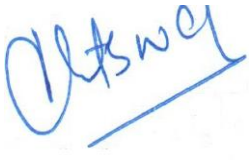
Prof. (DR.) Rajinder Singh Chauhan (Supervisor) **Date:** 18/2/2017

Dean (Biotechnology)

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, India



DR. Hemant Sood (Co-supervisor)

Assistant Professor (Senior Grade)

Department of Biotechnology & Bioinformatics

Jaypee University of Information Technology

Waknaghat, India

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LIST OF ABBREVIATIONS

ABC	ATP binding cassette
ACTH	acetoacetyl-CoA thiolase
AGPase	ADP-glucose pyrophosphorylase
AHC	Agglomerative hierarchical clustering
ARF2	Auxin responsive factor 2
DMAPP	Dimethylallyl pyrophosphate
DXPR	1-deoxy-D-xylulose 5-phosphate reductoisomerase
DXPS	1-deoxy-D-xylulose 5-phosphate synthase
FPKM	Fragments per kilobase of transcript per million mapped fragment
GDPS	Gerenyldiphosphate synthase
GMPase	GDP-mannose pyrophosphorylase
HDS	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase
HMGR	3-hydroxy-3-methylglutaryl-CoA reductase
HMGS	3-hydroxy-3-methylglutaryl-CoA synthase
HOG1	HOG1 protein kinase
HPLC	High performance liquid chromatography
IPPI	Isopentenyl pyrophosphate isomerase
ISPD	2-C-methylerythritol 4-phosphate cytidyltransferase
ISPE	4-(cytidine-5-diphospho)-2-C-methylerythritol kinase
ISPH	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase
MAP K	MAP kinase
MECPS	2-C-methylerythritol-2,4-cyclophosphate synthase
MEP	Methylerythritol 4-phosphate
MVA	Mevalonic acid
MVDD	mevalonatediphosphate decarboxylase
MVK	mevalonate kinase
NOP10	H/ACA ribonucleoprotein complex
PC	Plastocyanin
PCA	Principal component analysis
PEP C	Phosphoenolpyruvate carboxylase
PMK	Phosphomevalonate kinase

POP	Pyruvate orthophosphate
qPCR	Quantitative real-time PCR
RBX1	RING-box protein 1
SRF	SRF receptor kinase

ABSTRACT

Aconitum heterophyllum Wall (Ranunculaceae), popularly known as Atis, is a biennial herb native to North-Western and Eastern Himalayas of India. Its tuberous roots are commonly used as therapeutic ingredient in the Traditional Indian Medicinal System for curing dyspepsia, abdominal pain, diabetes and diarrhoea. Non-toxic active components like atisine, hetisine and heteratisine accumulating in tuberous roots of *A. heterophyllum* have wide pharmacological effects on immune, digestive and nervous systems. Extensive harvesting for pharmaceutical purposes, lack of organized cultivation and unorganized methods of uprooting has put this herb in the category of critically endangered species. No information exists as of today on molecular aspects of aconites biosynthesis, thereby, limiting genetic interventions towards genetic improvement of *A. heterophyllum*. Moreover, the molecular components contributing to the formation of tuberous roots - herbal biomass of *A. heterophyllum* are also lacking. The current study, therefore, investigated: (1) Identification of high aconites content accessions of *A. heterophyllum*. (2) Cloning and expression analysis of MVA/MEP pathways genes vis-à-vis aconites content in *A. heterophyllum*. (3) Dissecting biosynthetic machinery of tuberous roots- the herbal biomass of *A. heterophyllum*.

Atisine content ranged from 0.14-0.37% and total alkaloids (aconites) from 0.20-2.49% among 14 accessions of *A. heterophyllum*. Two accessions contained the highest atisine content with 0.30% and 0.37% as well as the highest alkaloids content with 2.22% and 2.49%, respectively. No atisine was detected in leaves and shoots of *A. heterophyllum*, thereby, suggesting that the biosynthesis and accumulation of aconite alkaloids occur exclusively in roots. Quantitative expression analysis of 15 genes of MVA/MEP pathways in roots versus shoots, differing for atisine content (0-2.2 folds) showed 11-100 folds increase in transcript amounts of 4 genes of MVA pathway; HMGS, HMGR, PMK, IPPI, and 4 genes of MEP pathway; DXPS, ISPD, HDS, GDPS, respectively. The overall expression of 8 genes decreased to 5-12 folds after comparative expression analysis between roots of high (0.37%) versus low (0.14%) atisine content accessions, but their relative transcript amounts remained higher in high content accessions, thereby implying their role in atisine biosynthesis and accumulation. PCA analysis revealed a positive correlation between MVA/MEP pathways genes and aconites content.

Furthermore, the transcriptomes of *A. heterophyllum* were analyzed to identify possible genes associated with tuberous root development by taking clues from genes implicated in other plant species. Out of 18 genes, 8 genes encoding GMPase, SHAGGY, Expansin, RBX1, SRF, β-amylase, AGPase and ARF2 showed higher transcript abundance in roots (13-171 folds)

compared to shoots. Comparative expression analysis of genes between tuberous root developmental stages showed 11-97 folds increase in transcripts in fully developed roots compared to young rootlets, thereby implying their association in biosynthesis, accumulation and storage of primary metabolites towards root biomass. Cluster analysis revealed a positive correlation with the gene expression data for different stages of tuberous root formation in *A. heterophyllum*.

The current study provides first report wherein partial sequences of 15 genes of MVA/MEP pathways have been cloned and studied for their possible role in aconites biosynthesis. It also demonstrated the apparent role of tuberous root development genes for biomass production in *A. heterophyllum*. The outcome of study will be of immense practical importance in planning a suitable genetic intervention strategy for the improvement of *A. heterophyllum*.

CHAPTER 1

INTRODUCTION

For centuries, the history of pharmacy has been interlinked with the history of pharmacognosy, which is obtained from natural sources – mostly plants [1]. Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health. In spite of the great advances made in modern medicine in recent decades, plants still make an important contribution to health care. It is estimated that about 25% of all modern medicines are directly or indirectly derived from higher plants [2]. According to the World Health Organization, because of poverty and lack of access to modern medicine, about 65–80% of the world's population that lives in developing countries depends essentially on plants for primary healthcare [3]. As per the estimates, the present demand for medicinal plants is ~US $30 billion a year and by the year 2050, it would be ~US $5 trillion. Herbal medicinal preparations are mostly popular in developing countries with a long tradition in the use of medicinal plants.

Ayurveda is perhaps, the most ancient of all medicinal traditions is probably older than the Traditional Chinese Medicinal System. It is considered to be the origin of systemized medicine. It is actually a practical and holistic set of guidelines used to maintain balance and harmony in the system. Dioscorides (who influenced Hippocrates) is thought to have taken many of his ideas from India. Ancient Hindu writings on medicine contain no references to foreign medicines whereas Greek and Middle Eastern texts do refer to ideas and drugs of Indian origin. Ayurveda is derived from the Indian words 'Ayar' (Life) and 'veda' (Knowledge or Science) and hence means the Science of Life. With such a rich history of Ayurveda, one of the major medicinal plant raw materials producing Asian country is India where out of 17,000 species of higher plants, 7500 are known for medicinal uses. This is the highest proportion of plants known for their medical purposes in any country of the world for the existing flora of that country.

The Himalayan region is rich in a variety of natural resources in which medicinal plants have important place. The Indian Himalayan region supports over 1748 (32.2% of India) plant species of known medicinal value [4]. Rich plant diversity of the Himalayas - over 8000 angiosperms, 44 gymnosperms, 600 pteridophytes, 1737 bryophytes, 1159 lichens, etc. - has been a source of medicine for millions of people in the country and elsewhere in the world [5]. In India, around 25,000 effective plant-based formulations are used in traditional and folk medicine. More than 2 million practitioners are using the traditional medicinal system for health care in India. It is estimated that more than 7800 manufacturing units are involved in

the production of natural health products and traditional plant-based formulations in India, which requires more than 2000 tonnes of medicinal plant raw material annually [6].

Unfortunately, the number of reports of people experiencing negative effects, caused by the use of herbal drugs, has also been increasing. There may be various reasons for such problems, poor quality of herbal medicines due to insufficient attention being paid to the quality assurance and control of these products. Although WHO has developed guidelines for the quality control of herbal drugs which provide a detailed description of the techniques and measures required for the appropriate cultivation and collection of medicinal plants, there is still a gap between this available knowledge and implementation, because farmers and other relevant persons like producers, handlers and processors of herbal drugs are not much aware of WHO's guidelines and they continue their work as before without any quality control measures which results in inferior quality of herbal drugs with lots of contaminants like heavy metals, pesticides and microbes.

The quantity and efficacy of an herbal drug formulation is influenced due to the presence of desired contents and combination of major chemical constituents, which in most of the cases are secondary metabolites. Since plants have to cope with a number of challenges, including engineering their own pollination and seed dispersal, local fluctuations in the supply of the simple nutrients that they require to synthesize their food, and the coexistence of herbivores and pathogens in their immediate environment, they have therefore evolved secondary biochemical pathways that allow them to synthesize a raft of chemicals, often in response to specific environmental stimuli, such as herbivore-induced damage, pathogen attacks, or nutrient deprivation [7]. These secondary metabolites can be unique to specific species or genera and do not play any role in the plants' primary metabolic requirements, but rather they increase their overall ability to survive and overcome local challenges by allowing them to interact with their environment. An indication of how essential these secondary metabolites are to plants' survival can be seen in the energy invested in their synthesis, which is usually far in excess of that required to synthesize primary metabolites. Some of the roles of secondary metabolites are relatively straight forward; for instance, they play a host of general, protective roles (e.g. as antioxidant, free radical-scavenging, UV light-absorbing, and antiproliferative agents) and defend the plant against microorganisms such as bacteria, fungi, and viruses. They also manage inter-plant relationships, acting as allelopathic defenders of the plant's growing space against competitor plants. The palette of secondary metabolites can be subdivided into a number of distinct groups on the basis of their chemical structure and

synthetic pathways. In this regard, the largest and most prevalent of phytochemical groups are the alkaloids, terpenes, and phenolic compounds.

The biosynthesis and accumulation of secondary metabolites occur in different tissues and organs of plants and is largely influenced by the developmental stage of a particular organ/tissue as well as external stimuli [8]. These factors play an important role, as the uniformity in the selection of plant material is essential for the preparation of herbal drug formulation. The quality of herbal ingredients can be affected by environmental factor like climate, altitude and other conditions under which they were cultivated. The optimization of these factors would, therefore, be helpful for the farmers, collectors of herbal raw material, researchers and herbal drug manufacturers in obtaining best quality plant material at a particular time when the active constituents remain at their maximum concentration.

Unlike most organisms, plants use two unrelated pathways for the production of phytochemicals in different cell compartments [9]. The methylerythritol 4-phosphate (MEP) pathway simultaneously produces both IPP and DMAPP from pyruvate and glyceraldehyde 3-phosphate, whereas the mevalonic acid (MVA) pathway synthesizes IPP from acetyl-CoA. IPP is then converted into DMAPP by the activity of IPP isomerases. It is now well established that all the MEP pathway enzymes are encoded by the nuclear genome and imported into plastids. Recent proteomics approaches have detected all the Arabidopsis MEP pathway enzymes in the stroma and have provided much-needed evidence on the subplastidial localization of enzymes involved in the production of specific types of isoprenoids, including chrorophylls, carotenoids, and prenylquinones such as plastoquinone, phylloquinone, and tocopherol [10], indicating the first step enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS) as rate-limiting. By contrast, the MVA pathway enzymes are distributed in different subcellular compartments. The main rate-determining enzyme of the pathway, hydroxymethylglutaryl-CoA reductase (HMGR), is anchored to the endoplasmic reticulum exposing the catalytic domain of protein towards the cytosol, whereas other enzymes of the pathway have been found in the cytosol and peroxisomes [11]. These observations have implications for increasing the accumulation of useful phytochemicals in other plant species.

Aconitum heterophyllum Wall (Ranunculaceae), popularly known as Atis, is a high value biennial herb native to North-Western and Eastern Himalayas of India (Fig. 1.1). It is found between 2400-3600 m altitudes above mean sea level. Its tuberous roots are commonly used

as therapeutic ingredient in the Traditional Indian Medicinal System for curing dyspepsia, abdominal pain, diabetes and diarrhoea [12]. Non-toxic active components like atisine, hetisine and heteratisine accumulating in tuberous roots of *A. heterophyllum* have wide pharmacological effects on immune, digestive and nervous systems [13-14]. The indiscriminate collection of plant material has led to considerable depletion of *A. heterophyllum* population, resulting in its inclusion in the category of 'critically endangered species' by the International Union for Conservation of Nature and Natural Resources [4].

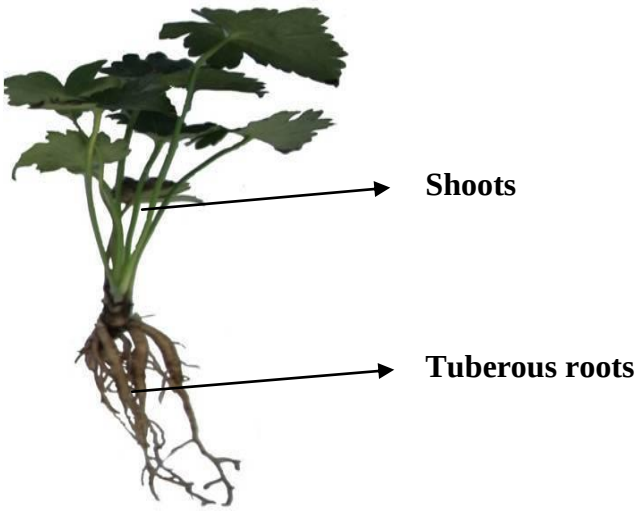

Fig. 1.1 Mature *A. heterophyllum* plant with fully developed tuberous roots (~3 years old)

The aconites, including atisine represent major constituents as well as marker compounds of *A. heterophyllum*. Aconites biosynthesis follows the common MVA/MEP route for isoprenoid production (Fig. 1.2) but no molecular data is available as of today on the relative importance of MVA/MEP pathways in the biosynthesis of these medicinally important moieties. The biosynthesis and accumulation of isoprenoids is controlled by various structural and regulatory genes [15]. Isopentenyl pyrophosphate (IPP) and dimethyl allyl pyrophosphate (DMAPP), the building blocks of isoprenoids, condense to form geranyl diphosphate (GPP) [16]. Plants utilize both MVA and MEP pathways for isoprenoid biosynthesis, even though they are localized in different compartments [17]. A total of 15 genes are involved, 8 coding for MEP pathway enzymes, and 6 for MVA pathway enzymes, and the final gene coding for IPPI that interconverts DMAPP and IPP in isoprenoid biosynthesis.

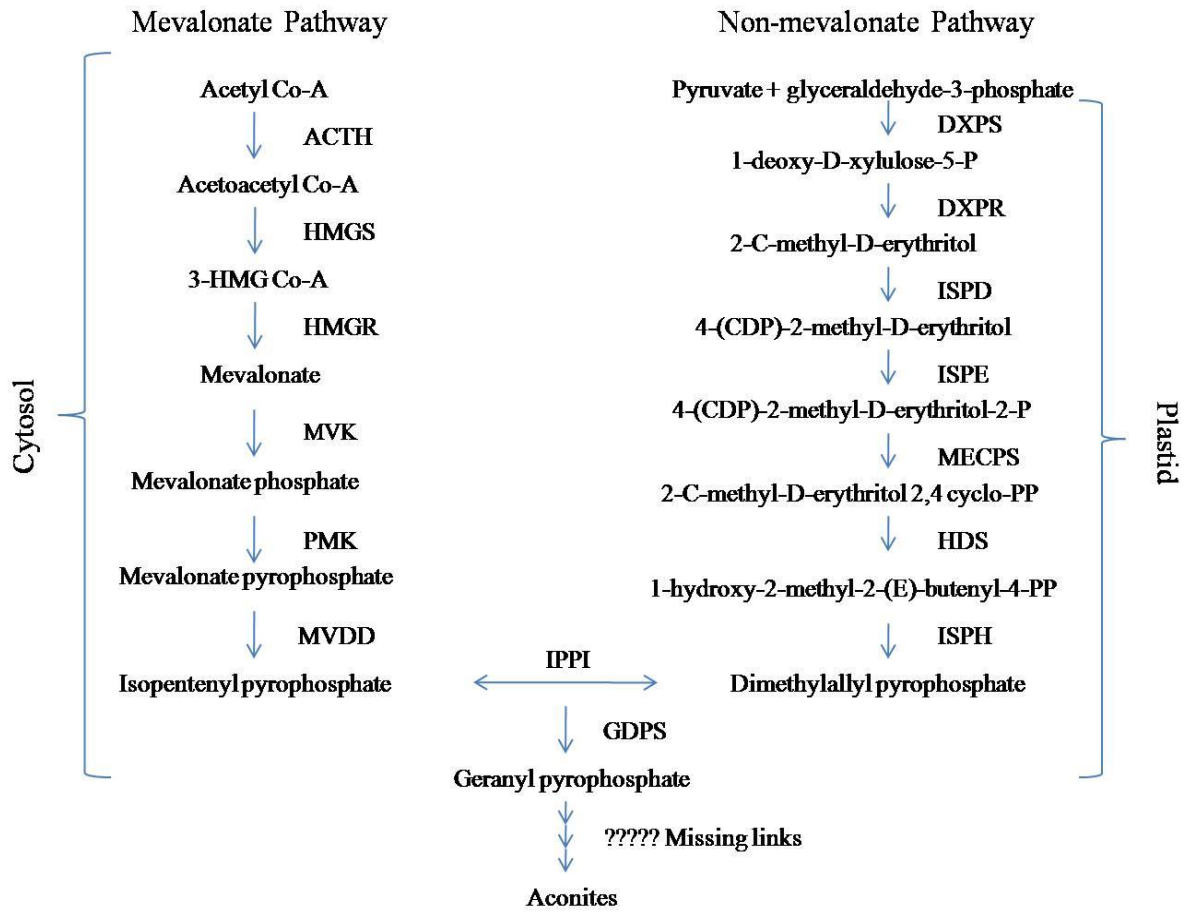

Fig. 1.2 Common isoprenoid pathway for aconites biosynthesis in *A. heterophyllum* [17]

No information exists as of today on genetic improvement of *A. heterophyllum* neither towards increased production of secondary metabolites or the biomass (tuberous roots) accumulating those metabolites. A genetic intervention strategy would require information on candidate genes contributing to the formation of tuberous roots. Generating whole genome transcriptome of *A. heterophyllum* would, therefore, be an ideal staring point to capture genetic components contributing to a trait of economic importance. The availability of whole transcriptome data can be used not only to discover candidate genes involved in tuberous root development and secondary metabolites production but also for understanding molecular basis of various biological processes.

