Shikonins are red naphthoquinone pigments, which possess anti-microbial, anti-inflammatory and anti-tumour activities (Kim et al., 2001; Papageorgiou et al., 1999). These are active ingredients in several pharmaceutical and cosmetic preparations, and are used as dye for fabrics and food items (Kim et al., 2001; Papageorgiou et al., 1999). First successful in vitro production of shikonin and its derivatives, collectively called shikonins, was reported from callus cultures of Lithospermum erythrorhizon by Tabata et al. (1974). Shikonins are commercially extracted from roots of Lithospermum erythrorhizon, which contain 0.14-1.09 % shikonins on dry weight basis (Chen et al., 2002).

Chemically, shikonins are composed of isoprenoid (GPP) and PHB moieties, which are also referred to as yield determining moieties (Yazaki et al., 2002). Plants have single PP pathway for the synthesis of PHB, whereas GPP is synthesised through MVA and MEP pathways. The preference for MVA versus MEP pathway depends upon the metabolites and the plant species under consideration (Sando et al., 2008; Totte et al., 2000). Coupling of GPP and PHB is mediated through the enzyme PGT.

Though there are several plants that yield shikonins, the roots of A. euchroma contain 1.58-1.94 % shikonins, the highest amongst the reported shikonins accumulating plants (Chen et al., 2002). A. euchroma has been listed as an ‘endangered’ species as per IUCN threat categories (Ved and Tandon, 1998) possibly due to indiscriminate harvesting coupled with lack of organized cultivation. Conservation of plant using biotechnological approaches including tissue culture is an alternative option for relieving A. euchroma from extinction pressure (Malik et al., 2010, 2011). Gene prospection is yet another route to conservation and utilization of plant resources (Bhardwaj et al., 2010; Ghawana et al., 2010; Wang et al., 2010). However, this aspect is completely lacking in A. euchroma. The objective of the present thesis was to bioprospect genes associated with shikonins biosynthesis. The production of shikonins depends on the GPP level, therefore the first objective of the thesis was to identify the pathway supplying GPP for shikonins biosynthesis.

Previous work on shikonins biosynthesis was carried out in Lithospermum erythrorhizon using one gene of the MVA pathway (HMGR), three genes of PP pathway (PAL, CAH, and 4-CL), and PGT. HMGR and PGT were shown to be regulatory genes in Lithospermum erythrorhizon for shikonins biosynthesis (Lange et al., 1998; Yazaki et al.,
Information on other genes in relation to shikonins biosynthesis, however, has not been reported so far.

### 5.1 Development of protocol for isolation of RNA

Isolation of good quality RNA is central to any molecular biology work involving RNA as starting material. Since the available protocols did not isolate RNA successfully from leaf tissues of *A. euchroma*, a phenol-based, guanidinium salt-free protocol was developed for the purpose (Ghawana et al., 2007, 2011). Reagents were selected based on their specific properties: phenol as a strong protein denaturant and inhibitor of RNase; SDS and EDTA are also inhibitors of RNase (Green, 1994; Kim et al., 2001; Farrell, 2005). This composition provided a cocktail of RNase inhibitors and protein denaturant. Further, pH of the solution was maintained in the acidic range to allow efficient and preferable partitioning of RNA in the aqueous phase leaving DNA in the phenolic phase; DNA prefers basic pH for its partitioning into the aqueous phase (Wallace, 1987). An appropriate concentration of NaOAc was included in the solution to aid precipitation of RNA in the presence of isopropanol. The addition of DEPC-treated ADW after addition of above solution rendered sufficient aqueous environment for partitioning of RNA into the aqueous phase.

The developed method successfully isolated RNA from leaf tissues of *A. euchroma* with a yield of 153.33 ±11 µg RNA/100 mg of tissue (Fig. 4.1). This was remarkable in the light that other protocols either failed to yield RNA or yielded extremely low quantity (Fig. 4.1). Though contamination of DNA cannot be completely avoided, as is also reported with most of the rapid protocols (Xu et al., 2010; Kiefer et al., 2000), digestion of RNA with RNase-free DNase took care of the contamination (Fig. 4.2). In fact, DNase digestion is a common step in protocols involving RNA analysis (Singh et al., 2004; Bhardwaj et al., 2010; Ghawana et al., 2010). The $A_{260}/A_{280}$ ratio of the isolated RNA ranged between 1.8-2.0, which indicated the RNA to be relatively free of proteins and contaminants. At times, low $A_{260/280}$ ratio ranging between 1.4-1.7 was also obtained, particularly when higher amount of the tissue was used. Distinct 28S and 18S rRNA bands were observed on 1% formaldehyde-agarose denaturing gel indicating intact RNA. Also, the isolated RNA was amenable to downstream applications e.g. RT-PCR, RACE and SSH.

The above method worked well with leaf tissues, however, did not work with root tissues, possibly due to the presence of colour and other interfering substances, such as naphthaquinones, which are potent enzyme inactivators (Heide and Tabata, 1987). None of
the available methods (Cox, 1968; Chirgwin et al., 1979; Chomczynski and Sacchi, 1987; Ausubel et al., 1994; Bugos et al., 1995; Mehra, 1998) describe on dealing with the tissues containing colours and the associated inhibitors. Therefore, yet another method was developed (Singh and Kumar, 2008) to remove colour and inhibitor compounds from the tissue prior to isolation of RNA. For this, RNase inhibitory property of SDS and EDTA was used (Green, 1994; Kim et al., 2001; Farrell, 2005). The solution also had ethanol, which allowed solubilization of colour and other inhibitory substances but left RNA and other components insoluble (section 3.2.2). After washing the colour and other inhibitory substances from root tissues, RNA from the pellet was extracted using the above discussed RNA isolation protocol (Ghawana et al., 2007, 2011). The quality and quantity of isolated RNA was acceptable as evidenced by appropriate $A_{260}/A_{280}$ ratio (1.81-2.03), distinct bands of 28S and 18S rRNA and RT-PCR (Fig. 4.3).