Tuberization in *A. heterophyllum* is a distinctive process from young rootlet to fully mature storage roots which are committed to the storage of primary as well as secondary metabolites. However, the knowledge on developmental pattern of tuberous roots in *A. heterophyllum* is

limited. The development of tuberous roots in this plant species undergoes simple, yet unique process. After seed germination, the primary roots grow immediately from the radicle and transform directly into tuberous roots rather formed into adventitious roots as in other plant species. The proliferation and swelling of primary tuberous root result in further branching and bulking of storage organ to accumulate primary metabolites such as starch and tuber proteins besides aconites with the passage of time. Therefore, the tuberous root formation in *A. heterophyllum* provides a unique system to explore mechanism of sink tissue formation and development vis-à-vis accumulation of medicinal metabolites.

Thus, to understand the relative role of MVA/MEP pathways gene(s) in aconites biosynthesis, the cloning and expression analysis of all 15 genes in relation to aconites content was studied for the first time in *A. heterophyllum*. Furthermore, as a first step to gain insight of tuberous root development, Illumina paired-end sequencing technology was utilized to characterize molecular components that are possibly involved in tuberous root formation in *A. heterophyllum*. Fragments per kilobase of transcript per million mapped fragment (FPKM) based comparative expression profiling study was done to systematically characterize the RNAs to identify differentially regulated genes involved in tuberous root formation. Next-generation sequencing (NGS) transcriptomes analysis of *A. heterophyllum* root and shoot tissues identified plausible candidate genes responsible for tuberous root formation and development, which were further assessed for their association with growth and developmental mechanism of tuberous roots. Additionally, genes pertaining to primary metabolism in plants such as starch production, photosynthesis, hormone metabolism and transcription factors were also studied to associate their role in storage organ development and root biomass production in *A. heterophyllum*. To fill the gaps in knowledge on biosynthesis of aconites and biosynthetic machinery of tuberous roots- the main herbal biomass of *A. heterophyllum*, the current study was undertaken with the following objectives:

(1) Identification of high aconites content accessions of *A. heterophyllum*.
(2) Cloning and expression analysis of MVA/MEP pathways genes vis-à-vis aconites content in *A. heterophyllum*.
(3) Dissecting biosynthetic machinery of tuberous roots- the herbal biomass of *A. heterophyllum*.

CHAPTER 2

REVIEW OF LITERATURE

The current status of literature on various aspects of *Aconitum* research has been reviewed as under:

2.1 Introduction

Out of many important medicinal plants cultivated in present era, *Aconitum* species finds a key position for conservation and cultivation. The genus *Aconitum* belongs to the family Ranunculaceae. These herbaceous biennial plants are chiefly natives of the mountainous parts of the Northern Hemisphere, growing in moisture retentive but well draining soils on mountain meadows. These plants are tall, with erect stem being crowned by racemes of large and eye-catching blue, purple, white, yellow or pink zygomorphic flowers with numerous stamens. The root is best harvested in the autumn as soon as the plant dies down and is dried for later use. There are over 250 species of *Aconitum* [18]. *Aconitum* in northwest Himalayas is represented by 10 species and two varieties. This is one of the important plant genuses that have been enlisted in Red Data Book. Some of the important species of *Aconitum* are *Aconitum balfourii, Aconitum japonicum, Aconitum heterophyllum, Aconitum napellus, Aconitum ferox, Aconitum violaceum, Aconitum dienorrhizum, Aconitum carmichaeli, Aconitum chasmanthum* and *Aconitum bisma*. In recent years, the demand for medicinal and aromatic plants has grown rapidly because of accelerated local, national and international interest. *Aconitum* is the centre of attraction in the field of herbal medicines because of its property of curing a wide range of diseases and hence the pressure on its natural habitat has increased.

2.2 Phytochemistry

The medicinal plant species of *Aconitum* are a rich source of alkaloids and flavonoids, many of which exhibit broad spectrum of pharmacological activity (Fig. 2.1). Moreover, there are reports on isolation and identification of various free fatty acids and polysaccharides.

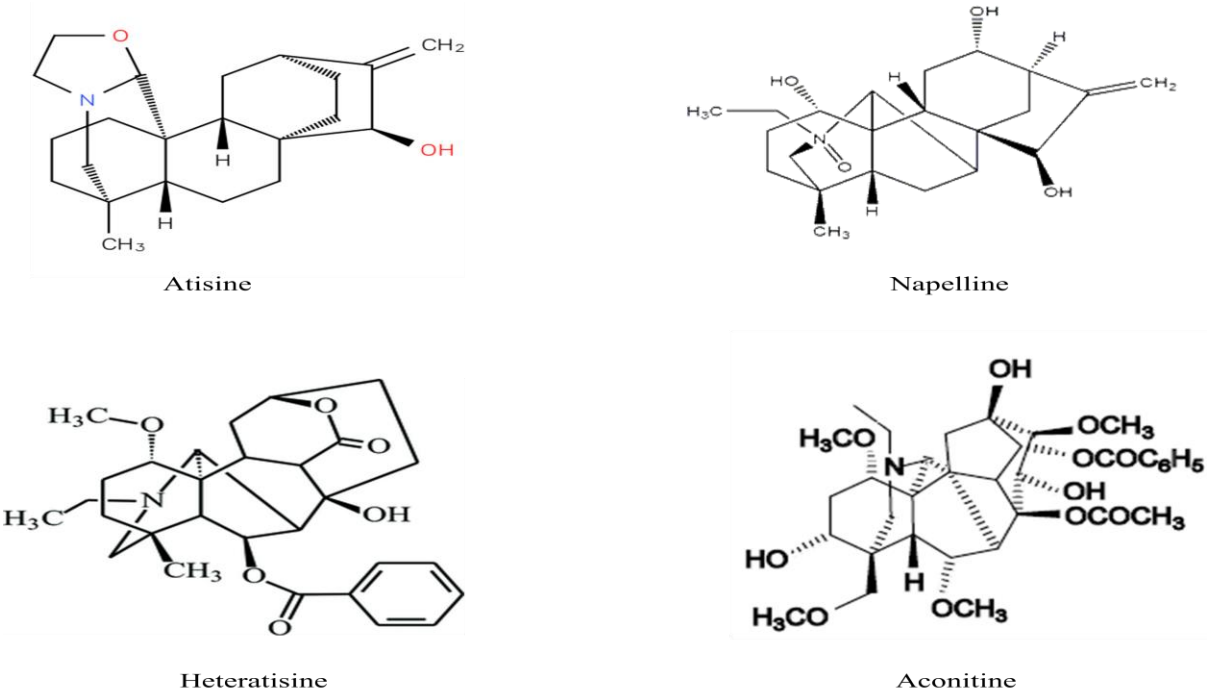

Fig. 2.1 Chemical structures of some pharmacologically important *Aconitum* compounds

2.2.1 Alkaloids

Alkaloids are a class of naturally occurring cyclic organic compounds containing nitrogen in a negative oxidation state [19]. The first alkaloid identified from *Aconitum* species was aconitine [20]. Systematic investigation of the *Aconitum* alkaloids was initiated in early half of 20th century [21]. The alkaloids benzoylmesaconine, mesaconitine, aconitine, hypaconitine, heteratisine, heterophyllisine, heterophylline, heterophyllidine, atidine, isotisine, hetidine, hetsinone and benzoylheteratisine have been isolated from tuberous roots of genus *Aconitum* [13-14]. New derivatives of different alkaloids have also been isolated from different species of *Aconitum* which includes isolation of 11 diterpene alkaloids [22]. Isoatisine and coryphine were found to be most active possibly because of the presence of oxazolidine rings. Investigation on alkaloidal constituents of *A. jaulense* led to the isolation of seven C-19 norditerpenoids and C-20 diterpene alkaloids [23]. 8-O-Azeloyl-14-benzoylaconine, a new alkaloid from roots of *A. karacolicum* subsp. *Rapcs*, was isolated by Chodoeva et al. [24].

Furthermore, two new aconitine-type norditerpenoid alkaloids namely 6-dehydroacetylsepaconitine and 13-hydroxylappaconitine along with three known

norditerpenoid alkaloids were isolated by Ahmad et al. [25], while two new diterpene alkaloids heterophylline A and heterophylline B along with two known alkaloids from the roots of *A. heterophyllum* Wall were isolated by Nisar et al. [26]. The diterpene alkaloids were reported for the first time in *A. spicatum* subsp. *Stapf* from which 13 norditerpenoids were isolated from the chloroform fraction of 90% ethanol extract of roots, of which two were new, namely spicatine A and spicatine B [27].

2.2.2 Flavonoids

Flavonoids are a class of low molecular weight phenolic compounds widely distributed in the plant kingdom. They are mainly involved in photo-protection from UV-rays involved in scavenging reactive oxygen species to prevent lipid peroxidation [28]. Only little information is available on the flavonoids composition of the *Aconitum* species. The few flavonoids studied in the last 10 years have been rather used as chemotaxonomic markers [29]. Some common *Aconitum* flavonoids including quercetin 7-*O*-(6-*trans*caffeoyl)- β -glucopyranosyl-(1→3)- α -rhamnopyranoside-3-*O*- β -glucopyranoside, kaempferol 7-*O*-(6-*trans*-caffeoyl)- β -glucopyranosyl-(1→3)- α -rhamnopyranoside-3-*O*- β -glucopyranoside and kaempferol 7-*O*-(6-*trans*-*p*-coumaroyl)- β -glucopyranosyl-(1 → 3)- α -rhamnopyranoside-3-*O*- β -glucopyranoside, together with β -3,4-dihydroxyphenethyl β -glucopyranoside, have been isolated from the flowers of *A. napellus* subsp. *Neomontanum* [30].

2.2.3 Free Fatty Acids

A number of free fatty acids have also been reported in *Aconitum* species. The esterification of the alcoholic extract followed by gas chromatography-mass spectrometry (GC-MS) analysis showed the abundance of three types of free fatty acids, predominantly linoleic acid followed by palmitic acid and oleic acid [31-32].

2.2.4 Polysaccharides

A water-soluble polysaccharide, FPS-1, was isolated from the root of *A. carmichaeli* subsp. *Debx* by hot-water extraction, anion exchange and gel permeation chromatography technique [31]. Four water-soluble polysaccharide fractions have also been isolated from the tubers of *A. kusnezoffii* subsp. *Reichb* [33].

2.3 Pharmacology

The pharmacological analysis of *Aconitum* species and their compounds have shown various therapeutic effects. The key points of the scientific research have been the effects of diterpene alkaloids on the central nervous system and the heart. Their antimicrobial and cytotoxic effects have also been studied.

2.3.1 Cardiovascular effects

The initial research focused on the cardiovascular (arrhythmogenic) toxicity of *Aconitum* alkaloids. The marked cardiac activity of diterpene alkaloids is mainly due to their effect on the voltage-gated Na^+ channels [34]. Depending on their mechanism of action, the *Aconitum* alkaloids action on cardiac function has been subdivided into arrhythmogenic and anti-arrhythmic alkaloids.

Arrhythmogenic alkaloids induce their effect by delaying the final repolarization phase of action potential in cardiac cells, which initiates premature or triggered excitations. The final inexcitability of the cells may cause heart arrest [35].

Anti-arrhythmic compounds are based on a variety of diterpene skeletons. Regarding the mechanism of action, inhibition of the voltage-dependent Na^+ channels and blocking of the delayed rectifier K^+ current are the key components. Na^+ channel-blocking diterpene alkaloids are competitive antagonists of the arrhythmogenic Na^+ channel activating alkaloids. These compounds block the voltage-dependent Na^+ channels. The high-frequency discharge of action potentials that can occur, for instance, during arrhythmia could be a prerequisite for the blocking action of these alkaloids [35].

2.3.2 Effects on central nervous system

Recent experimental findings indicate that some diterpene alkaloids act as selective antagonists on the bungarotoxin-sensitive nicotinic acetylcholine receptors (nAChR) or inhibit the delayed rectifier K^+ current. Several diterpene alkaloids also have either anti-epileptic or epileptiform effects, which are also related to the effect on the Na+ channels. The analgesic and anti-epileptiform action of the *Aconitum* alkaloids have been the most studied.

Alkaloids that activate voltage-dependent Na^+ channels are antinociceptive and have the potential to depolarize neurons permanently and hence block the neuronal conduction [36]. Na^+ channel blockers possess antinociceptive activity by inhibiting neuronal activity [36-37]. Numerous other diterpene alkaloids have also been demonstrated to have peripheral analgesic and antinociceptive properties in different test models [35].

2.3.3 Antimicrobial activity

On the basis of *in vitro* anti-proliferative effects of several atisine-type diterpene alkaloids against *Leishmania infantum*, the antiprotozoal activity of 43 C-19 diterpene alkaloids were tested on the extracellular and intracellular stages of the parasite. From among the tested compounds, three atisine-type *Aconitum* alkaloids inhibited the growth of *L. infantum* similarly to the reference drug, without being toxic to the host cells [38].

2.3.4 Cytotoxic activity

The cytotoxic activity of diterpene alkaloids on normal cells were first studied in experiments aimed at the insectrepellent effects of the compounds. A few of the tested 64 alkaloids, apparently randomly distributed among chemical classes, had a selective cytotoxic effect against insect-derived Sf9 cells; only 13-oxocardiopetamine was cytotoxic to mammalian CHO cells [39].

2.4 Toxicology

Aconitum has been considered as a mysterious herb due to its both healing and death causing properties [40]. In recent years, a large number of studies have investigated the toxicological characteristics of *Aconitum*, its main alkaloids and their derivatives [41]. It has been observed that the whole plant of *Aconitum* is highly toxic with the concentration of toxic compounds higher in roots and flowers than in leaves and stems [42]. The symptoms of toxicity affect mainly the central nervous system and the heart, with concomitant gastrointestinal signs. The cause of death is the development of ventricular tachyarrhythmia and heart arrest. No specific therapy exists for *Aconitum* poisoning although cardiovascular supportive treatment is usually applied [43]. The toxicity of *Aconitum* is mainly due to the diester diterpene alkaloids and monoester diterpene alkaloids such as deoxyaconitine, benzoylmesaconitine, jesaconitine, benzoylhypaconine and benzoylaconine [44]. Through various physical and chemical

methods of treatment, highly toxic *Aconitum* alkaloids could be transformed into less toxic derivatives.

2.5 Conservation

Several efforts have been carried out on conservation aspects of *Aconitum* species through micropropagation to save them from extinction. Clonal multiplication of *A. carmichaeli* by tip-tissue culture technique was done which showed less variation in alkaloid content of clonally propagated plant than those of the normally grown plants [45]. Similarly, microtubering in *A. carmichaeli* was enhanced at 15°C under dark rather than at 10-20°C [46]. *In vitro* propagation of *A. atrox* has also been conducted successfully at a lower elevation using tuber segments [47].

In another study, 20 primers were screened for the genetic analysis of *Aconitum* species and 14 were selected giving 51 polymorphic bands [48]. Furthermore, flavonoid profiles of species and subspecies of *Aconitum* showed significant correlation with genetic study [49]. Giemsa C banding pattern analysis showed that ancient Sudetic taxon of *Aconitum* may have contributed to the genome of Carpathian taxon, which was then supported by molecular ISSR + RAPD pattern that points to introgression between the Sudetic *Aconitum plicatum* and Carpathian *Aconitum firmum* subsp. *Maninese* [50].

2.6 *Aconitum heterophyllum* Wall

Aconitum heterophyllum is a rare, endangered, Himalayan species. Ayurveda classical texts of 15^{th}-16^{th} century introduced 'Abhava-Pratinidhi Dravya' concept, wherein it was categorized as an 'abhava dravya' (unavailable drug). Its roots are ovoid-conical, tapering downwards to a print, 2.0-7.5 cm long, 0.4-1.6 cm or more thick at its upper extremity, gradually decreasing in thickness towards tapering end, externally light ash-grey, white or grey-brown, while internally starch white, external surface wrinkled marked with scars of fallen rootlet and with a rosette of scaly rudimentary leaves on top.

A. heterophyllum is a biennial herb found in the North-Western Himalayan region above 2200 m amsl. It flowers in the second year. The flowers are helmet-shaped, bright blue or greenish blue in color and have a purple vein. For medicinal use, the roots from plants bearing fully developed tubers are collected. The tubers sometimes occur as a pair of mother and daughter tubers. In India, the plant is most commonly found in Jammu and Kashmir,

Himachal Pradesh, Uttarakhand, Sikkim and Arunachal Pradesh. As it is an endangered species, the Director General of Foreign Trade prohibits the export of *Aconitum* species of plants, plant portions and their derivatives and extracts obtained from the wild [51].

2.6.1 Medicinal properties

Sudarshana Churna, Balachaturbhadra Churna, Rasnerandadi Kwatha and Panchatiktaka Guggulu Ghrta are some of the popular multi-drug formulations in which *A. heterophyllum* is one of the main ingredients [52]. These drugs find common use in the treatment of fevers, diarrhea, indigestion, inflammation, helminthiasis, hyperlipidemia and other ailments. Some of the important medicinal properties of *A. heterophyllum* are listed in Table 2.1.

Table 2.1 Medicinal properties of *A. heterophyllum*

Use	Source
Anti-inflammatory	[53]
Antioxidant	[54]
Antihelminthic	[55]
Hypolipidemic	[56]
Abdominal distension	[57]
Aphrodisiac	[58]
Arthritis	[59]
Ant-diarrheal	[12]
Antibacterial	[60]
Anti-diabetic	[12]

2.6.1.1 Anti-inflammatory and antipyretic activity

In order to assess the antiinflammatory activity of *A. heterophyllum*, cotton-pellet induced granuloma method was used. The investigations showed that *A. heterophyllum* tuber (ethanolic extract) has significant anti-inflammatory activity, thereby providing scientific

evidence for a traditional medicinal claim as anti-inflammatory action [53]. The antipyretic effects of roots of *A. heterophyllum* in the form of aqueous, chloroform and hexane extracts were examined using the method of yeast induced pyrexia, with aspirin as a standard antipyretic agent for comparison. These studies showed that the extracts were nontoxic (up to 1.6 g/kg) and had no significant antipyretic activity.