5.2 Biosynthesis of shikonins predominantly uses MVA pathway for GPP supply

Shikonins are composed of PHB and an isoprenoid moiety derived from GPP. PHB is synthesized through PP pathway, whereas GPP can be synthesized through MVA (Newman and Chappell, 1999) and MEP (Lichtenthaler et al., 1997) pathways. Supply of GPP is critical in realizing the yield of isoprenoids (Nogués et al., 2006), therefore, study on regulation of gene expression in GPP biosynthesis is of immense significance. Depending upon the metabolite and species under consideration, the preference for the route to GPP biosynthesis might differ. For example, natural rubber relies on MVA pathway, whereas stevioside is derived through MEP pathway (Sando et al., 2008; Totte et al., 2000). Relative contribution of MVA versus MEP pathway towards shikonins biosynthesis was assessed by using mevinolin and fosmidomycin, which are inhibitors of HMGR (of MVA pathway) and DXR (of MEP pathway), respectively (Alberts et al., 1980; Schwender et al., 1999). For such a study, a cell suspension culture-based system was used wherein shikonins content increased from negligible quantity by 82.8 fold from day zero to day 10 of transferring the suspension cultures from LSPS to HSPS (Singh et al., 2010). Inclusion of mevinolin produced a severe inhibition (92.82%) in shikonins accumulation, whereas fosmidomycin produced a comparatively milder (49.97%) inhibition. Data implied a prominent role of MVA pathway in shikonins biosynthesis, though a role of MEP pathway cannot be completely ruled out. The results were slightly different from Lithospermum erythrorhizon.
in which mevinolin inhibited the shikonins biosynthesis by 98% (Gaisser and Heide, 1996). Involvement of both MVA and MEP pathway in terpenoid biosynthesis has also been reported for dolichols biosynthesis in *Coluria geoidea* (Skrupinska-Tudek et al., 2008), which might also be the case in *A. euchroma*.

### 5.3 Library screening yielded *HMGR* and *PGT*

To understand molecular basis of shikonins biosynthesis, cloning of genes of the pathway and their regulatory sequences occupies central role. To clone various genes of the pathway, the properties of LSPS and HSPS to accumulate low and high shikonins level, respectively was used. Since HSPS has high shikonins content as compared to LSPS, HSPS was presumed to have up-regulation of transcripts for shikonins biosynthesis. To clone these transcripts, SSH was performed between LSPS and HSPS.

SSH is a powerful approach to identify and isolate cDNAs of differentially expressed genes of both high and low abundance (Diatchenko et al., 1996; Rani et al., 2009). Though several methods exist to identify differentially expressed genes such as differential display of mRNA (Sharma and Kumar, 2005; Singh et al., 2009), serial analysis of gene expression (Matsumura et al., 1999; Jung et al., 2003) and cDNA-amplified fragment length polymorphism (Milioni et al., 2002; Van der Biezen et al., 2000), SSH has advantage of being less expensive and fast.

Analysis of AeFSL was detailed in section 4.4.2. Screening of SSH library yielded 407 ESTs in AeFSL (dbEST accession no. GR881945-GR882351; Table 4.1). ESTs for two genes of the pathway, *AeHMGR* and *AePGT* were obtained, and were also overexpressed in HSPS as compared to LSPS (Fig. 4.74). Details of analysis of AeRSL are mentioned in section 4.4.2. Screening of SSH library yielded 334 ESTs in AeRSL (dbEST accession no. GR882352-GR882685; Table 4.2). It was interesting that in contrast to AeFSL, none of the genes of shikonin biosynthesis pathway was present in the AeRSL suggesting an efficient subtraction.

While *AeHMGR* and *AePGT* are important genes of the pathway, the ESTs that were abundant in library and showed up-regulation in HSPS as compared to LSPS (Fig. 4.10) might also be implicated in shikonins biosynthesis. These were *SAMS*, *dark inducible proteins*, *Ca^{2+}-binding protein*, *Rab1-like small GTP-binding protein* and *PR defense protein*. *SAMS* catalyses the synthesis of SAM in a reaction involving L-methionine, ATP and H_{2}O. SAM is the donor for most biological methylations, including DNA, RNA,
proteins, lipids, and secondary metabolites and a precursor in ethylene biosynthesis (Schroder et al., 1997). Ethylene was considered as one of the positive regulatory elements in shikonins biosynthesis (Touno et al., 2005). Ethylene is known to induce PAL (Touno et al., 2005), which is an important enzyme of PP pathway involved in biosynthesis of shikonins (Singh et al., 2010).

ESTs encoding dark inducible protein shared a high similarity with dark inducible protein (LeDI-2) cloned from Lithospermum erythrorhizon, LeDI-2 was reported to show the strict dark-specific expression in cell suspension cultures (Touno et al., 2005) and was one of the candidates for the regulation of shikonins biosynthesis and specifically expressed in the roots (Touno et al., 2005). LeDI-2 was implicated in shikonins localization and imparting stability to shikonins (Yazaki et al., 2001).