2.6.1.2 Antibacterial activity

The new aconitine type nor-diterpenoid alkaloids 6-dehydroacetylsepaconitine and 13-hydroxylappaconitine, isolated from the tubers of *A. heterophyllum* along with the known alkaloids lycoctonine, delphatine and lappaconitine, were screened for antibacterial activity against different bacterial strains. They showed antibacterial activity against gram negative (diarrhea causing) bacteria *Escherichia coli, Shigella flexineri, Pseudomonas aeruginosa* and *Salmonella typhi* [25]. This report strengthens the prescription of *A. heterophyllum* as antimicrobial/anthelmintic. These tests were however not carried out using the plant extracts.

2.6.1.3 Immunomodulatory activity

The immunomodulatory activity of ethanolic extract of *A. heterophyllum* tubers along with other medicines of the Ayurveda and Unani systems of medicine were investigated on delayed-type hypersensitivity, humoral responses to sheep red blood cells, skin allograft rejection and phagocytic activity of the reticulo-endothelial system in mice. It was found that the extract appeared to enhance the phagocytic function and to inhibit the humoral component of the immune system. The results obtained from these preliminary studies showed that *A. heterophyllum* has immunomodulatory activity, which could possibly lead to new immunomodulating agents of herbal origin [61-62].

2.6.1.4 Effect on nervous system

A. heterophyllum has the ability to make the sympathetic nervous system more sensitive to physiological stimuli. It was found that while atisine had a hypotensive effect at every tested dose, the plant extract as a whole showed hypertensive properties. Hypertension produced by high doses of aqueous extract was attributed to the excitement of the sympathetic nervous system [63].

Two new diterpenoid alkaloids, heterophyllinines A and B, isolated from the roots of *A. heterophyllum* were about 13 times more selective in inhibiting the enzyme butyrylcholinesterase than acetylcholinesterase. These enzymes are involved in the transmission of nerve impulses [26].

2.6.1.5 Anthelminthic activity

Aqueous and alcoholic extracts of tubers of *A. heterophyllum* gave encouraging results when evaluated against *Pheritema postuma*, using piperazine citrate as standard. Time required for initial three paralytic attacks and death was used as parameters to evaluate the drug [55]. Though *A. heterophyllum* is considered to have anthelmintic property as per Ayurveda, the results obtained here are needed to be compared with other standard Ayurvedic anthelmintic agents like *Emblia ribes* to establish the utility of this drug in practice.

2.6.1.6 Antihyperlipidemic activity

The methanolic extract of tubers of *A. heterophyllum* had a hypolipidemic effect on diet induced obese rats. It was observed that the pharmacological effect was due to two factors; (i) inhibition of Hydroxymethylglutarate-Coenzyme A reductase (HMGR) and (ii) activation of Lecithin-cholesterol acyltransferase. This resulted in lowering of total cholesterol, low-density lipoprotein cholesterol, triglycerides and apolipoprotein B in blood serum, decrease in intestinal fat absorption and increase of high-density lipoprotein cholesterol and apolipoprotein A, supporting the classification of *A. heterophyllum* as a drug having scraping action with antihyperlipidemic properties [56].

2.6.1.7 Ant-diarrheal activity

The ant-diarrheal activity of roots of *A. heterophyllum* may be attributed to an antisecretory and anti-enteropooling type effect as a result of reactivation of Na^+ and K^+ ATPase activity mediated through nitric oxide pathway. They cause either a decrease in mucosal secretion or increase in mucosal absorption, which allows the faeces to become desiccated thus retarding its movement through the colon [12].

2.6.2 Chemical constituents

The main alkaloid reported in *Aconitum* is aconitine that is highly toxic [64]. However, among the reported ~250 species of *Aconitum*, *A. heterophyllum* is the only non-toxic species with therapeutic potential [65]. The pharmacological properties of *A. heterophyllum* are attributed to the non-toxic active constituents *i.e.* aconites, including atisine comprise the major alkaloid constituents of this plant species [4, 66]. These constituents make it a safer herb to use when compared with other *Aconitum* species since no purification process is mandatory for its purification or detoxification. Some of the important phytochemicals of *A. heterophyllum* are listed in Table 2.2.

Table 2.2 Major phytoconstituents of *A. heterophyllum*

Class	Chemical constituent	Source
Alkaloid	Atisine	[67]
	Heteratisine	[68]
	Atidine	[13]
	Heterophyllidine	[13]
	Hetisone	[13]
	Aconitine	[64]
	Lappaconitine	[25]
Terpenoid	Atisenol	[69]

The early investigations on the tubers of *A. heterophyllum,* beginning with Broughton (1873), Wasowicz (1879) and Wright (1879) have been documented by Jowett [70]. Broughton was first to isolate atisine. Different salts (sulphate, hydrochloride and platinichloride) were prepared from the alkaloid, and the molecular formula was deduced. Wasowicz showed that the aconitic acid is also present along with atisine besides suggesting slight modifications in the molecular formula of atisine. Wright proposed a new formula for atisine based on analysis of its aurichloride salt. Subsequently, investigations on the properties and composition of atisine and its salts were studied in great detail. No alkaloid other than atisine

was found in their study [70]. The structure of atisine and three other alkaloids hetisine, heteratisine and benzoylheteratisine were confirmed by Jacobs and Craig [67-68].

Detailed studies on hetisine, atisine and heteratisine helped in their structure elucidation [71-74]. Further investigations on *A. heterophyllum* led to the isolation and structure elucidation of additional new diterpene alkaloids; atidine, F-dihydroatisine, hetidine and hetisone as well as lactone alkaloids heterophyllisine, heterophylline and heterophyllidine [13, 75]. In 1982, a new entatisene diterpenoid lactone, atisenol, was isolated from the tubers of *A. heterophyllum* [69]. Moreover, the structure and most importantly, the stereochemistry of atisine and related alkaloids was established by Dvornik and Edwards [76].

Later on, work on *Aconitum* alkaloids led to the isolation of two new aconitine-type norditerpenoid alkaloids 6-dehydroacetylsepaconitine and 13-hydroxylappaconitine along with known norditerpenoid alkaloids lycoctonine, delphatine and lappaconitine [25]. Although aconitine, which is the major alkaloid of other *Aconitum* species, is not a major constituent of *A. heterophyllum*, High performance liquid chromatography (HPLC) studies carried out on the tubers from Kumaon and Garhwal regions of the Himalayas showed that aconitine is present in different populations and varies from 0.13% to 0.75% (dry weight basis) [77-78]. HPLC studies on quantification of aconitine from tubers of *A. heterophyllum* from the Kashmir valley have reported 0.0014-0.0018% aconitine [79]. Similarly, a study by Bahuguna et al. [80] reported higher content of atisine (0.35%) and aconitine (0.27%) in green house grown *A. heterophyllum* when compared with the naturally grown plants (0.19% and 0.16%, respectively). From the reported literature, it is evident that, alkaloids were the main focus of study in *A. heterophyllum*. Several pharmacological actions of *A. heterophyllum* have been attributed to their alkaloids [64].

2.6.3 Adulterants

Natural sources of medicinal plants are unable to meet demand for popular herbal products. Populations of many species have limited distribution in their natural habitats, requiring conservation strategies for protection. Unavailability of such medicinal plants has led to arbitrary substitution and adulteration in raw drug market. Adulteration is a practice of substituting the original crude drug partially or fully with other substances which are either free from or inferior in therapeutic and chemical properties or addition of low grade or entirely different drug similar to that of original drug substituted with an intention of

enhancement of profits. *A. heterophyllum* has been substituted by *Cyperus rotundus* in herbal processing methods, thus affecting the quality of the herbal drug formulations [81].

2.6.4 Tissue culture

The use of tissue culture techniques has been employed for conservation of this medicinally important plant species. Plants of *A. heterophyllum* were obtained via somatic embryogenesis in callus derived from *in vitro* raised leaf and petiole explants [82]. In another study, a protocol was developed for *in vitro* shoot proliferation from callus cultures of *A. heterophyllum* [83], but thereafter, not much has been reported.

Prolonged seed dormancy, high seedling mortality and ecological constraints have made *A. heterophyllum* endangered. Some of the efforts made in this regard corresponds to optimization of conditions for *in vitro* seed germination and shoot multiplication as reported by Solanki and Siwach [84]. In another study, Rana and Sreenivasulu [85] found 80% ethanol induced seed germination in *A. heterophyllum*. They also identified 40 differentially expressed proteins from ethanol treated and untreated seeds through comparative two-dimensional electrophoresis (2-DE) protein profiling.

A method for the production of hairy roots of *A. heterophyllum* was developed by Giri et al. [86]. Embryogenic callus cultures were successfully transformed using *Agrobacterium rhizogenes* strains viz. LBA 9402, LBA 9360, and A4 for the induction of hairy roots. Total alkaloid (aconites) content of transformed roots was found to be 2.96%, which was 3.75 times higher compared to 0.79% in the non-transformed (control) roots. Furthermore, thin layer chromatography (TLC) analysis of aconites in the transformed roots revealed the presence of heteratisine, atisine, and hetidine in *A. heterophyllum*.

The resources pertaining to molecular machinery of tuberous roots – the herbal biomass of *A. heterophyllum*, committed to storage of therapeutic metabolites have not been reported till date. Several studies on morphogenesis have been carried out to understand tuberous root development in various plant species such as *Ipomoea batatas* and *Manihot esculenta* [87-88]. The fundamental genetic mechanism controlling their formation in *Rehmannia glutinosa* has been studied to decipher the role of tuberous root development genes [89]. Moreover, primary metabolism has been found to be activated in storage roots of *I. batatas* and *Raphanus sativus* [90-91]. With the advent of new technologies, a comprehensive analysis of

transcriptome and proteome datasets in *R. glutinosa* provided new insight on the mechanisms for the formation of storage organ [92].

The complete review of literature therefore, highlights the following gaps in our understanding on this highly valuable medicinal herb:

(1) No information is available on biology of aconites biosynthesis.
(2) No information is available on molecular components regulating root biomass yield.
(3) No genetically defined accessions are available for commercial use.

CHAPTER 3

MATERIALS AND METHODS

The present investigation was carried out in the Jaypee University of Information Technology, Waknaghat, Himachal Pradesh, India. The material used and the methodologies adopted to achieve objectives of the investigation are described here under:

3.1 Plant material

Plants of *A. heterophyllum* were procured from the Himalayan Forest Research Institute (HFRI) at Shilaru, Himachal Pradesh, India (2450 m altitude, 31°23′ N, 77°44′ E) (Fig. 3.1). Roots and shoots of different age groups (1-3 years) were collected, frozen immediately in liquid nitrogen, and stored at -80°C. Fourteen accessions were collected from different locations of Himachal Pradesh, India under the supervision of scientists at HFRI and planted in the green house of Jaypee University of Information Technology under controlled conditions (Table A1). They were also segregated into root and shoot tissues for further use.

Fig. 3.1 Field plantation of *A. heterophyllum* at HFRI, Shilaru, Himachal Pradesh, India

3.2 Optimization of differential conditions for aconites biosynthesis

To regulate the quality of herbal drug formulations, it was important to understand the sites of biosynthesis and accumulation of aconites in *A. heterophyllum* corresponding to different growth and development stages.

3.2.1 Preparation of samples for atisine analysis

All tissues (roots and shoots) of *A. heterophyllum* were thoroughly washed under running tap water and ground to fine powder using a pestle and mortar in liquid nitrogen. Powdered samples (100 mg) were extracted in 10 ml 80% HPLC grade methanol. The samples were then vortexed to mix properly and extracted overnight at room temperature. Samples were centrifuged at 10,000 rpm for 15 min. and the supernatant was filtered through 0.22 µm filter (Millipore, USA) and used for HPLC analysis.

3.2.2 Quantification of atisine and total alkaloids

The quantification of atisine was carried by reverse phase HPLC (Waters 515) through C18 (5 µm) 4.6 x 250 mm Waters Symmetry Column using PDA detector (Waters 2996). The filtrate was diluted 10 X and 20 X and injected into above-mentioned column. Two solvent systems were used for running the test samples i.e. Solvent A (2 g sodium heptane sulfonic acid, 2 ml triethylamine in l l distilled water with pH adjusted to 3 by orthophosphoric acid) and Solvent B (100% acetonitrile). The column was eluted in isocratic mode with a flow rate of 1 ml/min. Atisine was detected at an absorbance of 270 nm wavelength. The cycle time of analysis was 30 min. at 30°C. The compound was identified on the basis of retention time and comparison of UV spectra with the isolated atisine standard from dried tuberous roots of *A. heterophyllum*. Atisine was isolated as per the method of Jacobs and Craig [67] and quantified in HPLC.

Total alkaloids (aconites) content was estimated by benzene-bromocresol green extraction method [93]. 100 mg sample was dissolved in 1 ml of 1N HCl, followed by extraction with 5N NaOH and benzene (pH>10). 100 µl of bromocresol green was used, followed by ethanol treatment (repeated thrice). Absorbance was measured at 630 nm wavelength and total alkaloid content was calculated. Fourteen accessions of *A. heterophyllum* were assayed for atisine and total alkaloids content in roots.

3.3 Selection of high versus low metabolite content accessions

On the basis of metabolite quantification (Fig. 3.2), high and low content accessions differing for atisine/aconites content were selected. They were subjected to molecular analysis for determination of genetic factors controlling biosynthesis and accumulation of aconites.

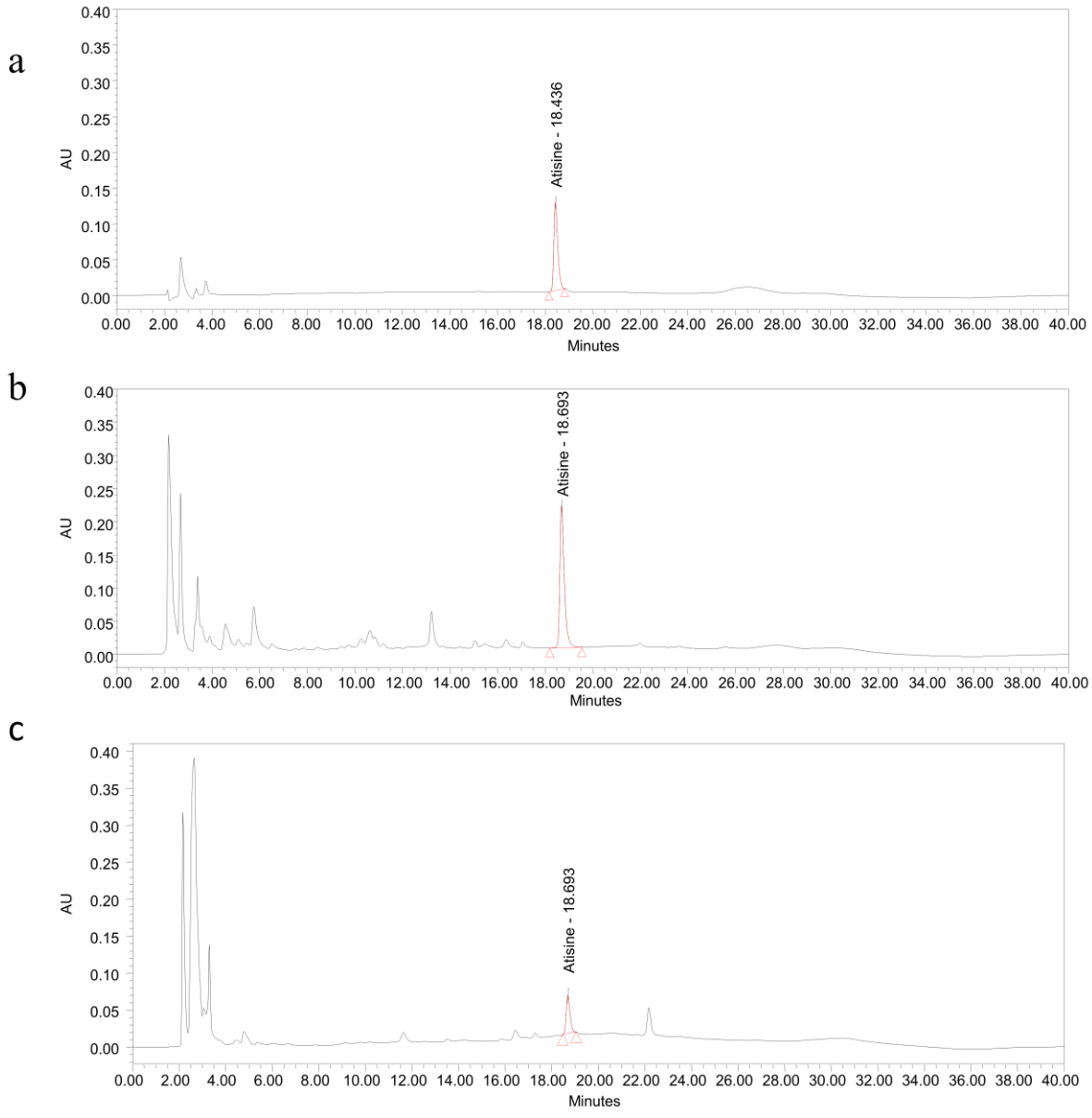

Fig. 3.2 HPLC chromatograms for atisine standard (a), high atisine content accession (b) and low atisine content accession (c) of *A. heterophyllum*

3.4 Isolation of genomic DNA and total RNA

Genomic DNA was isolated from leaves of *A. heterophyllum* following the protocol of Murray and Thompson [94]. Total RNA was isolated from *A. heterophyllum* samples by using RaFlex™ Total RNA isolation kit (Bangalore Genei Pvt Ltd, India) by following manufacturer's instructions. The quality of RNA was checked in 1% (w/v) ethidium bromide-stained agarose gel and through absorbance spectrum at wavelengths 260 nm and 280 nm.