Ca$^{2+}$-binding proteins related to signal transduction pathway, encoded protein that interacts with Ca$^{2+}$ ions, thereby functions as a secondary messenger and controls many biological processes (Schäfer and Heizmann, 1996). There are several Ca$^{2+}$-binding proteins, for example, calmodulin, a small Ca$^{2+}$-binding protein that transduces secondary messenger signals into a wide array of cellular responses (Zielinski, 1998). One class of Ca$^{2+}$ binding protein possesses a common Ca$^{2+}$-binding motif, the EF-hand proteins (Schäfer and Heizmann, 1996). EF-hand protein is involved in the process of elicitation of various Ca$^{2+}$- and calmodulin-dependent protein kinases by increasing the level of free Ca$^{2+}$ in the cytoplasm. This triggers the cellular response to alter gene expressions (Sudha and Ravishankar, 2002). Ca$^{2+}$-binding proteins could play a role in shikonins biosynthesis by modulating the intracellular-free Ca$^{2+}$ level (Zielinski, 1998). In Onosma paniculatum, cytoplasmic Ca$^{2+}$ carries the elicitor signal to regulate the biosynthesis of shikonins in cell culture (Ning et al., 1998).

Rab1-like small GTP-binding protein encodes membrane and cellular transport related proteins, which could be of immense interest in understanding intracellular transport of shikonins. This protein belongs to Rab family, a large family of GTPases that plays a role in vesicular trafficking between specific compartments of eukaryotic cells (Bischoff et al., 1999). Biosynthetic site and transport of shikonins is poorly understood. It has been suggested that all steps including coupling of PHB and GPP takes place in a special vesicle or biosynthetic site, which facilitated not only the orderly biosynthetic reactions but also the transport and secretion of the secondary metabolite (Yamaga et al., 1993). Role of Rab family of proteins cannot be ruled out in transport of vesicles containing shikonins from site
of synthesis to secretion site at cell wall. However, none of Rab family protein is yet characterized in relation to shikonins transport.

Gene encoding defense-related proteins (*pathogenesis-related protein* PR2, *hypersensitive-induced response protein*, LeDI-1) may play a role in shikonins biosynthesis. These were also reported in shikonin-producing cells of *Lithospermum erythrorhizon*. These were overexpressed in response to signal molecules associated with the plant defense response system (Hwang *et al*., 2003). Plants respond to pathogen attack and wounding by producing several compounds, such as ethylene, jasmonic acid, SA and ROS, which induce the expression of genes encoding PR proteins (Touno *et al*., 2005). In *Lithospermum erythrorhizon* cell cultures, expression of LeDI-1, which showed high similarity to the genes encoding parsley PR1-2 protein, exhibited a positive correlation with biosynthesis of shikonins (Yazaki *et al*., 1995). Ethylene and MJ induced by wounding could induce the expression of genes encoding PR protein, such as LeDI-1 and shikonins synthesis (Touno *et al*., 2005).

5.4 Cloning of *AeACTH, AeHMGS, AeMVK, AePMVK, AeMVDD, AeIPPI, AeGDPS, AePAL, AeC4H* and *Ae4-CL* was achieved by degenerate primers

Inhibitor studies suggested a predominant role played by the MVA pathway in synthesis of shikonins. MVA pathway *per se* involves eight genes namely, *ACTH, HMGS, HMGR, MVK, PMVK, MVDD, IPP1* and *GDPS* for the biosynthesis of GPP (Fig. 1.2). However, screening of the library yielded only two genes namely, *AeHMGR* and *AePGT*. Using degenerate primers, cloning of *AeACTH, AeHMGS, AeMVK, AePMVK, AeMVDD, AeIPPI, AeGDPS, AePAL, AeC4H* and *Ae4-CL* was achieved. The partial genes were cloned to full-length cDNA by RACE.

Intensive bioinformatics analyses showed that all the twelve genes contained the putative functional/conserved domains and predicted secondary structures, which are essential to render characteristic functionality. These domains were present as described previously in other plant systems (Table 5.1). Result section (4.4.4) discussed the bioinformatics analyses in detail for these genes *vis-à-vis* the genes reported from other plant system and hence are not being detailed in this section. However, it is pertinent to mention here that out of the twelve genes cloned in the present work, eleven genes from other plant systems were validated for functionality by various groups. It was worth noting
that the functional domains and predicted secondary structures present in the validated genes were also present in the genes cloned by us (Tables 4.3 and 5.1). This suggested that the genes cloned in the present work were also the functional genes. These genes were ACTH from *Raphanus sativus* (Vollack and Bach, 1996); HMGs from *Brassica juncea* (Nagegowda et al., 2004); HMGR (Learned and Fink, 1989), MVK (Riou et al., 1994), MVDD (Cordier et al., 1999), IPPI (Campbell et al., 1998), 4-CL (Hamberger and Hahlbrock, 2004), and PAL (Cochrane et al., 2004) from *Arabidopsis thaliana*; GDPS from *Abies grandis* (Burke and Croteau, 2002), C4H from *Populus trichocarpa* x *Populus deltoides* (Ro et al., 2001), and PGT from *Lithospermum erythrorhizon* (Yazaki et al., 2002).