3.5 Cloning and sequencing of MVA/MEP pathways genes in *A. heterophyllum*

Comparative genomics was utilized to clone MVA and MEP pathways genes in *A. heterophyllum* (Fig. 3.3). Nucleotide and protein sequences for all 15 genes of MVA/MEP pathways were retrieved from different plant species in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and multiple sequence alignments (MSA) were done to identify conserved sequence regions (Table 3.1). Though the extent of sequence similarity was low in coding regions of genes, short patches of conserved sequences were identified. Primer pairs were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) from conserved regions of gene sequences and tested on genomic DNA and cDNA of *A. heterophyllum* (Table 3.2).

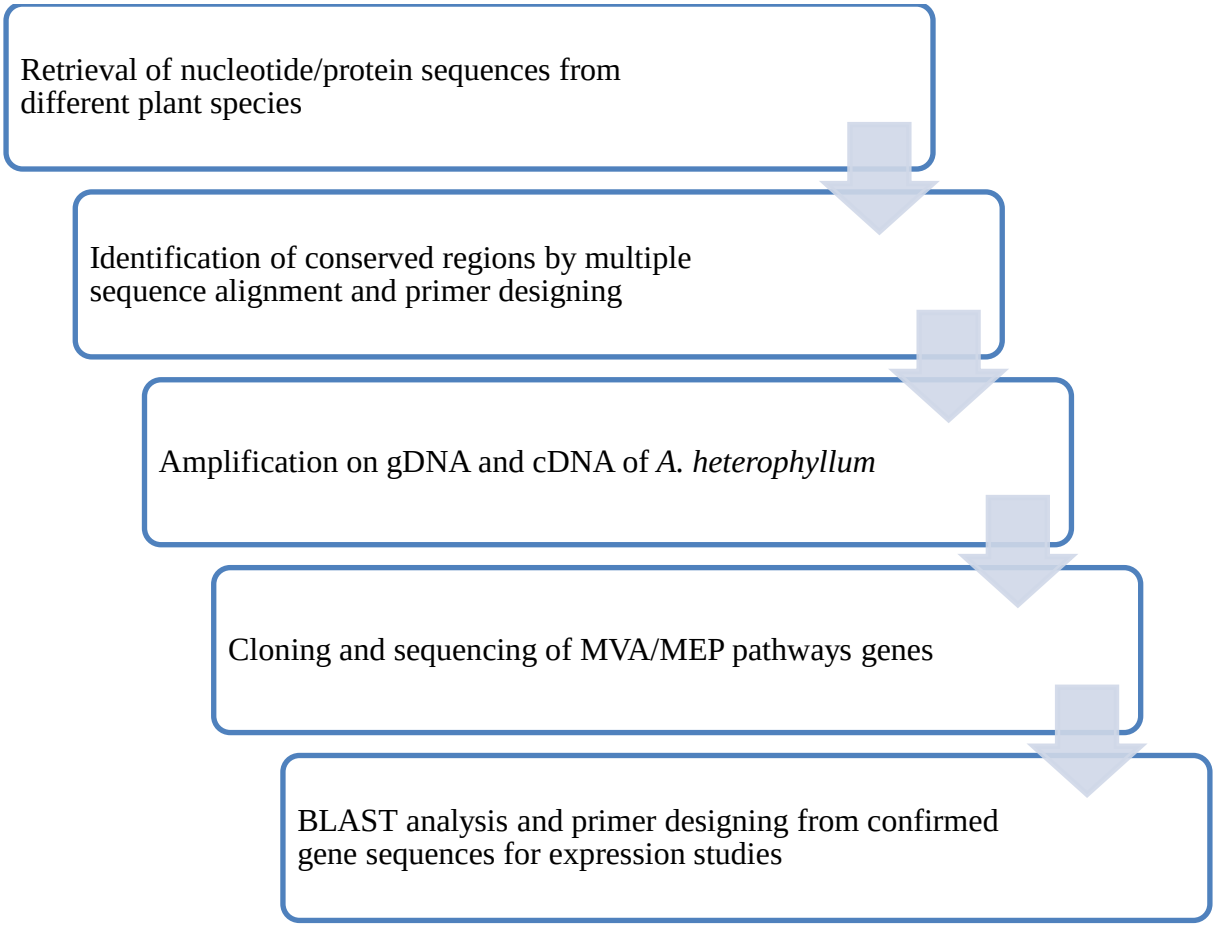

Fig. 3.3 Cloning of MVA/MEP pathways genes through comparative genomics

For amplification, the PCR was performed on 30 ng of genomic DNA and cDNA separately with varying amounts of primer pairs, Mg^{2+}, dNTPs and Taq DNA polymerase. Amplification programs included 94°C for 3 min, 30 cycles of 94°C for 30 s, annealing temperature (46-60°C) for 45 s, 72°C for 2 min and a final extension of 7 min at 72°C. 10 µl of each PCR product was mixed with 2 µl of 6 X gel loading dye (0.2% bromophenol blue, 0.2% xylene cyanol dye and 30% glycerol) and electrophoresed in a 1.2% agarose gel prepared in 0.5 X Tris acetate-EDTA (TAE) buffer. The gels were analyzed using gel documentation system Alpha Imager EP (Alpha Innotech Corp, USA). PCR products were cloned in pGEMT vector (Promega, USA) and sequenced. Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST) was used to calculate sequence similarities. Primer pairs for expression analysis were designed from confirmed gene sequences (Table 3.3).

Table 3.1 GenBank information on 15 genes of MVA/MEP pathways in other plant species used for cloning in *A. heterophyllum*

Gene	Plant species	GenBank ID
ACTH	*Arabidopsis thaliana*	AF364059
	Hevea brasiliensis	AB294687
	Nicotiana tabacum	AY748245
	Raphanus sativus	X78116
	Arnebia euchroma	DQ395086
HMGR	*A. thaliana*	AAR83122
	N. tabacum	AAB87727
	R. sativus	AA48610
	Lycopersicon esculentum	AAB62581
	Vitis vinifera	CAO47551
HMGS	*A. thaliana*	X83882
	Medicago truncatula	DQ452574
	Campotheca acuminate	EU677841
	Salvia miltiorrhiza	DQ243700
	Panax ginseng	GU565098

MVK	*H. brasiliensis*	KM272630
	Bacopa monierri	JQ670899
	Panax notoginseng	JQ957844
	Siraitia grosvenorii	HQ128557
	Nervilia fordii	KP280082
PMK	*A. thaliana*	NM_001198195
	S. miltiorrhiza	JN831095
	A. euchroma	EU315068
	H. brasiliensis	AF429385
	Catharanthus roseus	HM462020
MVDD	*A. thaliana*	NM_115285
	A. euchroma	DQ631830
	Picrorhiza kurroa	EU590912
	Theobroma cacao	XM_007010255
IPPI	*N. tabacum*	AB049816
	A. euchroma	DQ453138
	H. brasiliensis	AB294697
	Solanum lycopersicum	EU253957
	Periploca sepium	AB091677

DXPS	*Antirrhinum majus*	AY770407
	Tagetes errecta	AF251026
	Ricinus communis	EQ974568
	Stevia rebaudiana	FJ214107
DXPR	*N. tabacum*	DQ839130
	A. majus	AY770406
	S. rebaudiana	FJ214108
	Ginkgo biloba	AY494186
	Hordeum vulgare	AJ583446
ISPD	*A. thaliana*	AF230737
	S. miltiorrhiza	EF534309
	S. rebaudiana	DQ269452
	V. vinifera	XM_002267283
	C. roseus	DQ848671
ISPE	*A. thaliana*	AF288615
	N. tabacum	EF474476
	H. brasiliensis	FJ217703
	S. miltiorrhiza	EF534309
	S. rebaudiana	DQ269453

MECPS	*A. thaliana*	NM_180640
	M. truncatula	XM_003611014
	H. brasiliensis	AB294705
	S. miltiorrhiza	JN831097
	Citrus jambhiri	AB266583
HDS	*A. thaliana*	NM_125453
	S. miltiorrhiza	JN831098
	S. rebaudiana	DQ768749
	Artemisia annua	FJ479720
	Populus trichocarpa	EU693024
ISPH	*A. thaliana*	AAW82381
	S. rebaudiana	ABB88836
	Solanum tuberosum	ABB55395
	Oryza sativa	NP_0015467
GDPS	*N. tabacum*	EF382626
	H. brasiliensis	AB055496
	S. rebaudiana	DQ432013
	T. errecta	AF251012
	Daucus carota	AB027705

Table 3.2 Primer sequences* used to clone fifteen genes of MVA and MEP pathways in *A. heterophyllum*

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Fragment size (bp)
ACTH	GGHAAYGTHCTHTCNGCHAAY	GCYTTNGGDATDGCNAGDGCN	48	450
HMGS	GATGGHGCAAGYAAAGGRAARTAYA	GGRTATTCACTNGCAAGHTTGGGC	48	400
HMGR	TWYTNGGRCARTGYTGYGARATG	AGRTTYTTRAGCRTGTTYAGCTCBAC	60	600
MVK	TCAGTCTAAGACGAGACCTCA	TCGAGAGCCCATTTATTTAG	48	400
PMK	TGGMWGTRGTKGCBTCKGCKCCKGG	GTMARAGGSAGWCCACRHGCTTCAA	60	400
MVDD	GTAACTCTGGATCCTGACCACCT	CACATGAGCGAACTTCCTTAAGAT	48	400
IPPI	ATTRCTYCAGCAWCGTTCTGC	AGRAGRTAATCYAATTCYTGT	52	350
DXPS	GCTCYCCCAYATGDTCGMCCT	TGSTAYAGCTGAHCAGCATGC	57	400
DXPR	TGGTCYGTTTGTTCYTCCTC	GGCACYAAGAAYTCCGGTCA	57	400
ISPD	GAGAAAAGTGTHTCTGTGRTTCTTYG	RATNACCTGWGGWGTYTGCATTTCC	53	650
ISPE	CCYTGMAHGWTAMATGTYTTCTT	AGYWAYTGTACAWTDTGCTGC	47	500
MECPS	ATCTATAGCGGCAAACCTACAC	ACTTTAGAGAGGGATGGAGGG	59	350
HDS	ATGCCYTTTAAGGAYCTKGCAACWG	GGAGCACCACCRACATAHCCAAART	46	400
ISPH	GGNTTYTGYTGGGGNGTNGA	CCNARDATRTARTCRCANACRTA	47	500
GDPS	GARTCCATGMGGTAYTCYCTT	CACYACYTGAAAMAGMARYCC	60	350

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

Table 3.3 Primer sequences used in expression analysis

Gene/Accession number	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Fragment size (bp)
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58.5	500
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56	215
ACTH/KF961192	AACCACCACTCTTTGTTGTC	TTTGAGATTGCTTTTGGAAT	52.5	110
HMGS/KF961193	GCAAGCTTTCTCAGACTTGT	CAGAAACTCTGTAGGCAACC	54	110
HMGR/KF961194	CGCTTACTTTCTCCTCCAC	TAAAGCAAATCGGAACCATA	52	120
MVK/KF961195	TCGTTTCTTTCTTTCTCAGC	TGAGACATTTGATTCCACAA	50	110
PMK/KF961196	ATTTCAAGCTCGTTCTTCAG	GAAGCCAAATTCCAAGATAC	48	100
MVDD/KF961197	CTCCCTCAGACACTCACAGT	CAGTAGGGAAAATGGTAGGA	56	120
IPPI/KF961198	GAAAGTTTCAAACCCTCCTC	ACGACGATAGTACCGATAGC	51.5	110
DXPS/KF961199	TTGCACTTCATCAATTCAAC	ATCTTGAAGAAGTTGCGATG	55	100
DXPR/KF961200	AGTATGTGTTGGTCCCAAAG	GGAATTTCTGGCTTGTGTAG	49	100
ISPD/KF961201	TCCTGTTGTTGTACAGGTCA	ATGAGAGGTTTGGAGGGTAT	54	120
ISPE/KF961202	GTTGGCACCTTCTTATCAAC	TTCTTGGAGCTCTTTTCTTG	50.5	110
MECPS/KF961203	GATTTTATTTGCCATCAAGC	TGGTTTCTCTGAAGCAGATT	54	100
HDS/KF961204	TTCGTTTTACCACCGTACTT	TCGAGAGATAGAGGGACAGA	58	120
ISPH/KF961205	GCTCAGTGTCATCAAACAAA	GTAGCCAGTTCTCTTTCTCG	50.5	120
GDPS/KF961206	TGCTTGGTTACAAAAATCCT	ACAGATGCGTACGAGAAAAT	51	170

3.6 Expression analysis of MVA/MEP pathways genes

3.6.1 cDNA synthesis and quantitative real-time PCR (qPCR) analysis

The cDNA was prepared from 5 µg of RNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (Bangalore Genei) with oligo-dT primer. Equal sample quantities were verified by measuring the amount of RNA with a spectrophotometer. The cDNAs were then separated by electrophoresis, stained with ethidium bromide to further verify equal concentrations. The qPCR was performed using gene specific primers in triplicate on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with the iScript one step RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 48-59°C, followed by one elongation step for 20 s at 72°C. 26S rRNA and GAPDH were used as internal controls for calculating transcript abundance. The significant differences between treatments were statistically evaluated by standard deviation.

3.6.2 Principal component analysis (PCA)

PCA is a technique that takes a set of correlated components and linearly transforms those components into a set of uncorrelated components. It was performed to correlate the expression of pathway genes and amount of alkaloids as per the method of Hotelling [95]. The PCA analysis determined relationships between each module of roots versus shoots (C1), high aconites content accession (C2), low aconites content accession (C3) and their assessments for all the three conditions for all 15 genes of MVA/MEP pathways. The analysis of three correlated components into three uncorrelated components was done without any loss of information. Furthermore, the proportion of each component as a measure of variance by each component was examined.

3.7 Selection of tuberous root developmental stages

Tuberous roots are known to be the storehouse of starch and aconites in *A. heterophyllum*, therefore, understanding their growth and development was a perquisite to plan any genetic intervention strategy for increasing biomass/metabolites in *A. heterophyllum*. Seeds of *A. heterophyllum* were germinated in the nursery of HFRI under natural conditions. As the plant starts developing tuberous roots immediately after seed germination, roots of different age

groups (6-36 months old) comprising young, intermediate and mature stages were harvested. The root growth stages were classified as R1, R2, R3, R4 and R5 for 6, 12, 18, 24 and 36 months old tuberous roots, respectively (Fig. 3.4).

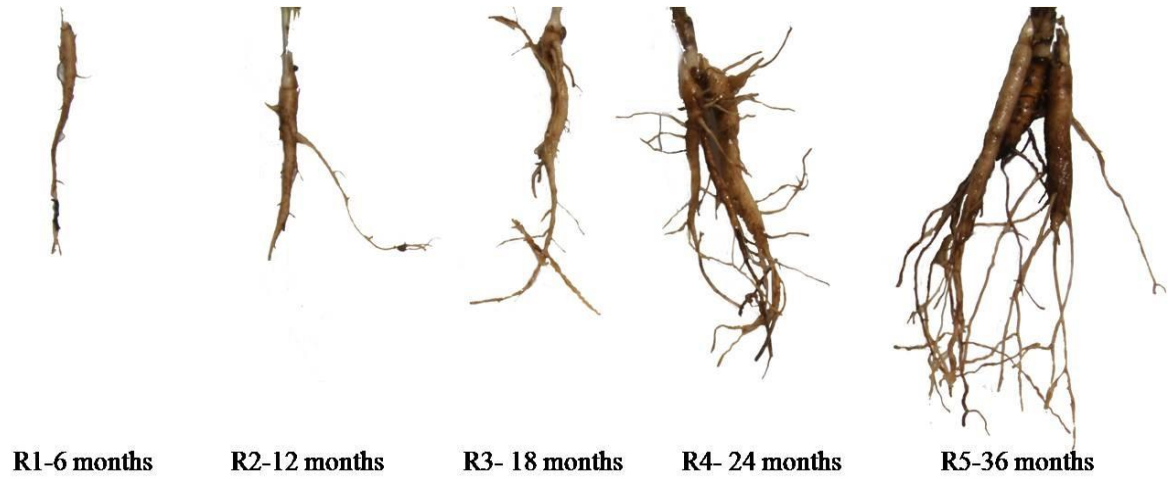

Fig. 3.4 Tuberous root developmental stages in *A. heterophyllum*. Tuberous roots of 6 and 12 months old (young stage) are represented by R1 and R2; 18 and 24 months old (intermediate stage) are represented by R3 and R4; 36 months old (mature stage) are represented by R5

3.8 Generation of *A. heterophyllum* transcriptomes

A genetic intervention strategy would require information on candidate genes contributing to the formation of tuberous roots. Generating whole genome transcriptome of *A. heterophyllum* would, therefore, be an ideal staring point to capture genetic components contributing to traits of economic importance. Hence, root and shoot transcriptomes of *A. heterophyllum*, designated as AHSR and AHSS, respectively were generated by Illumina paired-end sequencing technology (Table 3.4). It was done by Xcelris Labs Ltd (India) as per protocol. Illumina TruSeq RNA Library was prepared starting with mRNA fragmentation followed by reverse transcription, second-strand synthesis, pair-end adapter ligation, and finally ended by index PCR amplification of adaptor-ligated library. Quantification and qualification was performed on Caliper Lab Chip GX using HT DNA High Sensitivity Assay Kit.

Table 3.4 Transcriptome assembly statistics for *A. heterophyllum*

Description	Root transcriptome	Shoot transcriptome
Best kmer	kmer-51	kmer-43
Number of transcripts	75,548	39,100
Total transcript length	52,568,225	34,586,788
Transcript N50	1,059	1,239
Max transcript size (bp)	12,376	19,757
Min transcript size (bp)	200	200
GC content (%)	42	42

3.8.1 Transcriptome mining for identification of tuberous root development genes

Total 18 genes were selected for investigating their role in tuberous root development in *A. heterophyllum* (Table 3.5). The criterion for selection of genes was on the basis of various physiochemical processes required for tuberous root growth and biomass enhancement in other plant species. FPKM approach was used to detect expression level of even poorly expressed transcripts using fragment count. The transcripts sharing maximum homology with functionally characterized sequences were selected on the basis of transcript abundance and literature survey. The transcripts were analyzed by using BLASTX algorithm with E-value threshold of 1e-5.

Table 3.5 Genes implicated in root biomass productivity in different plant species

Category	Sub-category	Genes	Plant species	Source
Tuberous root formation		GMPase SHAGGY NOP10 Expansin Early Nod RBX1 MAP kinase SRF	*Ipomoea batatas* *Manihot esculenta* *Rehmannia glutinosa*	[88-89, 96-100]
Primary metabolism	Starch pathway	AGPase β-amylase	*M. esculenta* *I. batatas*	[90, 101]
	Photosynthesis	PEP C POP PC RCA	*Oryza sativa* *Solanum tuberosum*	[102-103]
	Hormonal regulation	HOG1 ARF2	*Arabidopsis thaliana*	[104-105]
	Transcription factors	NAC1 ANT	*A. thaliana*	[106]

3.8.2 Transcriptome mining for identification of ABC transporters

ATP binding cassette (ABC) transporters constitute largest class of protein families implicated in the transport of various metabolites. Hence, for the identification of ABC transporters, AHSR and AHSS transcriptomes were subjected to functional annotation. It was performed by aligning them to non-redundant database of NCBI using BLASTX program. Domain architecture present in a protein can be used to predict its functional class; hence, for identification of ABC transporters, the protein sequences were scanned for their domain architecture using Pfam domain database. The comparative analysis of AHSR versus AHSS transcriptomes for ABC transporters resulted in identification of 5 transcripts specific to tuberous root development in AHSR transcriptome.

3.9 Expression analysis of tuberous root development genes

3.9.1 cDNA synthesis and qPCR analysis

Primer pairs for 18 genes were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and tested on genomic DNA and cDNA of *A. heterophyllum* (Table 3.6). cDNA synthesis was done using Verso cDNA synthesis kit (Thermo Scientific) from total RNA (5 µg) as per manufacturer's instructions. The cDNAs were then separated by electrophoresis, stained with ethidium bromide to further verify equal concentrations (100 ng each). The reaction was performed in triplicate on a CFX96 system (Bio-Rad) with the iScript one step RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 48-52°C, followed by one elongation step for 20 s at 72°C. 26S rRNA and GAPDH were used as internal controls for calculating transcript abundance. The significant differences between treatments were statistically evaluated by standard deviation.

Table 3.6 Primer sequences used in qPCR analysis of tuberous root development genes

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Fragment size (bp)
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58	500
GAPDH	TTGCCATCAATGACCCCTTCA	CGCCCCACTTGATTTTGGA	56	215
GMPase	TTGAGGCCTTTGACACTCAG	ATCTTGATCCCAACCTTTGC	50	140
SHAGGY	ACGACCAAGTGGATCAACAA	TCTGCCCACTTGAGATCTTG	49	170
NOP10	AGCAAGGAGAGGAGAAACCA	AGATGAGCACAACTGCCAAG	50	170
Expansin	ACTCCCACCTCCACTAATGC	TTCTAGTCTGGAGCCCAGGT	50	120
Early Nod	TACATTGTCGCTCGAGGAAG	AAAGCGACCAAGCGAAGTAT	48	100
RBX1	CTGCAATTGAATCCCCTTTT	TTTTCCGTTGCTCAGTCAAG	51	110
MAP K	TACCACGATCCCTCTGATGA	TCTTGTTCGTTCGCTTTGTC	50	100
SRF	CCTAAGCCCAACGACAATTT	AGGCTTCAGAAGGAGGTTGA	50	100
β-amylase	CTGAGTGCAGGTTGGAGAGA	TTGGTGGTCCGTCTTTGTTA	50	140
AGPase	CAATTGGTGGATGCTACAGG	TATATGTGCGTGCAATGTGG	50	150
PEP C	GCCATCGAGATGATGTATGC	TGGTCCTGATCTTCCAACAA	49	150
POP	TGAGCACCGTTGTTTCTAGC	GGGTCAACAGGAGAAGTGGT	48	110
PC	TCCCAACAACTTTACGGTGA	TAGAAATCTTGGCAGCATCG	52	110
RCA	GTGGGGTTTGGAAGAAGAAA	TTGCAAATGCTACCAACCAT	50	110
HOG1	AGACCCTGCAGGAGTACTGG	TGGATCAAAAGCGTAGCATC	50	110
ARF2	TCCACCCAGTTGTAAGCAAA	GAATCCACCTTGGCAAGAAT	50	110
NAC1	TTCACCACCCTCAAGAACAA	ACCCTAAGAAAGGAGCAGCA	52	170
ANT	CCTTCACCCATCCTCAGATT	CCAGTAGTGGAGGTGGAGGT	50	120

3.9.2 Statistical analysis

Cluster analysis was done for gene expression profiles obtained from qPCR data with the help of K-means clustering and Agglomerative hierarchical clustering (AHC) methods [107]. Its aim was to establish correlation between abundance of the transcripts and the corresponding tuberous root developmental stages. K-means clustering aims to partition N observations into K clusters in which each observation belongs to the cluster with the nearest mean, serving as a prototype of the cluster. AHC generates dendrogram which represents a hierarchy of partitions. It is then possible to choose a partition by truncating the tree at a given level, the level depending upon user-defined constraints.

3.10 Expression analysis of ABC transporter genes

Primer pairs for 5 genes were designed using Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) and tested on genomic DNA and cDNA of *A. heterophyllum* (Table 3.7). The cDNA was prepared from 5 µg of RNA (RNA was treated with 2U of DNase I), reverse transcribed by using M-MuLV reverse transcriptase (GeNei™) with oligo-dT primer. Equal sample quantities were verified by measuring the amount of RNA with a spectrophotometer. The cDNAs were then separated by electrophoresis, stained with ethidium bromide to further verify equal concentrations. The reaction was performed in triplicate on a CFX96 system (Bio-Rad Laboratories, Hercules, CA, USA) with the iScript one step RT PCR kit (Bio-Rad). The PCR protocol was as follows: denaturation for 5 min at 94°C, followed by 40 cycles each of denaturation for 20 s at 94°C, annealing for 30 s at 51-59°C, followed by one elongation step for 20 s at 72°C. 26S rRNA was used as internal control for calculating transcript abundance. The significant differences between treatments were statistically evaluated by standard deviation.

Table 3.7 Primer pairs used in qPCR analysis of ABC transporter genes

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Annealing temperature (°C)	Fragment size (bp)
26S	CACAATGATAGGAAGAGCCGAC	CAAGGGAACGGGCTTGGCAGAATC	58.5	500
ABC1	TATGTCAGGTGGATGGCGTA	AATCTTGGGAATGGGAAACC	55.5	110
ABC2	GCCACAAAACTGAGGGAAAA	TTTGGGTTTCGATGAAGAGG	54	120
ABC3	AGGTGCTTGGGGAACTCTTT	TGTCGATGTCTTCCCTTTCC	55	110
ABC4	AGAGGCGGAGCCATTTATCT	AGCTGCTCTAGCTGCTTCGT	58.5	150
ABC5	CCAGAGTAGGCGAACGAGAC	CGCCGGTACATACTCGAACT	51	110

CHAPTER 4

RESULTS AND DISCUSSION

The findings of the current investigation are explained under the following headings:

4.1 Quantification of atisine and total alkaloids content

4.2 Cloning and sequencing of MVA/MEP pathways genes in *A. heterophyllum*

4.3 Expression analysis of MVA/MEP pathways genes vis-à-vis atisine/aconites content

4.4 Identification of genes associated with tuberous root formation in *A. heterophyllum*

4.5 Expression analysis of tuberous root development genes through qPCR

4.6 Identification of ABC transporters

4.7 Expression status of ABC transporter genes in root versus shoot transcriptomes

4.1 Quantification of atisine and total alkaloids content

Different growth and developmental stages of *A. heterophyllum* were discerned to know which particular stage accumulates higher content of atisine. Atisine content was estimated in root and shoot tissues of different age groups. Atisine content in roots increased from 0.14% in 1 year old plants to 0.22% in 2 years old plants and then decreased to 0.08% in the roots of 3 years old plants (Fig. 4.1). High atisine content in roots of 2 years old plants suggested that roots of this stage are suitable raw material for the preparation of herbal drug formulations. Although the precise reasons for variation in atisine content in roots of different ages are not clear, we assumed that concentration of atisine decreased in 3 year old roots due to seizure of certain metabolic processes as plant attained maturity with the passage of time. Being a biennial plant, *A. heterophyllum* will pursue towards senescence, thereby causing decline in atisine content. However, contents of secondary metabolites are known to increase with the age of storage organs. For example, picrosides, podopyllotoxin and berberine content were found to increase in roots with increase in age of *Picrorhiza kurroa* [108], *Podophyllum hexandrum* [109] and *Berberis darwinii* [110], respectively. Atisine was not detected in shoots of *A. heterophyllum*, thereby, suggesting that its biosynthesis and accumulation occur only in the roots.

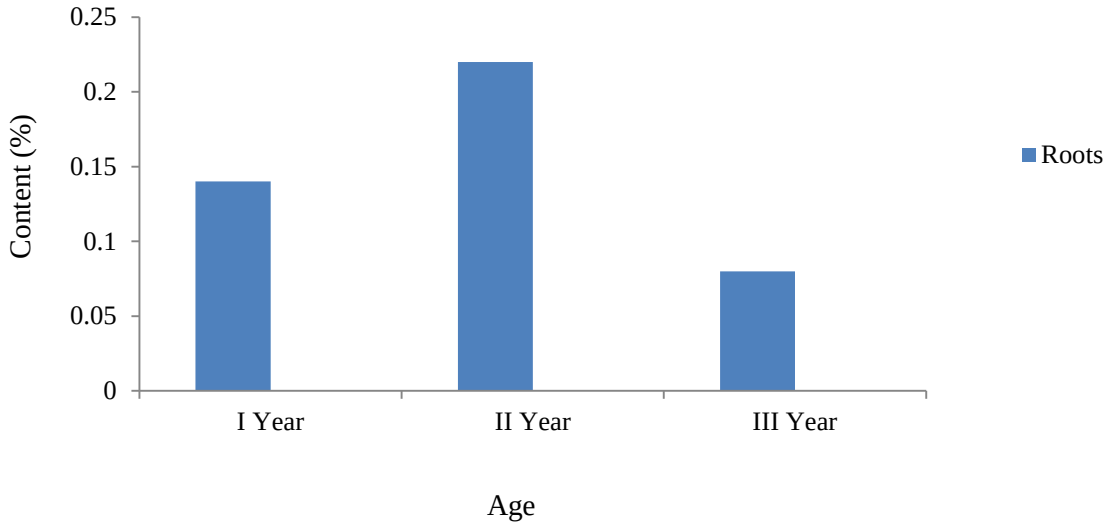

Fig. 4.1 Atisine content in roots of different ages in *A. heterophyllum*

For the identification of elite chemotypes with high content of aconites, 14 accessions of *A. heterophyllum* (2 years old) were screened for variation in concentration of aconites. Atisine content in roots of 14 accessions ranged from 0.14-0.37% and total alkaloids (aconites) from 0.20-2.49% (Fig. 4.2). Two accessions, namely AHCR and AHSR showed the highest atisine content of 0.30% and 0.37% as well as the highest total alkaloids content of 2.22% and 2.49%, respectively. The differences in atisine/aconites content among different accessions of *A. heterophyllum* are most likely due to variation in their genetic potential, not the environmental factors because all accessions were planted under controlled conditions in the green house so as to provide uniformity in growing conditions. Most of the studies on characterization of variation in chemical constituents in different plant species have been done in accessions collected from different geographical locations that differ for altitude and environmental components, thereby, limiting the determination of genetic variations. The effect of climate, altitude and geographical components have been found to significantly influence the production of metabolites in plants such as hyperforin and hypercin content in *Hypericum perforatum* [111], picrosides content in *P. kurroa* [112], camptothecin content in *Nothapodytes nimmoniana* and reserpine content in *Rauwolfia serpentina* [113].

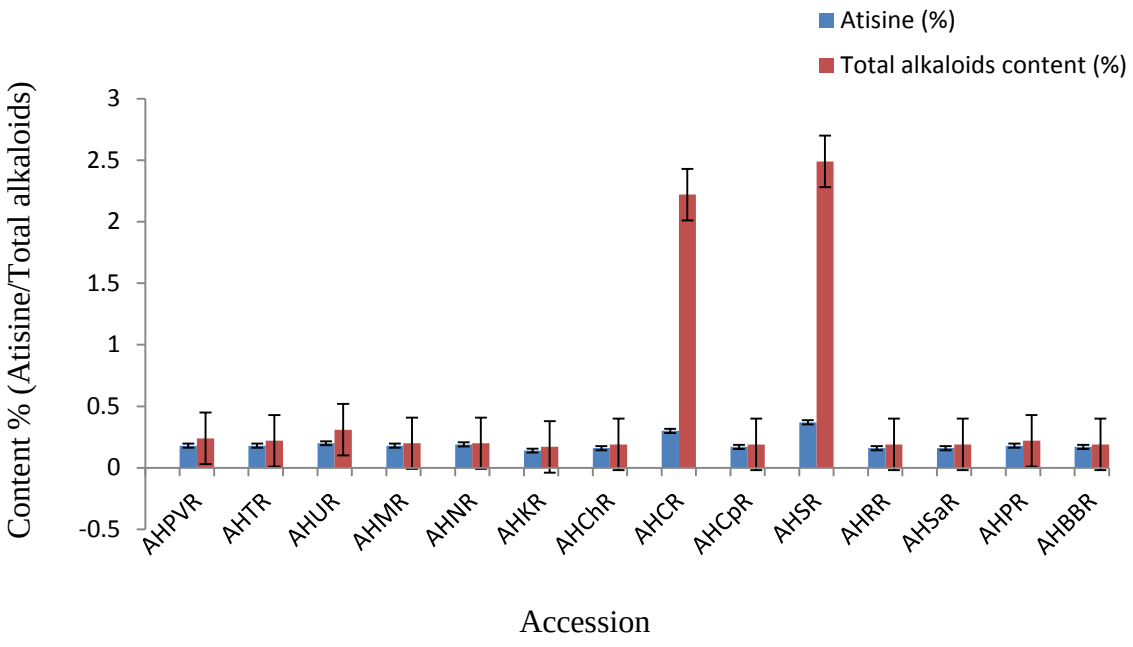

Fig. 4.2 Atisine and total alkaloids content in different accessions of *A. heterophyllum*. AHSR (0.37% and 2.49%) and AHCR 0.30% and 2.22%) represents highest atisine and total alkaloids content accessions. Total alkaloids include aconitine, indaconitine, atidine, atisine, heteratisine, heterophyllinine, hetidine and hetsinone

4.2 Cloning and sequencing of MVA/MEP pathways genes in *A. heterophyllum*

To determine variation in the genetic make-up of high and low aconites content accessions of *A. heterophyllum*, partial gene sequences of 15 genes encoding enzymes of MVA and MEP pathways were cloned from *A. heterophyllum* through comparative genomics using degenerate primers designed from conserved gene regions of the nucleotide sequences retrieved from the GenBank (see Materials and methods). Multiple sequence alignments of PMK gene from MVA pathway and MECPS gene from MEP pathway have been shown in Figs. 4.3 and 4.4, respectively. The amplicons for all gene fragments, ranging from 350-650 bp, were sequenced and annotated for biological function (Fig. 4.5). The partial sequences of all 15 genes have been submitted to the GenBank under accession numbers KF961192-KF961206. The sequences were utilized for designing primers for gene expression analysis.

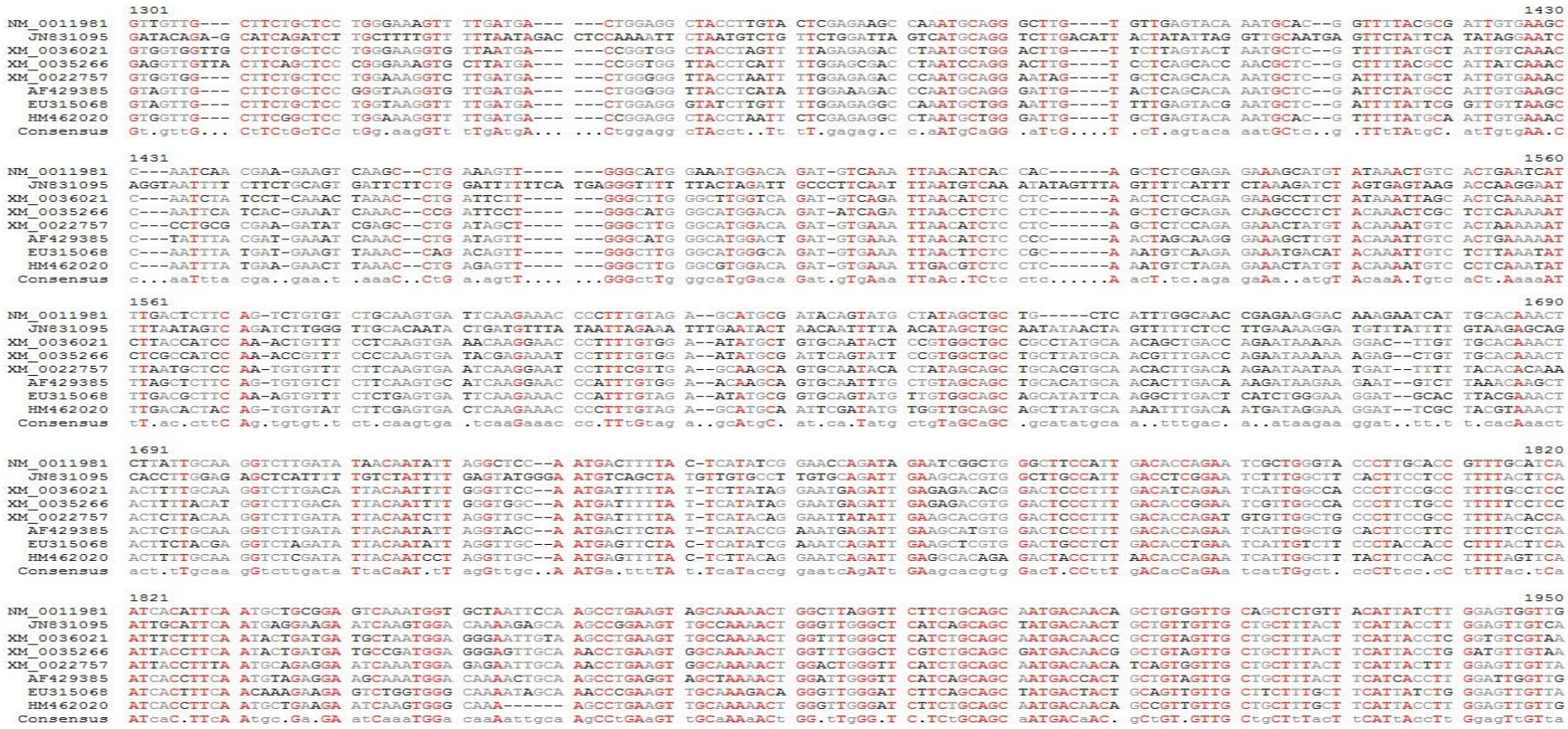

Fig. 4.3 Multiple sequence alignment of PMK gene from different plant species. Red color indicates the conserved sequence region