### Table 5.1 Bioinformatics analyses and functional validation of genes from other plants

<table>
<thead>
<tr>
<th>Gene</th>
<th>Functional / conserved domain</th>
<th>Predicted secondary structure</th>
<th>Predicted TM domains</th>
<th>Validation method</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH from <em>Raphanus sativus</em></td>
<td>Thialase acetyl enzyme intermediate signature, VNKCSAGMLAV, and NvSmGsaVStHgPStcSg, Thialase active site, GVGVCNGGcAcA</td>
<td>n-helices (183 aa), β-turms (34 aa), extended strands (54 aa) and random coils (135 aa)</td>
<td>None</td>
<td>Overexpression in yeast (Vollack and Bach, 1996)</td>
</tr>
<tr>
<td>HMGs from <em>Brassica juncea</em></td>
<td>Hydroxymethylglutaryl-coenzyme-A synthase active site: NIDVEGCVAMNAYG</td>
<td>n-helices (206 aa), β-turms (24 aa), extended strands (66 aa) and random coils (165 aa)</td>
<td>None</td>
<td>Overexpression in E. coli (Nagegowda et al., 2004)</td>
</tr>
<tr>
<td>HMGR from <em>Arabidopsis thaliana</em></td>
<td>Hydroxymethylglutaryl-coenzyme-A reductase signature, 1 RCCxTGxGxGmGmNml, signature 2 YGvYG, signature 3 ALAAGLxRSHMLY</td>
<td>n-helices (259 aa), β-turms (30 aa), extended strands (82 aa) and random coils (196 aa)</td>
<td>3</td>
<td>Heterologous expression in yeast (Learned and Fink, 1989)</td>
</tr>
<tr>
<td>MVK from <em>Arabidopsis thaliana</em></td>
<td>GSHMP ximases putative ATP-binding domain LPyGcGSGSIA</td>
<td>n-helices (161 aa), β-turms (17 aa), extended strands (62 aa) and random coils (138 aa)</td>
<td>1</td>
<td>Complementation in the yeast (Riou et al., 1994)</td>
</tr>
<tr>
<td>MVDD from <em>Arabidopsis thaliana</em></td>
<td>GSHMP ximases N terminal domain</td>
<td>n-helices (158 aa), β-turms (21 aa), extended strands (73 aa) and random coils (187 aa)</td>
<td>1</td>
<td>Heterologous expression in yeast (Cordier et al., 1999)</td>
</tr>
<tr>
<td>IPPI from <em>Arabidopsis thaliana</em></td>
<td>Nudix hydrolase domain profile</td>
<td>n-helices (127 aa), β-turms (6 aa), extended strands (33 aa) and random coils (125 aa)</td>
<td>None</td>
<td>Enzyme activity assay (Campbell et al., 1998)</td>
</tr>
<tr>
<td>GDPS from <em>Abies grandis</em></td>
<td>PalA/prexyn transferase signatures, signature 1 LIDDVHovxMntHhRGG, signature 2 YGvYG, signature 3 ALAAGLxRSHMLY</td>
<td>n-helices (249 aa), β-turms (27 aa), extended strands (17 aa) and random coils (90 aa)</td>
<td>1</td>
<td>Heterologous expression in E. coli and enzyme activity assay (Burke and Croteau, 2002)</td>
</tr>
<tr>
<td>PAL from <em>Arabidopsis thaliana</em></td>
<td>Phenylalanine and histidine ammonia-lyase signature, GTTGASGDLxPLysxG</td>
<td>n-helices (409 aa), β-turms (42 aa), extended strands (60 aa) and random coils (214 aa)</td>
<td>2</td>
<td>Heterologous expression in E. coli and enzyme activity assay (Cochrane et al., 2004)</td>
</tr>
<tr>
<td>C4H from <em>Populus trichocarpa</em> x <em>Populus deltoides</em></td>
<td>Cystochrome P450 cytochrome heme-iron ligand FGvxGRSCPG</td>
<td>n-helices (250 aa), β-turms (25 aa), extended strands (54 aa) and random coils (176 aa)</td>
<td>2</td>
<td>Over-expression in yeast and <em>Arabidopsis thaliana</em> (Ro et al., 2001)</td>
</tr>
<tr>
<td>4CL from <em>Arabidopsis thaliana</em></td>
<td>Putative AMP-binding domain signature, MPYSSGTGIPK</td>
<td>n-helices (149 aa), β-turms (40 aa), extended strands (104 aa) and random coils (195 aa)</td>
<td>2</td>
<td>Heterologous expression in E. coli and enzyme activity assay (Hamberger and Hahlbrock, 2004)</td>
</tr>
<tr>
<td>PGT from <em>Lithospermum erythrorhizon</em></td>
<td>UKA prexyn transferase family signature, NdyxGmFDkVeOrTkxRPluxG</td>
<td>n-helices (139 aa), β-turms (15 aa), extended strands (66 aa) and random coils (87 aa)</td>
<td>4 or 5</td>
<td>Heterologous expression in yeast and enzyme activity assay (Yazaki et al., 2002)</td>
</tr>
</tbody>
</table>
5.5 Mevinolin down-regulated expression of genes of shikonins biosynthesis pathway