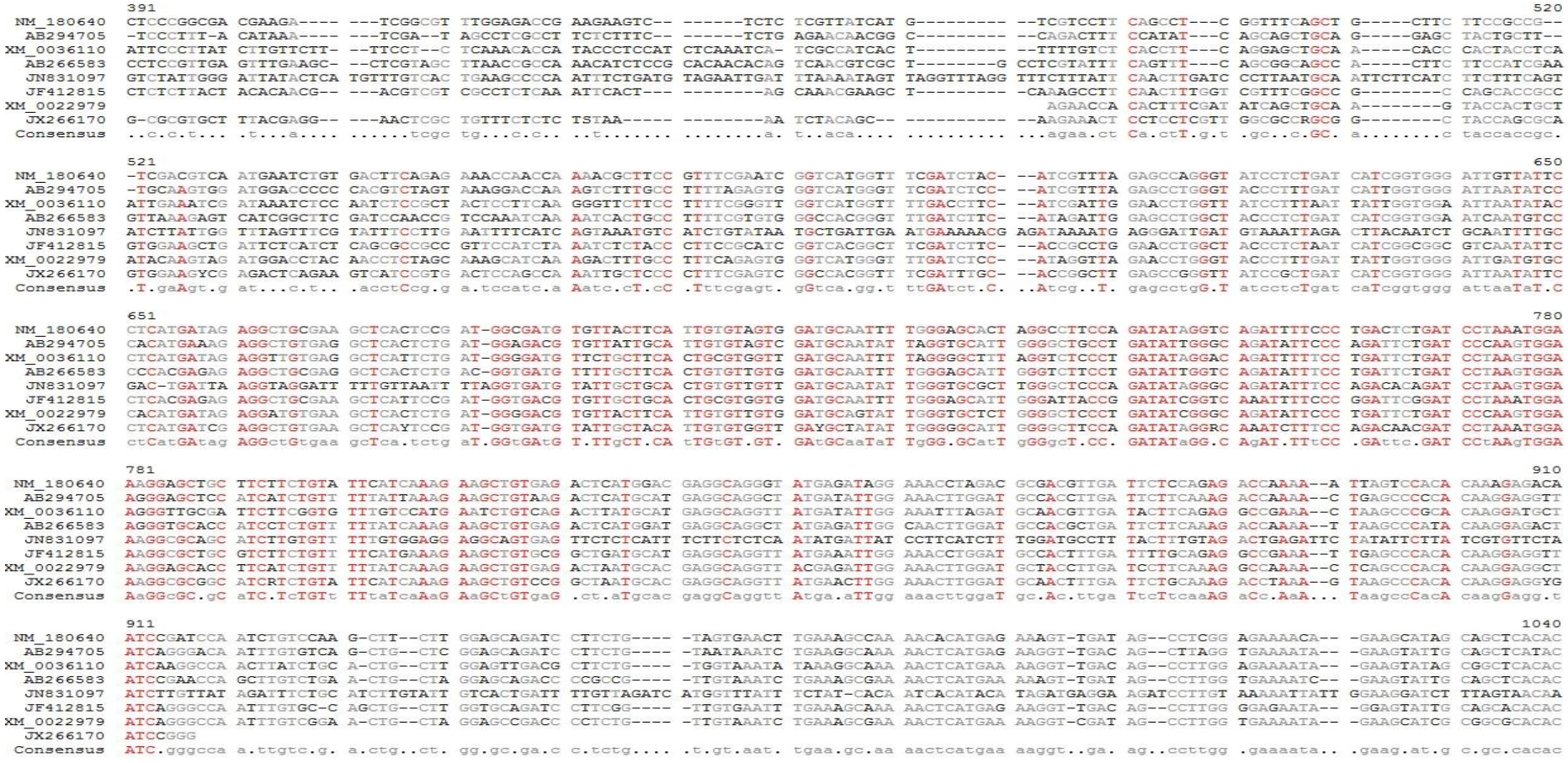

Fig. 4.4 Multiple sequence alignment of MECPS gene from different plant species. Red color indicates the conserved sequence region

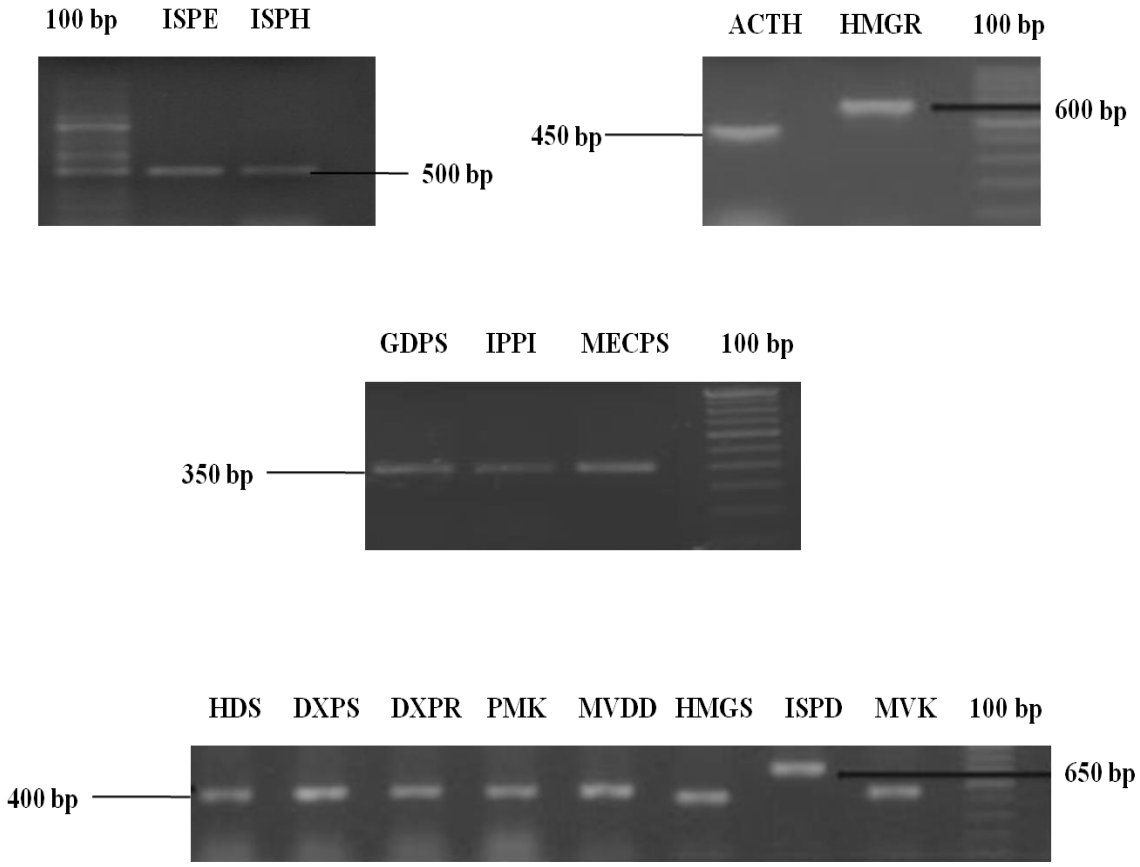

Fig. 4.5 Amplification of MVA/MEP pathways genes on genomic DNA of *A. heterophyllum*

4.3 Expression analysis of MVA/MEP pathways genes vis-à-vis aconites content

qPCR analysis revealed that most genes of MVA and MEP pathways had relatively higher expression in roots of *A. heterophyllum* (AHSR) compared to shoots. Four genes of MVA pathway; HMGS, HMGR, PMK, IPPI, and 4 genes of MEP pathway; DXPS, ISPD, HDS, GDPS showed elevated levels of transcripts in roots (11-100 folds) compared to shoots (Fig. 4.6). The transcript of HMGR gene showed highest increase (96-fold) along with IPPI gene with 39-fold in roots. Relatively higher expression of genes coding for HMGR and IPPI enzymes was positively correlated to their role in shikonin plastidial monoterpenes biosynthesis in *Arnebia euchroma* [114], *Lithospermum erythrorhizon* [115] and petals of Snapdragon flowers [116]. The transcript of DXPS gene increased 30-fold in roots compared to shoots. The MEP pathway is regulated by the activity of DXPS, the first enzyme in the MEP pathway [117-118]. DXPS is known to be involved in the production of DXP which is further bestowed in plastidic isoprenoid biosynthesis [118] along with thiamin and pyridoxol

production [119-120]. DXPS as a candidate gene has been shown to be responsible for the monoterpenoid production and Muscat flavour in *Vitis vinifera* [121]. Additionally, over expression of this gene upregulated monoterpene production in *Nicotiana tabacum* [121]. The gene coding for GDPS enzyme showed 39-fold transcript increase in the roots of *A. heterophyllum*. The biosynthesis of tanshinones in roots of *Salvia miltiorrhiza* has also been found to be regulated by GDPS enzyme [122].

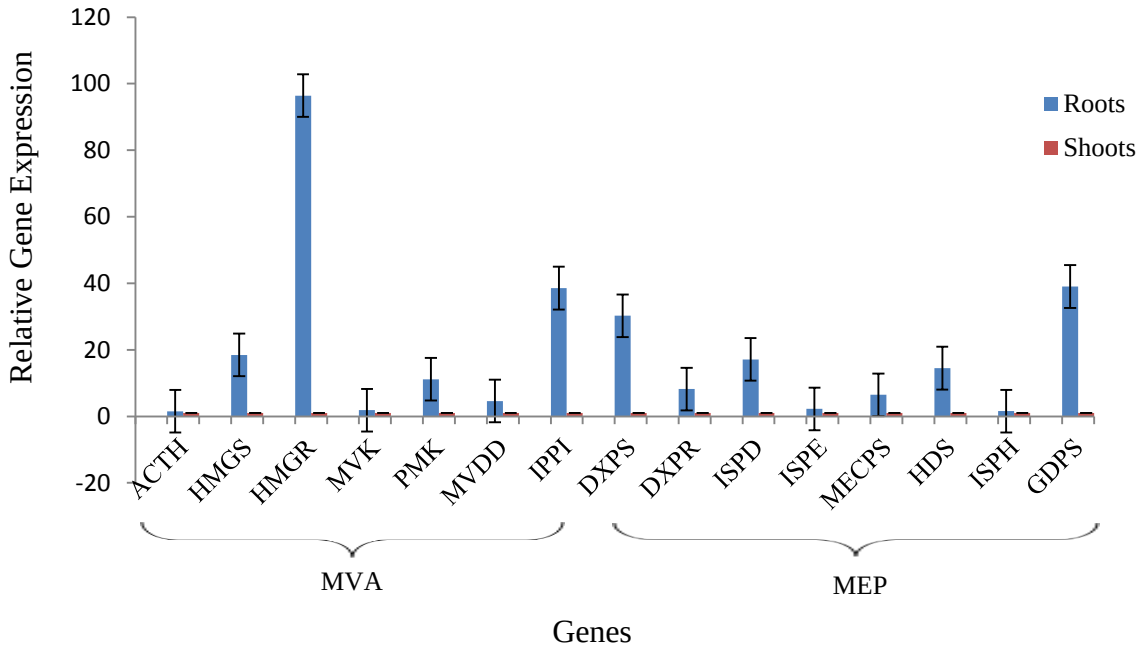

Fig. 4.6 Expression status of MVA/MEP pathways genes in roots (0.22% atisine) compared to shoots (0.00% atisine) of *A. heterophyllum*

The higher expression of MVA/MEP pathways genes in roots compared to shoots may be due to their involvement in the biosynthesis of several other metabolites, in addition to atisine and aconites. Therefore, to ascertain whether the elevated levels of transcripts of MVA/MEP pathways genes are only affecting the biosynthesis of atisine uniquely present in the roots compared to shoots and to minimize the tissue-type variations, the expression status of 8 genes, HMGS, HMGR, PMK, IPPI, DXPS, ISPD, HDS and GDPS along with other MVA/MEP pathways genes was further studied between roots of *A. heterophyllum* accessions that were varying for atisine and aconites contents. Two high atisine content

accessions, AHSR (0.37%) and AHCR (0.30%), and two low content accessions, AHKR (0.14%) and AHChR (0.16%) were used in comparative expression analysis. The expression of 4 genes of MVA pathway; HMGS, HMGR, PMK, IPPI, and 4 genes of MEP pathway; DXPS, ISPD, MECPS, HDS, and GDPS decreased to 5-12 folds between high versus low content roots compared to 11-100 folds between roots versus shoots. There was overall decrease in expression of genes when compared between roots of high versus low content accessions (Fig. 4.7), relative to fold increase in roots versus shoots. The expression level of HMGR and IPPI genes decreased from 96-fold to 5-fold and from 38-fold to 12-fold, respectively. Similarly, the expression of DXPS and ISPD genes decreased from 30-fold to 9-fold and 17-fold to 8-fold, respectively in roots of high content accessions (Table 4.1). The significant drop in folds expression of gene coding for HMGR enzyme compared to others was indeed an unforeseen finding since HMGR is known to act as an important control point for plant metabolites synthesis via MVA pathway [123]. The decrease in expression level of gene can be attributed to the role of HMGR as a feeder to GDP, thus regulating MVA pathway as described in previous reports on phytosterols biosynthesis [124-128]. Although the expression level of genes encoding HMGR, IPPI, DXPS and ISPD decreased significantly, the regulatory pattern remained similar to what has been observed for isoprenoid production in other plant species [129-130]. These genes have been reported to regulate the biosynthesis of isoprenoids, sesquiterpenoids, phytoalexins, alkaloids and other metabolites in *Solanum tuberosum*, *Arabidopsis thaliana*, *Lycopersicon esculentum* and *Catharanthus roseus* [118, 129-131]. Overexpression of these genes have led to increased production of terpenoids and alkaloids, whereas their suppression led to decreased amounts of end-products in *A. thaliana* and *C. roseus* [132-133]. Multiple genes such as DXPS, DXPR, ISPD, MECPS, and GDPS are known to regulate alkaloids and secoiridoids biosynthesis in *Gentiana macrophylla* and *C. roseus* [134]. The transcripts of HMGR, IPPI and ISPD have been found to regulate nicotine biosynthesis in *Nicotiana* species [135]. Overall, multiple genes of MVA/MEP pathways showed elevated expression in relation to atisine/aconites content in *A. heterophyllum*. The increased amounts of transcripts of DXPS, ISPD, MECPS and PMK genes have been correlated with increase in picrosides content in *P. kurroa* [136-137]. These genes were also implicated in the production of anticancer compounds like paclitaxel [138] and were also found to collectively regulate monoterpenes biosynthesis in peppermint oil gland secretory cells [139], apocarotenoids in monocot roots [140], and carotenoids in pepper and tomato fruit [130, 141]. The relatively higher expressions of DXPS and MECPS genes have been reported in *C. roseus* for the production

of vinblastine and vincristine [142-144]. The accumulation of artimisinin in *Artimisia annua* [15] and higher content of taxol in *Taxus media* [145] have also been correlated with the higher expression of DXPS and MECPS genes. Overall, the expression of IPPI gene was highest among all genes. The gene is well established as a basic building block for isoprenoid biosynthesis, although using different precursors in the form of other MVA/MEP pathways genes [146-147].