Mevinolin is a known inhibitor of HMGR. Down-regulation of expression of HMGR was reported in *Lithospermum erythrorhizon* in response to mevinolin treatment (Lange et al., 1998a). However, there is no information on other genes expression inhibited by mevinolin. Our data showed that mevinolin down-regulated expression of all the eight genes involved in MVA pathway, all the three gene of PP pathway and *AePGT*. Application of fosmidomycin had no effect on expression of these genes. Data suggested a substrate/product mediated feed-back and feed-forward regulation of the genes under study. For HMGR, its post-transcriptional and post-translational feedback regulation was suggested in the experiments using *Arabidopsis thaliana* treated with lovastatin (analogous to mevinolin) (Kobayashi et al., 2007). Previously, Dixon et al. (1990) reported that endogenous cinnamate (product of PAL) caused inhibition of both transcription and enzymatic activity of PAL. Rani et al. (2009) reported catechin-mediated down-regulation of several genes of PP pathway. In animal system, MVA pathway involved in the biosynthesis of cholesterol, has long been known to be regulated by end-product feedback inhibition and this regulation has been attributed to direct regulation of the expression of several cholesterol biosynthetic genes by the sterol sensing SRE binding protein-2 (SREBP-2) (Goldstein et al., 2006). In yeast and higher plants, MVK was subjected to feed-back inhibition by GPP and FPP (Gray, 1987; Oulmouden and Karst, 1991), suggesting feedback inhibition by prenyl phosphates as a general regulatory mechanism to modulate the activity of MVK. Existing literature supported the view of substrate mediated feedback inhibition, possibly that has been observed for HMGR, MVK and PAL. Overall down-regulation of other genes in response to mevinolin and fosmidomycin needs further investigations on substrate/product mediated feed-back and feed-forward regulation of the gene expression.

5.6 Not all the genes involved in shikonins biosynthesis exhibited up-regulation in HSPS

LSPS and HSPS provided a convenient system to study the possible regulatory genes in shikonins biosynthesis. Analysis of all the twelve genes showed that *AeHMGR* (of MVA pathway), and *AePGT* (involved in coupling of GPP and PHB) exhibited evident up-regulation in HSPS as compared to LSPS from day 2 onwards. Generally, an up-regulation
of all the genes of PP pathway was noticed in HSPS as compared to the LSPS. Earlier reports suggested HMGR to play a key role in the control of isoprenoid biosynthesis in plants (Stermer et al., 1994; Bach et al., 1997).

Lange et al. (1998) proposed the importance of HMGR as an early enzyme in isoprenoid biosynthesis to control the metabolic flux into the MVA pathway, in contrast to the stringently regulated later enzymes controlling the biosynthesis of specific end products. Our data also suggested that AeHMGR could be an important regulatory gene in shikonins biosynthesis, since a strong positive correlation (r, 0.95) was obtained between its expression and the shikonins content (Fig. 4.74).

A requirement of higher PP pathway activity appeared to be a general feature for shikonins biosynthesis in A. euchroma as evidenced by higher expression of the three genes (Fig. 4.74). PP supplies PHB which is a substrate involved in shikonins biosynthesis (Inouye et al., 1979; Schmid and Zenk, 1971). While in Lithospermum erythrorhizon, genes of PP pathway did not exhibit any specific trend in relation to shikonins biosynthesis (Yamamura et al., 2003), data on A. euchroma appears to be in line with the previous reports on tea (Rani et al., 2009; Singh et al., 2009) wherein all the three genes of PP pathway were reported to be regulatory. AePGT encodes for PGT involved in GHB biosynthesis. This gene also showed up-regulation (three times) in HSPS and was positively correlated (r, 0.91) with shikonins content. Heide and Tabata (1987) reported high (thirty five times) activity of PGT in shikonins producing culture extracts of Lithospermum erythrorhizon as compared to the non-shikonins producing system suggesting PGT to be important in regulation of biosynthesis of shikonins.

5.7 Genes involved in shikonins biosynthesis exhibit evident up-regulation in older leaves

Young and mature leaves have different capacities for secondary metabolite production (Rosenstiel et al., 2002; Mayrhofer et al., 2005; Sun et al., 2010). This difference could be primarily because of sink-source relationship between young and mature leaf. In early phases of leaf growth, young leaves act as a net carbon importer (sink). In late growth phase, fully expanded mature leaves act as a net carbon exporter (source) (Turgeon and Webb, 1975; Marchi et al., 2005). Higher expression of targeted genes in older leaf (Fig. 4.75) is in agreement with the finding by Suzue et al. (2006), who showed that the expression of S6PDH in older leaves was higher than folded and younger
leaves in pear. $S6PDH$ is involved in sorbitol biosynthesis, which is transported from leaves to the sink organs. Several genes related to terpenoid and flavonoid biosynthesis including $HMGR$, $HMGS$, 4-CL cinnamoyl–CoA reductase and isoflavone reductase exhibited higher expression in older leaf of $Ginkgo biloba$ as compared to young leaf (Wang et al., 2010). In fact, higher shikonins biosynthesis has been reported from older and more mature cells of $Lithospermum erythrorhizon$ (Tani et al., 1992). Data on gene expression in young and mature tissues is suggestive of the importance of mature leaf in supplying substrates that are possibly conjugated in the roots.

5.8 Genes involved in shikonins biosynthesis exhibit down-regulation during noon in roots, but not in leaves

It has been reported in several plant systems that most secondary metabolites such as linamarin (cyanogenic glucoside) in $Manihot esculenta$ (Selmar, 1994) and saponins in $Achyranthus bidentata$ (Li and Hu, 2009) are produced in leaves and stored in roots. $Geranylgeranyl diphosphate synthase$ for biosynthesis of forskolin in $Coleus forskohlii$, is mainly synthesized in leaves, subsequently allocated to stems and finally accumulated in stem bases and roots (Engprasert et al., 2004). Storage tissues such as roots are also known to have capabilities to synthesise the metabolites (Shimomura et al., 1991; Takase et al., 2007). Therefore, it was of interest to study the various genes involved in shikonins biosynthesis in leaf as well as root tissues.

The expression of all the twelve genes in $A. euchroma$ did not exhibit any variation in leaf during morning (6:00 h; light intensity, 0.08 µE m$^{-2}$ s$^{-1}$), noon (13:00 h; light intensity, 553.05 µE m$^{-2}$ s$^{-1}$) and evening (19:00 h; light intensity, 0.51 µE m$^{-2}$ s$^{-1}$), (Fig. 4.76). Root tissues, however, exhibited distinct down-regulation of all the twelve genes during noon (Fig. 4.76). Metabolite and transcript abundance profiling at different time point of the day in potato ($Solanum tuberosum$) leaves showed several of the Kreb cycle intermediates and minor organic acids namely citrate, isocitrate, maleate, caffeate, maltose and chlorogenate pools declined at noon (12:00 h) and rose steadily during the dark period. Also the transcript levels of $acyltransferase$, $anthocyanin 3'-glucosyltransferase$, $caffeic acid o-methyltransferase$, $glucosyl transferase$, $orcinol o-methyltransferase$, $PAL$ and $phytoene synthase$ were elevated in the dark period (Wochniak et al., 2005). Down-regulation of gene expression observed in root tissues of $A. euchroma$ during noon envisaged the possibility of transmission of photosynthesis-related signal from leaf to root.
James *et al.* (2008) showed that a photosynthesis-related signal from the shoot synchronizes the root circadian clock and differs markedly from shoot circadian clock in *Arabidopsis thaliana*. It is also likely that altered gene expression during different time point of the day might be a strategy to match with substrate/product availability and enzyme activities at that point of time (Mayrhofer *et al*., 2005).

Except for noon time, the level of expression of all the genes was higher in leaf tissues. It is worth noting that the transcripts of *PGT* were not detectable in leaf tissue of *Lithospermum erythrorhizon* (Yazaki *et al*., 2002), whereas the gene expressed well in *A. euchroma*. Since PGT is involved in coupling of GPP and PHB, it is likely that in *A. euchroma*, both leaf as well as root tissues might be actively involved in shikonins biosynthesis.

### 5.9 Effect of external cues on shikonins content and gene expression

ABA enhanced accumulation of shikonins upto 146.3 % at day 2 (Fig. 4.72). ABA is reported to play role as signal transducer for wound and microbial defense responses in plants (Hildmann *et al*., 1992; Klessig and Malamy, 1994). ABA is also used as an elicitor of secondary metabolic pathways in cell culture systems (Shirasu *et al*., 1997). ABA has been shown to increase the intracellular concentration of Ca$^{2+}$ by two pathways: inositol 1, 4, 5-trisphosphate-mediated Ca$^{2+}$ release and cyclic ADP-ribose-mediated Ca$^{2+}$ release (Allen *et al*., 1995). ABA was reported to enhance the production of paclitaxel (known commercially as Taxol®) in cell suspension cultures of *Taxus chinensis* (Luo *et al*., 2001). However, in cell culture of *Onosma paniculatum*, addition of ABA significantly decreased shikonins formation (Sun *et al*., 2007). Contrary results of the effect of ABA in *A. euchroma* and *Onosma paniculatum* might be due to plant specific response.

Gene expression analysis suggested that enhanced shikonins production by ABA in *A. euchroma* could be the result of up-regulation of regulatory genes of the pathway i.e. *AeHMGR* and *AePGT* (Fig. 4.77). In fact ABA mediated enhancement in artemisinin content in *Artemisia annua* was attributed to up-regulation of the expression of *HMGR*, *farnesyl diphosphate synthase*, *amorpha-4,11-diene c-12 oxidase* and *CYP reductase*, the important genes in the artemisinin biosynthetic pathway (Jing *et al*., 2009).

MJ also enhanced shikonins accumulation in *A. euchroma* (Fig. 4.72) as reported the case in *Lithospermum erythrorhizon* as well (Yazaki *et al*., 1997b; Matsuno *et al*., 2002). MJ also elicited massive accumulation of caffeoylputrescine in *Lycopersicon*
esculentum leaves. This was attributed to the upregulation of genes of PP and polyamine pathways (Chen et al., 2006). Paclitaxel (a terpenoid) production was also increased in response to MJ elicitation in cell culture of Taxus x media, Taxus canadensis and Taxus cuspidata and other Taxus species (Mirjalili and Linden, 1995; Yukimune et al., 1996; Ketchum et al., 1999). Ketchum et al. (1999) showed that MJ was most effective as elicitor when added to cell culture at day 7 or 8 after inoculation rather than at the time of inoculation, possibly due to greater cells biomass, taxadiene synthase activity and pool of taxoid precursors at day 7 or 8 (Hezari et al., 1997).