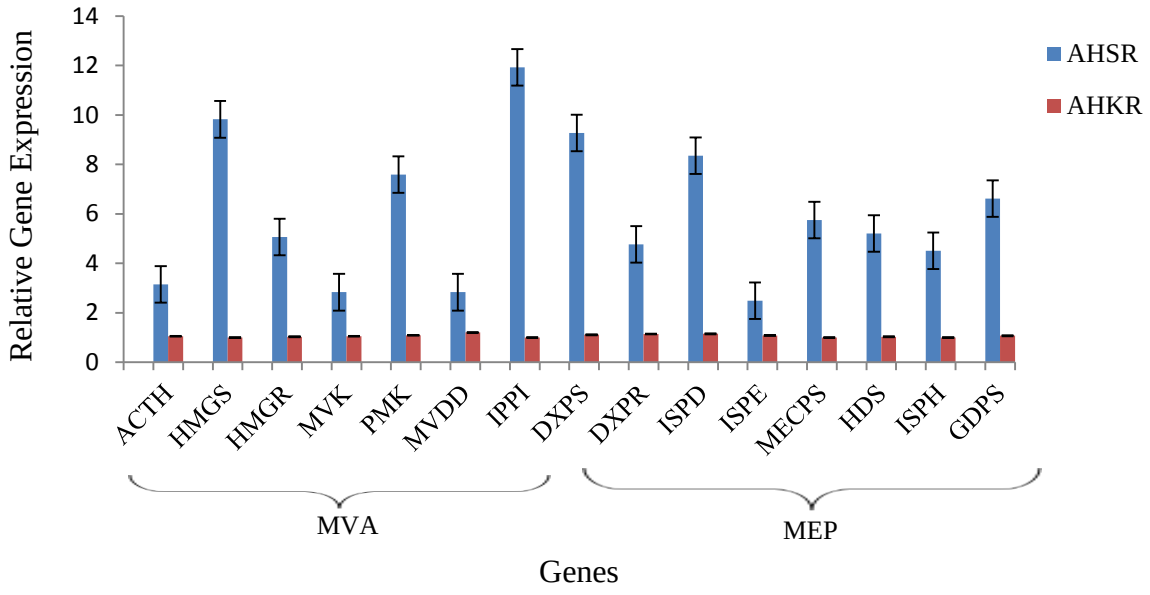

Fig. 4.7 Expression status of MVA and MEP pathways genes in roots of high (AHSR) versus low (AHKR) aconites content accessions of *A. heterophyllum*

Table 4.1 Folds difference in expression status of MVA/MEP pathways genes between A (roots vs shoots) and B (roots of high vs low content accessions)

Gene	A	B	Folds difference (X)
ACTH	1.52	3.15	+2.07
HMGS	18.47	9.82	-1.88
HMGR	96.40	5.06	-19.05
MVK	1.84	2.83	+1.53
PMK	4.61	2.83	-1.62
MVDD	11.15	7.59	-1.46
IPPI	38.56	11.92	-3.23
DXPS	30.24	9.27	-3.26
DXPR	8.20	4.77	-1.71
ISPD	17.12	8.35	-2.05
ISPE	2.22	2.49	+1.12
MECPS	6.47	5.75	-1.12
HDS	14.47	5.21	-2.77
ISPH	1.56	4.51	+2.89
GDPS	39.04	6.62	-5.89

The relative contribution of MVA and MEP pathway routes to aconitine-type alkaloids biosynthesis in *A. heterophyllum* couldn't be inferred through the comparison of folds expression pattern of genes. Previous studies have suggested that cross-talk and compartmentation of the metabolites biosynthesis occur at IPPI/DMAPP in MVA/MEP pathways where the coordinated metabolite exchange is bidirectional but the biological implications for such phenomenon are not yet fully understood [148]. The synthesis of GPP moiety via MVA/MEP pathways depends upon the metabolite and the plant system under consideration. The GPP is then converted into FPP which further forms GGPP which is regarded as the major precursor for biosynthesis of aconitine-type alkaloids as per information provided in the KEGG database (http://www.genome.jp/kegg-

bin/show_pathway?map01066). Earlier reports have shown that the plant isoprenoids like ent-kaurene, carotenoids and chlorophyll are synthesized via GGPP in plastid which is the center for MEP pathway activity [149-150]. So in order to infer the relative contribution of MVA/MEP pathways, the whole data set was arranged into 3 components with component 1 R&S (C1) for roots versus shoots, component 2 AHSR (C2) for high aconites content roots and component 3 AHKR (C3) for low aconites content roots (Table 4.2). PCA analysis of 3 components showed that C1 interacted with C2 and C3 with similar patterns while contribution of C1 was found more than other two components. C1 accounted for 94% variation among three components. While analyzing the individual genes in each component, C2 contributed maximum with 48.5% while C1 and C2 cumulatively accounted for 74.5% of the three components. Overall variability of 100% indicates successful variance of uncorrelated components without loss of any information. Plot between variability, Eigen-values and principal components indicates this scenario very well (Fig. 4.8). Biplot was generated for PCA analysis of 15 genes as observations 1-15 (Fig. 4.9). C1 was found to play an important role in both MVA and MEP pathways while C2 and C3 contributed significantly in MEP and MVA pathways, respectively. We found that both C1 and C2 are significantly associated with HMGR gene, which is analogous to previous reports where the production of alkaloids, sesquiterpenes and phytoalexins in *C. roseus* [151], *Solanum xanthocarpum* [152] and potato tubers [153] was enhanced by HMGR gene activity. While comparing data set with atisine (%) and total alkaloids content (%), we applied two-step refinement process to prove correlation amongst the observations for all 3 components and their respective genes w.r.t. squared cosine values. In the first process, we analyzed genes with the squared cosine value >0.8 and in the last process, we analyzed genes with the squared cosine value >0.9 to obtain relevance in contribution of MVA/MEP pathways. Two genes of MVA pathway; IPPI, PMK and 4 genes of MEP pathway; MECPS, HDS, ISPH, GDPS showed higher significance than other genes.

Table 4.2 Data sets for PCA analysis

Gene	R&S (C1)	AHSR (C2)	AHKR (C3)
ACTH	1.52	3.15	1.05
HMGS	18.47	9.82	1
HMGR	96.40	5.06	1.03
MVK	1.84	2.83	1.05
PMK	4.61	2.83	1.09
MVDD	11.15	7.59	1.2
IPPI	38.56	11.92	1
DXPS	30.24	9.27	1.11
DXPR	8.20	4.77	1.14
ISPD	17.12	8.35	1.15
ISPE	2.22	2.49	1.08
MECPS	6.47	5.75	1
HDS	14.47	5.21	1.03
ISPH	1.56	4.51	1
GDPS	39.04	6.62	1.07

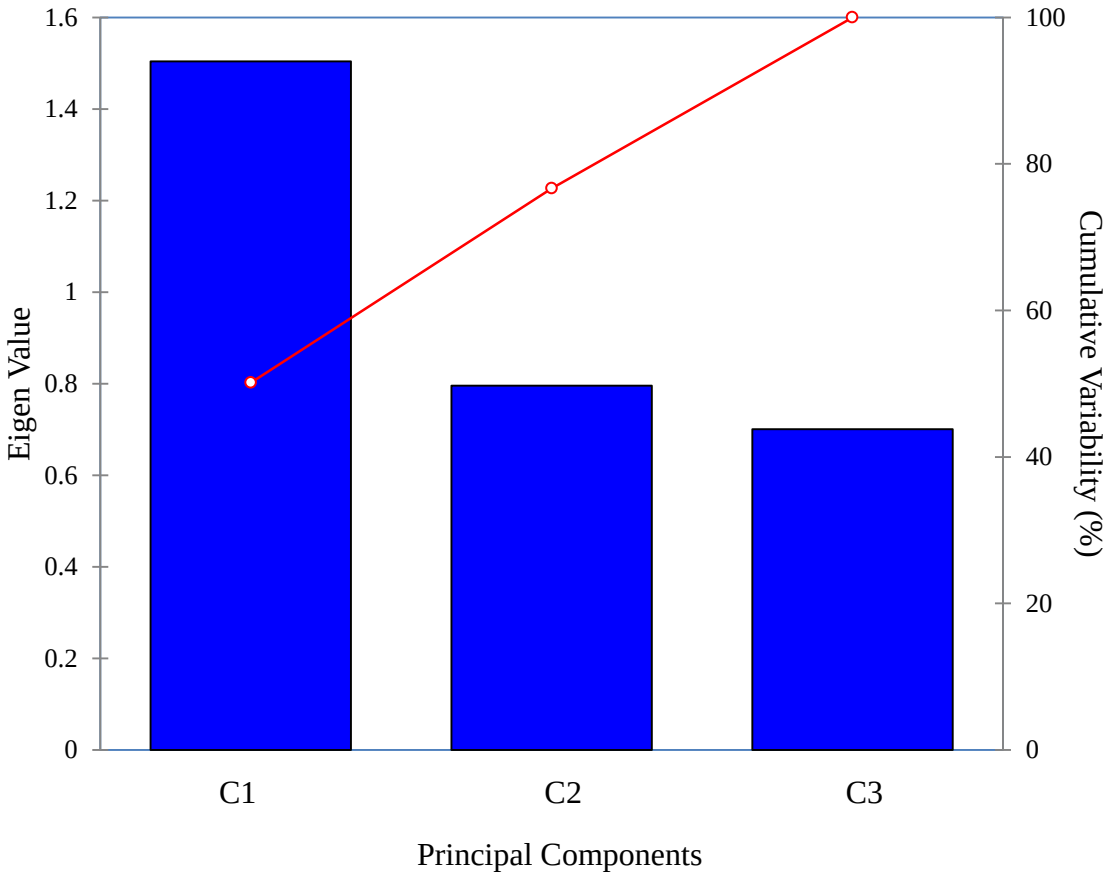

Fig. 4.8 Screen plot for principal components (C1 - C3), their respective Eigen values, and cumulative variability. C1 (R&S) shows most significant appearance in all three components as 94%; low significance levels of C2 (AHSR) and C3 (AHKR) are shown as 50% and 40% of their respective contribution

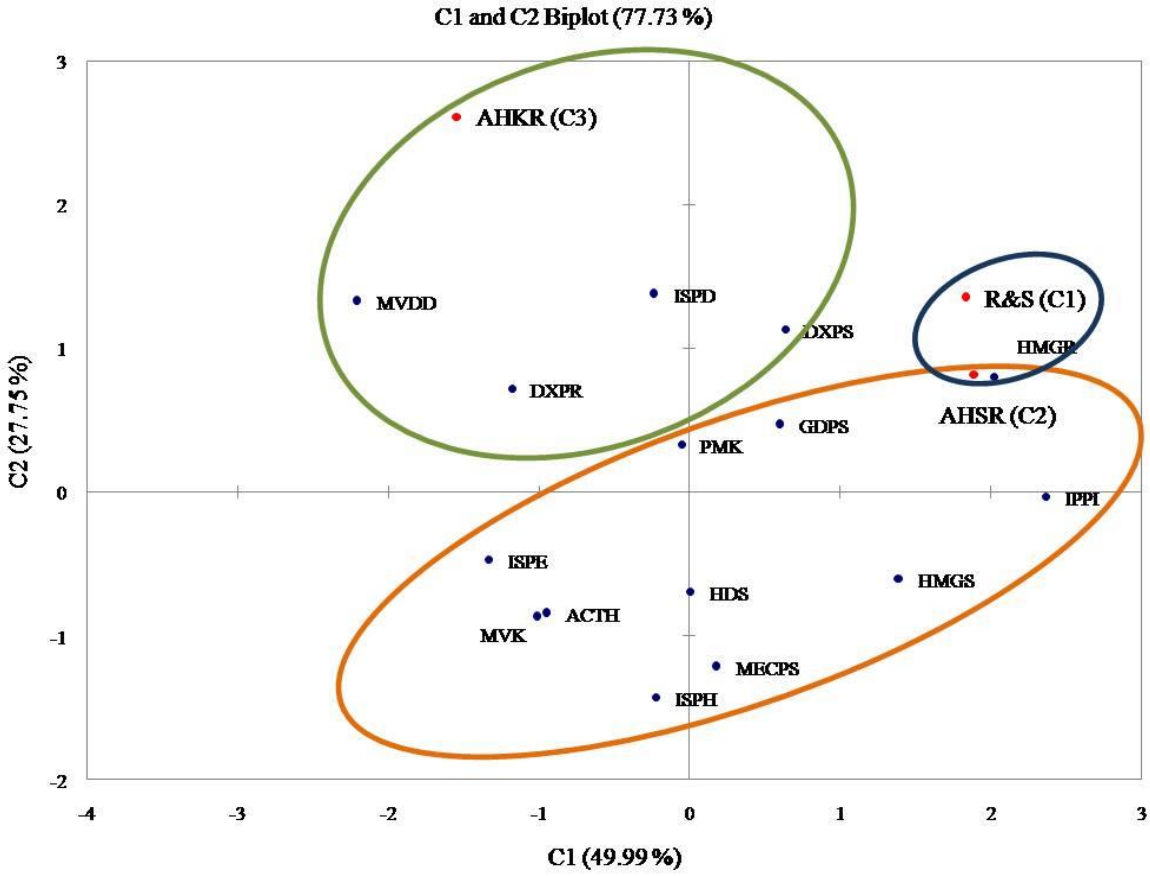

Fig. 4.9 Biplot for the PCA analysis of three different conditions; R&S (C1), AHSR (C2), and AHKR (C3). Observations for 15 genes are indicated in blue (R&S), orange (AHSR) and green (AHKR) ellipses

The qPCR in conjunction with PCA analysis implied contribution of both MVA and MEP pathways in aconites biosynthesis in *A. heterophyllum*. The combinatorial role of MVA/MEP pathways in alkaloids and diterpenoids production was correlated to their involvement in tanshinones biosynthesis in *S. miltiorrhiza* [154]. Both the pathways have been implicated in biosynthesis of withanolides, phytosterols and terpenoids biosynthesis in *Withania somnifera* [155], *Croton sulyratus* [156] and *C. roseus* [143], respectively.

4.4 Identification of genes associated with tuberous root formation in *A. heterophyllum*

Tuberous roots of *A. heterophyllum* are known to be the source of therapeutic metabolites - aconites. Hence, their formation in *A. heterophyllum* provides a distinctive system to explore mechanism of sink tissue formation and development vis-à-vis accumulation of primary

metabolites as well as aconites. The overall picture for defining parameters of tuberous root formation and development is still not clear in *A. heterophyllum*. On the basis of literature survey, genes belonging to various physicochemical processes required for tuberous root growth and biomass enhancement were identified in the transcriptomes of *A. heterophyllum* on the basis of high sequence similarity with known genes (≥40% and E-value threshold of 1e-5). In addition to 9 genes implicated in tuberous root development in different plant species (GMPase, SHAGGY, NOP10, Expansin, Early Nod, RBX1, MAP kinase, β-amylase), 9 genes belonging to starch pathway (AGPase), photosynthesis (PEP C, POP, PC, RCA), hormone metabolism (HOG1, ARF2) and transcription factor (NAC1, ANT) families responsible for biosynthesis and accumulation of primary metabolites towards biomass production in plants were identified (See Materials and methods).

4.5 Expression analysis of tuberous root development genes through qPCR

In order to gain an insight into the molecular basis of sink organ i.e. tuberous root development in *A. heterophyllum*, quantitative expression of 18 genes was studied in different tissues by using transcript abundance analysis. qPCR analysis revealed that 15 out of 18 genes of corresponding classes had relatively higher expression in roots of *A. heterophyllum* compared to shoots. Eight out of 15 genes encoding GMPase, SHAGGY, Expansin, RBX1, SRF, β-amylase, AGPase and ARF2 showed elevated level of transcripts in roots (13-171 folds) compared to shoots (Fig. 4.10). The transcripts of AGPase, β-amylase and SRF genes showed highest transcript abundance of 171-fold, 85-fold and 57-fold, respectively in roots. Relatively higher expression of genes coding for AGPase and β-amylase enzymes was positively correlated to their role in starch biosynthesis in *M. esculenta* [101, 157], *I. batatas* [88] and model crop species like *Oryza sativa* and *Zea mays* [158]. Additionally, the starch production is regulated by the activity of AGPase, the first enzyme of the starch biosynthetic pathway [100]. The transcript of SRF gene increased 57-fold in roots compared to shoots. The gene has been found to developmentally regulate storage root formation in *I. batatas* by actively initiating cell division process, thereby causing thickening of tuberous roots [98]. The higher expression of this gene has been shown to regulate plant carbohydrate metabolism in *I. batatas* [159]. The genes encoding for RBX1 and Expansin enzymes showed 24-fold transcript increase in the roots of *A. heterophyllum*. The ubiquitination activities, which are primarily active during tuberous root development, are controlled by RBX1 enzyme [99].

Similarly, Expansin is well known regulator of cell wall extension during sink organ development in *R. glutinosa* [89].

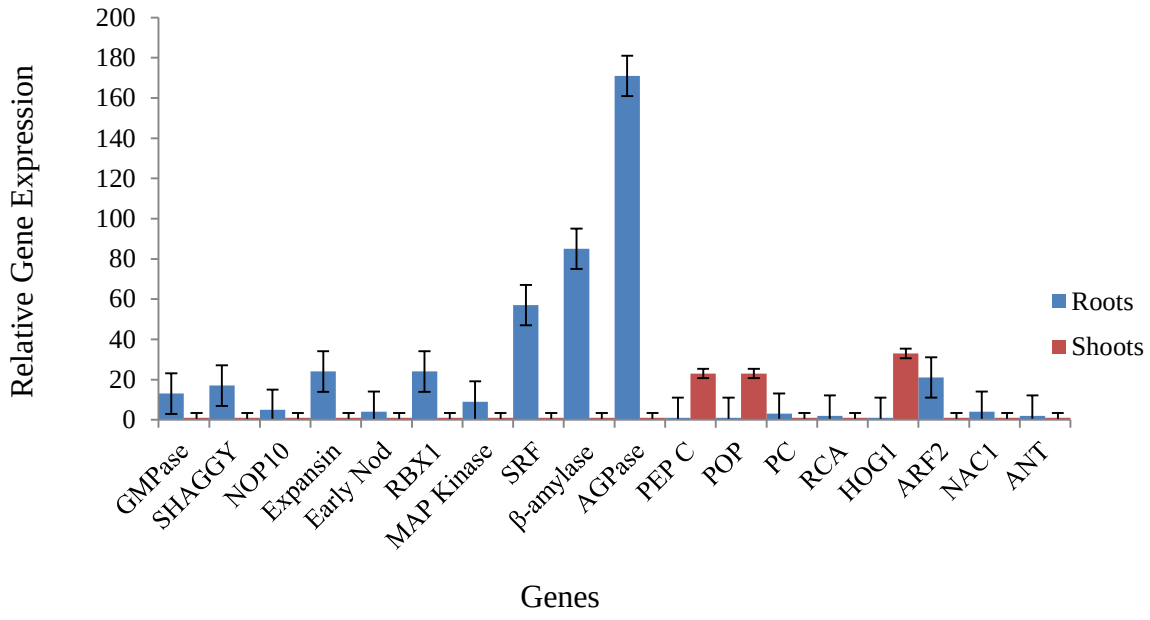

Fig. 4.10 Expression status of tuberous root development genes in different tissues (roots and shoots) of *A. heterophyllum*

Not to our surprise, 3 genes encoding PEP C, POP and HOG1 showed highest transcript abundance of 23-fold, 23-fold and 33-fold, respectively in shoots of *A. heterophyllum* compared to roots which was quiet evident as far as whole plant development is concerned. These results were comparable to the role of major pathways of primary metabolism towards plant growth and development. Photosynthesis genes, PEP C and POP have been shown to influence photosynthetic capacity and seed yield in *O. sativa* [102]. Furthermore, over expression of these genes regulated cytosolic enzymatic levels of photosynthetic pathway resulting in tuber yield enhancement in *Solanum tuberosum* [103]. Cytokinin production in *Arabidopsis thaliana* has been found to be regulated by HOG1 gene which significantly controls biomass yield [105].