SA inhibited the accumulation of shikonins at day 6 onwards (Fig. 4.72). Yazaki et al. (1997c) reported no significant change on shikonins production after two weeks of SA treatment in Lithospermum erythrorhizon cell culture. SA induced the expression of genes related to biosynthesis and production of phytoalexins, these are secondary metabolites involved in defense response of the plant against pathogens (Taguchi et al., 2001). The induction mechanism of defense is generally thought to be related to the elevation of ROS including H$_2$O$_2$, which could serve as secondary messengers in defense signaling pathway (Ebel and Mithofer, 1998; Qian et al., 2006; Jannat et al., 2011). The inhibitory effect of SA on shikonins production in A. euchroma needs further analysis.

The inhibitory effect of PHB and MVA on shikonins accumulation persisted almost throughout the culture period, while GPP initially decreased shikonins content at day 4 and 6, followed by enhancement at day 8 and 10 of the treatment (Fig. 4.72). PHB, GDP and MVA are intermediates of the shikonins biosynthesis pathway. PHB and GPP are substrates for PGT, while MVA is the product of HMGR (Fig. 1.2). The substrate/product mediated feed-forward and feed-back inhibition of PGT and HMGR and other enzymes of shikonins biosynthesis pathway could be the reason for decrease in shikonins accumulation. We have discussed the possibility of substrate/product mediated feed-back and feed-forward regulation of the genes of shikonins biosynthetic pathway in response to mevinolin in section 5.5. Feed-back and feed-forward regulation of biosynthetic pathways is well known mechanism in plants and animals (Goldstein et al., 2006; Oulmouden and Karst, 1991). The enzymes of MVA/PP pathway namely HMGR (Kobayashi et al., 2007), MVK (Gray, 1987; Oulmouden and Karst, 1991) and PAL (Dixon and Lamb, 1990) undergo such kind of inhibition as discussed in section 5.5. In Lithospermum erythrorhizon, exogenous supply of PHB at low concentration (1-10 µM) enhanced shikonins production by about 50%, while there was no significant effect at 100 µM (Yazaki et al., 1997c). This result is somewhat different from our result possibly due to
different solution used for administering PHB in cell cultures of *Lithospermum erythrorhizon* (as dimethyl sulfoxide solution) and *A. euchroma* (as ethanol solution) or the difference in the metabolism of the two tissues.

H$_2$O$_2$ inhibited shikonins biosynthesis in the present work (Fig. 4.72). H$_2$O$_2$ activates many other important signal molecules such as Ca$^{2+}$, SA, ABA, JA, ethylene, NO and different transcription factors, which in turn regulate gene expression and cell-cycle processes (Desikan *et al.*, 2003; Slesak *et al.*, 2007; Quan *et al.*, 2008). In *Taxus cuspidata* cell suspension culture, H$_2$O$_2$ was reported to enhance taxol content (as discussed by Zhang *et al.*, 2011). Possibly, H$_2$O$_2$ in our study led to programmed cell death (Desikan *et al.*, 2002) which later led to down-regulation of genes of the shikonins biosynthesis pathway (Fig. 4.78) leading to inhibition of shikonins biosynthesis. In *Arabidopsis thaliana*, H$_2$O$_2$ generated in cell in response to elicitors induced the expression of defense-related genes as well as initiated programmed cell death (Desikan *et al.*, 2000).

### 5.10 CYP family genes offer opportunity for gene discovery

The genes for the enzymes involved in terminal steps in shikonins biosynthesis i.e. steps leading from GHB to shikonin are yet to be characterized. These reactions involve oxidation and reduction reaction catalysed by redox enzymes. Redox enzymes, in general, are encoded by CYP family genes. CYPs are hence vital to aid to the discovery of genes involved in this pathway. CYPs are membrane-bound, heme-iron containing enzymes, which require NADPH$+H^+$ as a co-substrate. CYPs catalyze a large number of reactions such as hydroxylation, epoxidation, reduction, oxidation, demethylation, desulfonation, peroxidation, deamination and dehalogenation reactions in plants (Lupien *et al.*, 1999; Rendic and Dicarlo, 1997; Bouwmeester *et al.*, 1999; Ralston *et al.*, 2001; Isin and Guengerich, 2007). Limonene 6-hydroxylase and amorphadiene oxidase involved in the production of *trans*-carveol and artemisinic acid, respectively are CYP group of enzymes (Kirby and Keasling, 2009). Yamamoto *et al.* (2000) reported a CYP-dependent monooxygenase (GHQ3$^*$-hydroxylase) which produces GHQ-3$^*$$-OH$, a new intermediate of shikonins biosynthesis, thus strengthening the possibility of CYPs role in the terminal stages of shikonins biosynthesis.

To clone CYPs, primer based approach was followed. This approach worked well in *Lolium rigidum* and *Ocimum basilicum* (Fischer *et al.*, 2001). In the present work, 36 putative CYPs (including novel) were cloned. Ten CYPs exhibited expression in accordance
with the expression of regulatory genes of the pathway, for example, *HMGR*, *PGT* and genes of PP pathway (Fig. 4.74, 4.80). Since it was a correlative study, functional evaluation of each gene would be essential to ascertain their role in shikonins biosynthesis.

### 5.11 Identification of putative regulatory cis-elements of selected pathway genes

Biosynthesis and regulation of secondary metabolites are influenced by several environmental cues (Yazaki *et al.*, 2002; Touno *et al.*, 2005) including signaling network involving H$_2$O$_2$, NO, Ca$^{2+}$, cAMP, cGMP, MAPK cascades, SA, MJ, ethylene and ABA signaling (Li and Xue, 2010; Zhao *et al.*, 2005). Transcriptional regulation of gene is achieved through binding of transcription factors to cis-acting regulatory elements. These are usually located in the promoter regions, which are located upstream of the coding sequences (Udvardi *et al.*, 2007). Generally, genes having similar expression patterns contain common motifs in their promoter (Klok *et al.*, 2002) to ease regulation of various genes of a metabolic pathway by a common transcription factor. There were no information on genes and their upstream sequences in *A. euchroma*. In the present thesis, upstream sequences of six genes namely, *AeACTH*, *AeHMGR*, *AeMVDD*, *AeIPPI*, *AeGDPs* and *AePGT* were cloned and analysed (section 4.7). In addition to a number of motifs identified for basal transcription, several putative regulatory motifs were identified in the upstream sequences. These included motifs related to light, ABA, LT, drought/dehydration, gibberelins, cytokinin, auxin, signal transduction, leaf/plastid specificity, cell division and biotic factors (Table 4.5-4.10).

*A. euchroma* grows in cold deserts of high altitudes, which is characterized by the prevalence of high light intensity and LT, and low soil moisture and low relative humidity. *In silico* analysis did show the prevalence of light, drought, and LT responsive elements throughout the upstream regions of the six genes. LREs such as GATA box, GT-1, TATA box, and I box were implicated in light-dependent gene regulation (Gilmartin *et al.*, 1990; Terzaghi and Cashmore, 1995; Tjaden *et al.*, 1995; Villain *et al.*, 1996; Zhou, 1999; Reyes *et al.*, 2004). GT element in the light-repressed *Oryza sativa PHYA* gene was reported to play essential role in the dark activation of this gene by modulating a dark specific promoter element and the repression requires the presence of specific photoreceptors (Zhou, 1999). Analysis of upstream sequences of *AeACTH*, *AeHMGR*, *AeMVDD*, *AeIPPI*, *AeGDPs* and *AePGT* showed the presence of I box, GT1 and GATA box. The role of these elements
could be envisaged in shikonins biosynthesis. The role of light in modulating a range of terpenoids and the corresponding transcripts has been documented (Zhao et al., 2001; Souret et al., 2003; Kawoosa et al., 2010). Effect of light on the activity of HMGR promoter has been reported that explained the light mediated alteration in HMGR transcripts (Learned and Connolly, 1997). It is worth mentioning that in *A. euchroma*, all the genes expressed under light as well as dark e.g. in leaf all the twelve genes of the pathway (Fig. 4.75 and Fig. 4.76) and in HSPS which is maintained in dark (Fig. 4.74). Therefore, the role of these elements in light mediated gene regulation requires critical analysis.

LTRE such as CBF, ABA responsive elements, and MYC and MYB recognition sites are reported to be involved in LT-mediated regulation of several genes (Baker et al., 1994; Choi et al., 2000; Suzuki et al., 2005). These were also found in upstream regions of all the six genes in *A. euchroma*. LT-mediated increase in secondary metabolites in *Arabidopsis thaliana* has mainly been attributed to the transcriptional up-regulation of genes of secondary metabolism, which in turn, has been suggested due to the over-expression of relevant transcription factors at LT (Hannah et al., 2005; Kawoosa et al., 2010). Since *A. euchroma* is present in niche of cold desert location, the presence of LTRE suggested the role of low temperature role in modulating shikonins biosynthesis.

ABREs could be important, keeping in view the pivotal roles played by ABA in response to stress and secondary metabolism have been discussed in section 5.9 (Shinozaki and Yamaguchi-Shinozaki, 2000; Finkelstein et al., 2002; Xiong et al., 2002). ABA plays role in signal transduction of plant defense responses, and used as elicitor of secondary metabolic pathways (Hildmann et al., 1992; Klessig and Malamy, 1994; Shirasu et al., 1997). Role of ABA in plant secondary metabolism and its implication in shikonins biosynthesis has been discussed in section 5.9. ABA regulates the expression of regulatory genes (*AeHMGR* and *AePGT*) involved in shikonins biosynthesis (Fig. 4.77).

W-box elements present in the upstream regions could be important in plant defense response against pathogen. HMGR was reported to play a role in plant defense response, for example, in potato, HMGR genes were activated in response to pathogen (Yang et al., 1991). Role of W-box elements needs further investigation in relation to growth and development of plant. Further to establish function of the above elements, analysis by EMSA followed by transgenic analysis would be required.
5.12 Conclusions and future prospects

The present work is the first endeavor to understand the molecular basis of shikonins biosynthesis in *A. euchroma*. Importantly, twelve genes of the pathway were cloned and the expression analysis identified *AeHMGR, AePGT* and the genes of PP pathway as possible regulatory genes in shikonins biosynthesis (Fig. 5.1). Concomitantly, thirty six *CYPs* and upstream regulatory elements of six genes were identified. The knowledge so generated has
Chapter 5

Discussion

implications to understand molecular basis of shikonins biosynthesis in *A. euchroma* and is a first steps towards metabolic engineering for this important moiety. The present work could be followed in future as:

1. Functional characterization of genes by heterologous expression followed by enzymatic assay or complementation assay.
2. Identification of candidate genes involved in terminal steps of shikonins biosynthesis.
3. Functional characterization of upstream sequences/promoters.
4. Strategies could be devised to transplant the shikonins biosynthesis pathway in heterologous systems.