The relative role of 15 genes showing higher expression in roots was further validated on different developmental stages of tuberous root formation in *A. heterophyllum*. This was done to ascertain their significance in root biomass increase by screening all stages, from young

rootlet to fully developed tuberous roots. Comparative expression analysis of tuberous root development stages (see Materials and methods) revealed that almost all genes showed increase in transcript abundance in R4 and R5 stages compared to R1, R2 and R3 stages. The results showed non-significant changes in the expression levels of 8 genes coding for GMPase, SHAGGY, NOP10, Expansin, RBX1, AGPase, β-amylase and SRF enzymes from stages R1-R3, significant increase in intermediate R4 stage (6-39 folds) and dramatic increase in transcript levels of these genes in fully developed mature tuberous roots of R5 stage (11-97 folds) in *A. heterophyllum* (Fig. 4.11). The precise reasons for variation in transcript abundance of these genes are attributed towards enhanced capacity of the sink organ to biosynthesize and accumulate primary metabolites with age of the plant. For example, carbohydrate metabolism and starch accumulation were found to increase with tuberous root development in *R. sativus* [91] and *I. batatas* [160], respectively. The transcript of AGPase gene showed 97-fold expression in R5 stage compared to R1 stage. This could be related to the formation of fully developed sink tissue with increased storage ability for accumulation of starch and other sugars [157]. Being a rate-limiting enzyme of starch biosynthesis, the expression pattern of AGPase gene was consistent with the published reports [161]. Similarly, the 28-fold increase in transcript abundance of a gene coding for β-amylase enzyme corresponds to its regulatory role in starch biosynthetic pathway [162]. The expression of SRF gene showed 28-fold increase in R5 stage compared to R1 stage. Previous reports have suggested that the mature storage roots of *I. batatas* [98] accumulate carotenoids, therefore, it is obvious that the expression of SRF gene increases with the formation of fully developed tuberous roots having utmost rate of primary metabolism. Interestingly, the change in expression pattern of Expansin transcript was indeed an astonishing finding. It showed 39-fold increase in R5 stage than R1 stage while its expression remained 24-fold in roots compared to shoots. The increase in its transcript level is attributed to its involvement in various biochemical and physiological processes for tuberous root development including root hair formation for rapid cell proliferation in various plant species [163-164]. Moreover, the transcripts coding for SHAGGY and NOP10 genes showed 24-fold and 18-fold increase in expression level in R5 stage compared to R1 stage. SHAGGY has been known to be involved in plant developmental processes [97] while NOP10 regulates mRNA splicing and ribosome biogenesis [165] but its exact role in plant processes remains to be elucidated.

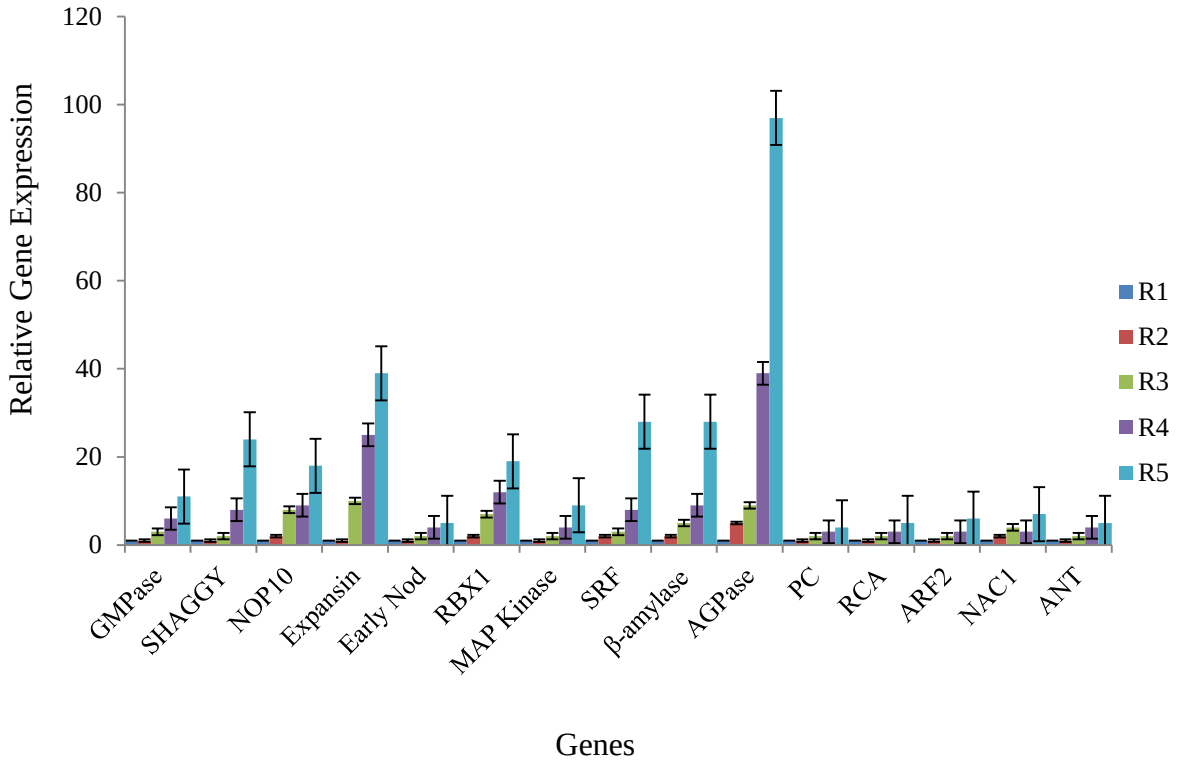

Fig. 4.11 Expression status of tuberous root development genes in tuberous root developmental stages (R1-R5) of *A. heterophyllum*

For complete corroboration of the current findings, the data set was subjected to statistical analysis. The study showed positive correlation with the gene expression data for different stages (R1-R5) of tuberous root development in *A. heterophyllum*. Clustering of the whole data set revealed close relationship between R1, R2 and R3 stages showing more or less similar expression pattern while R4 and R5 stages exhibited increase in transcript abundance as plant attained maturity. The observed profiles of 8 genes viz. GMPase, SHAGGY, NOP10, Expansin, RBX1, SRF, β-amylase and AGPase were found to be distinct and well separated from each other in R5 stage compared to R4 stage. The results obtained by K-means clustering and Agglomerative hierarchical clustering (AHC) demonstrate this scenario very well (Fig. 4.12). Profile plot through K-means clustering indicated increased profiles for R4 and R5 stages while bottom up approach for clustering in AHC clearly reflected the close proximity of R1-R3 stages. Overall, multiple genes of different families exhibited an important role in tuberous root architecture for biosynthesis and storage of primary

metabolites, thus paving the way towards increasing the biomass yield through genetic modification in *A. heterophyllum*.

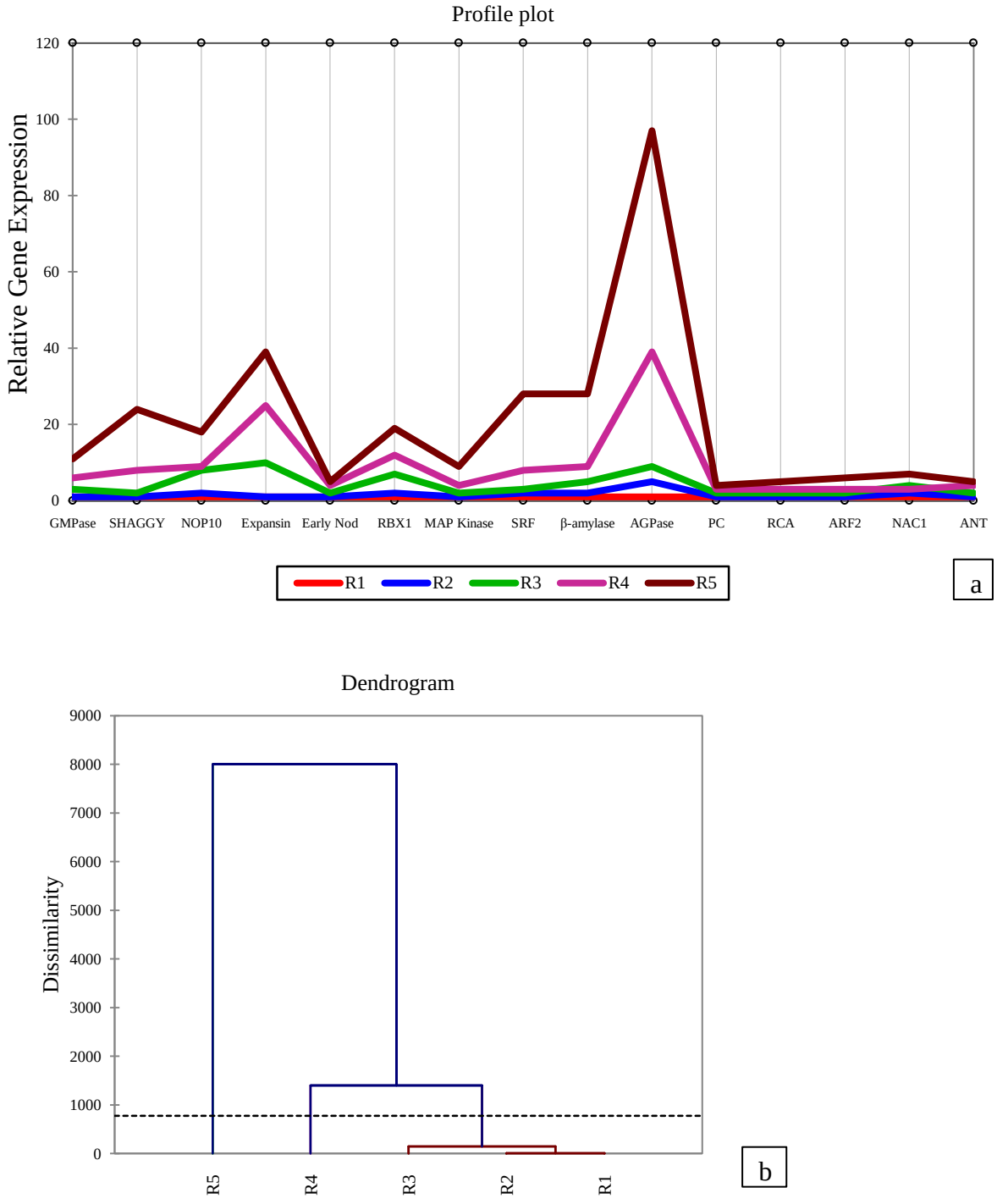

Fig. 4.12 Cluster analysis of tuberous root development-related genes in *A. heterophyllum* through a) K-means clustering b) Agglomerative hierarchical clustering

4.6 Identification of ABC transporters

ABC (ATP binding cassette) transporters constitute largest class of protein families implicated in the transport of various metabolites towards root biomass production. These are known to regulate metabolites biosynthesis and accumulation in a spatial and temporal manner [166]. They regulate transportation, organ growth, plant nutrition, plant development, response to abiotic stress and secondary metabolites production. A total of 234 and 165 protein sequences having ABC-type transporters domains were identified in AHSR and AHSS transcriptomes, respectively using Pfam domain database.

4.7 Expression status of ABC transporter genes in root versus shoot transcriptomes

The comparative analysis of AHSR versus AHSS transcriptomes for ABC transporters resulted in the identification of 9 transcripts specific to biomass accumulation in *A. heterophyllum* root transcriptome. Five out of 9 transcripts were experimentally validated through comparative qPCR analysis. The expression profile for 5 out of 9 ABC transporters showed similar pattern of expression in qPCR and transcriptomes (Fig. 4.13). Knowledge of transporters provides a platform to gain insight into the molecular mechanisms behind the transportation and accumulation of a metabolite in different compartments of plants. Previous reports on *Thalictrum minus* and *Coptus japonica* have demonstrated the importance of ABC transporters in alkaloids accumulation which may also be true for *A. heterophylum* since it contains diterpene alkaloids [14].

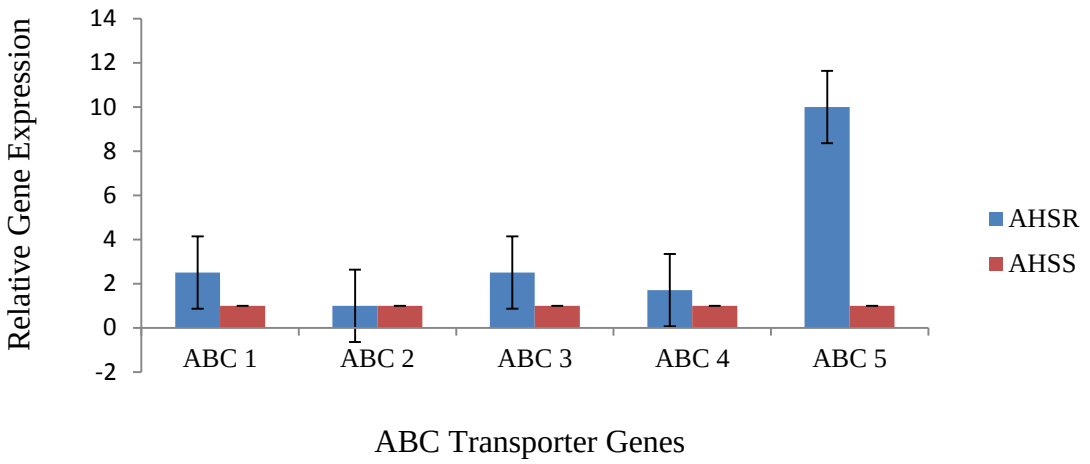

Fig. 4.13 Expression status of ABC transporter genes in root versus shoot transcriptomes

The results and discussion section has suggested some promising genes associated with the biosynthesis of atisine and the growth and development of tuberous roots in *A. heterophyllum*, which can be further pursued through various gene function approaches to ascertain their definite biological role and potential future usage.

SUMMARY

Understanding the biology of aconites biosynthesis has provided insights about the sites of biosynthesis and accumulation of aconites in *A. heterophyllum*. The present study has, therefore, suggested that the biosynthesis and accumulation of atisine/aconites occur exclusively in the roots of *A. heterophyllum*. Also the *A. heterophyllum* accessions collected from different locations of Himachal Pradesh were utilized for the identification of high aconites content accessions. The idea was to identify superior strains for further exploitation and breeding programme. The current study was also taken up for understanding the molecular basis of aconites biosynthesis in *A. heterophyllum*. Furthermore, the molecular components regulating tuberous root formation – herbal biomass of *A. heterophyllum* were also dissected. The current findings would be helpful for planning genetic intervention strategies for *A. heterophyllum*.

The estimation of atisine in roots of plants of different age groups (1-3 years) showed variation in atisine content. It increased from 0.14% in 1 year old plants to 0.22% in 2 years old plants and then decreased to 0.08% in the roots of 3 years old plants. Atisine was not detected in shoots of *A. heterophyllum*. High atisine content in roots of 2 years old plants (0.22%) is, therefore, important in the selection of raw material for the preparation of herbal drug formulations. This can help in maintaining uniformity in the raw material, which would otherwise affect the amount of aconites and the quality of herbal drug efficacy.

Atisine/aconites content were estimated through HPLC and bromocresol green extraction method in 14 accessions of *A. heterophyllum* collected from different locations of Himachal Pradesh. The significant variation for atisine/aconites content among 14 accessions of *A. heterophyllum* was observed. Atisine content in roots of 14 accessions of *A. heterophyllum* ranged from 0.14-0.37% and total alkaloids (aconites) from 0.20-2.49%. Two accessions, namely AHCR and AHSR showed the highest atisine content of 0.30% and 0.37% as well as the highest total alkaloids content of 2.22% and 2.49%, respectively. The identification of elite chemotypes with high content of aconites can be helpful in commercial cultivation of genetically uniform planting material of *A. heterophyllum*.

The present research led to the cloning of 15 genes of aconites biosynthetic pathway and also analyzed the expression pattern of those genes in relation to atisine/aconites content in *A. heterophyllum*. Multiple genes of MVA/MEP pathways showed elevated expression in high atisine/aconites content accession as compared to low atisine/aconites content accession. This was the first attempt towards molecular understanding of atisine/aconites biosynthesis in *A.*

heterophyllum. Eight genes viz. HMGS, HMGR, PMK, IPPI, DXPS, ISPD, HDS and GDPS with elevated expression in relation to aconites content could be used as suitable targets for developing gene markers for genetic improvement of *A. heterophyllum*. The cloning of promoter regions of these genes in *A. heterophyllum* can provide insights into what determines their differential expression between different accessions.

With progress in modern technologies, transcriptomics has emerged as a powerful tool to capture traits of economic importance. The availability of whole transcriptome data could be used not only to discover candidate genes involved in tuberous root development and secondary metabolites production but also for understanding molecular basis of various biological processes. The biosynthetic machinery of tuberous roots was, therefore, discerned to identify plausible key genes towards root biomass development by utilizing transcriptome data sets of *A. heterophyllum*. Four genes viz. AGPase, β-amylase, SRF and Expansin showed maximum contribution towards tuberous root development. There is possibility of altering the expression levels of these genes for improving tuberous root (biomass) yield for herbal drug industries. These results can be further explored to dissect the molecular regulation of tuberous root formation and growth in *A. heterophyllum*. Besides this, ABC5 transporter gene showed highest transcript abundance in tuberous roots.

Overall, the superior strains of *A. heterophyllum* can be commercially utilized while functional validation of key genes controlling aconites biosynthesis and tuberous root growth in *A. heterophyllum* can aid in designing a suitable genetic intervention strategy in *A. heterophyllum*.

APPENDIX

Table A1 Accessions of *A. heterophyllum* from different locations of Himachal Pradesh

Accession	District	Altitude (amsl)
AHPVR	Lahaul & Spiti	3500 m
AHTR	Lahaul & Spiti	2600 m
AHUR	Lahaul & Spiti	2800 m
AHMR	Kullu	3100 m
AHNR	Kinnaur	3150 m
AHKR	Kinnaur	2800 m
AHChR	Kinnaur	3450 m
AHCR	Shimla	2900 m
AHCpR	Shimla	2850 m
AHSR	Shimla	2400 m
AHRR	Shimla	2750 m
AHSaR	Chamba	3350 m
AHPR	Chamba	3000 m
AHBBR	Kangra	3100 m

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GenBank Submissions

KF961192, KF961193, KF961194, KF961195, KF961196, KF961197, KF961198, KF961199, KF961200, KF961201, KF961202, KF961203, KF961204, KF961205, KF961206.