CHAPTER 5

DISCUSSION
DISCUSSION

Plant isoprenoids comprise a structurally diverse group of compounds that can be divided into classes of primary and secondary metabolites. Isoprenoids that are primary metabolites include sterols, carotenoids, growth regulators, and the polyprenol substituents of dolichols, quinones, and proteins. These compounds are essential for membrane integrity, photoprotection, orchestration of developmental programs, and anchoring essential biochemical functions to specific membrane systems, respectively. Isoprenoids classified as secondary metabolites include monoterpenes, sesquiterpenes, and diterpenes. Compounds within this latter category are considered secondary because they are not essential for viability. They however, mediate important interactions between plants and their environment such as plant-plant (Stevens, 1984), plant-insect (Gibson and Pickett, 1983), and plant-pathogen (Stoessl et al., 1976) interactions. Mevalonate is a six-carbon intermediate in the isoprenoid biosynthetic pathway, arising from the sequential condensation of three acetyl-COA units to generate HMG-COA, which is then converted to mevalonate in an irreversible reaction catalyzed by HMG-COA reductase (HMGR). Due to the irreversible nature of this reaction, early workers correctly surmised that this step was a likely regulatory point of sterol biosynthesis in mammalian systems and eventually correlated the absolute rate of cholesterol biosynthesis with the level of this enzyme activity (Goldstein and Brown, 1990). Whether this enzyme plays a similar rate-limiting role in controlling plant isoprenoid biosynthesis remains unresolved (Choi et al., 1992; Narita and Gruissem, 1989; Bach, 1986).

The mevalonate, synthesized by the catalytic action of HMG-Co A reductase, is at the beginning of isoprenoid biosynthesis pathway and serves as the common precursor for the production of a number of natural products e.g. sesquiterpinoids, triterpenoids, sterols,
carotenoids and phytoalexins. All terpenoid synthases are almost similar in their physical characteristics and chemical properties (Li et al., 2006). Based on their similar reaction mechanism, conserved structural and sequence characteristics, including the amino acid sequence homology, conserved sequence motifs, intron number and exon size, several research groups have suggested that the plant terpene synthases share a common evolutionary origin (Trapp and Croteau, 2001). Due to insufficient information dealing with the structure-function correlation, it has been difficult to predict the cellular and biochemical functions of a terpene synthase solely on the degree of sequence identity (Trapp and Croteau, 2001).

The production of isoprenoids is supposed to be tightly regulated by the activities of rate limiting enzymes and they are thought to play a regulatory role in the biogenesis of isoprenoids. Based on the previous studies, it has been suggested that the mevalonate pathway operating in cytosol provides the carbon for the synthesis of artemisinin as well as other isoprenoids (Maujiram et al., 2010; Towler and Weathers, 2007; Akhila et al., 1987; Kudakasseril et al., 1987). To understand the role of HMGR in artemisinin biosynthesis, we carried out a series of feeding experiments and also used transgenic approach to modulate its activity. Based on the results of these studies, we showed that its activity is co-related with cellular MVA pool and artemisinin accumulation in A. annua L. plants (Maujiram et al., 2010; Nafis et al., 2010). The increased HMGR activities through over-expression of hmgr in transgenic plants have also been reported to enhance the level of many isoprenoid compounds such as artemisinin, lycopine, phytoalexins, phytosterols etc. (Nafis et al., 2010; Aquil et al., 2009; Ayora-Talavera, 2002; Argolo et al., 2000; Chappell et al., 1995).

As there are now increasing evidences that mevalonate synthesised through mevalonate pathway is utilized in the biosynthesis of many
other isoprenoids (Arsenault et al., 2010), there could be a strong competition among these pathways for available mevalonate. It, therefore, becomes a limiting factor for the biosynthesis of these isoprenoid including artemisinin. Moreover, in the biosynthesis pathway of terpenes, various terpene synthases catalyze cyclization reactions, converting a few allylic diphosphates (FPP, DMAPP, etc.) into a surprising array of cyclic products (Chen et al., 1995). The first step of artemisinin biosynthesis linking it to mevalonate pathway is the cyclization of farnesyl diphosphate (FPP) by an enzyme, amorpha-4, 11-diene synthase (ADS) to produce the amorpha-4,11-diene (Alam et al., 2010; Wallaart et al., 2001), which is subsequently converted into dihydroartemisinic acid through a series of enzyme catalyzed reactions (Chang et al., 2000). Finally, the conversion of dihydroartemisinic acid into artemisinin consists of non-enzymatically catalyzed reactions starting with the photo-oxidation (singlet oxygen, $^{1}\text{O}_2$) of dihydroartemisinic acid, which proceeds through a classical diene reaction with rearrangement of the endocyclic double bond, yielding dihydroartemisinic acid hydroperoxide. Ring cleavage is induced by air oxidation (triplet oxygen, $^{3}\text{O}_2$) and then re-formation of the ring structure in artemisinin is completed as an end product (Wallaart et al., 2001). Therefore, the cyclization of FPP catalyzed by an amorpha-4, 11-diene synthase is the essential step of the artemisinin biosynthesis (Fig. 6; Wallaart et al., 2001).

As both HMGR and ADS catalyzing the rate limiting steps in mevalonate pathway and artemisinin biosynthesis pathway, respectively, we have cloned the genomic DNA sequences encoding these enzymes from C. roseaus (Aquil et al., 2009) and A. annua L. (Alam et al., 2010) respectively, with an aim to transform A. annua L. for enhancing the biosynthesis of artemisinin and its accumulation. Further, we have also analyzed the nucleotide sequence of $ads$ and amino acid sequence of the protein derived from...
from this gene employing bioinformatics tools before transformation experiments. The results obtained in these studies are discussed in following sections.

5.1 Analysis of the Nucleotide and Protein Sequences of ADS

Amorpha-4, 11-diene synthase belongs to the class of enzymes referred as sesquiterpene synthases (sesquiterpene cyclases). These are very similar in physical and chemical properties. Based on their similar reaction mechanism, conserved structural and sequence characteristics including amino acid sequence homology, conserved sequence motif, introns number and exon size, several groups have suggested that plant sesquiterpene synthases have a common evolutionary origin. Because of lack of the knowledge of structure-function correlation, it was impossible to predict the function of terpene synthases solely on the degree of sequence identity until now (Trapp and Croteau, 2001). The cloned ads gene sequence (FJ432667) from a high yielding strain of A. annua by us, have shown 98 % identity with nucleotide and amino acid sequences derived from earlier published A. annua ads gene sequences. The genomic organization of ads gene comprises of 7 exons (17-103; 218-448; 2126-2508; 2685-2903; 3032-3170; 3268-3516 and 3629-3922) and 8 introns (1-16; 104-217; 449-2125; 2509-2684; 2904-3031; 3171-3267; 3515-3628 and 3923-3963), respectively (Fig. 14). The deduced mass and the pI of the encoded protein are 62.2 kDa and 5.25, respectively.

Multiple sequence alignment analysis of ADS gene with seven ads gene already reported has revealed 98 % homology. On amino acid scale, ADS (533 aa) protein has shown more than 98 % identity with amorpha-4, 11-diene synthase proteins of other strains of Artemisia annua L. (Fig. 15). Phylogenetic tree analysis has shown relatively higher homology of ads gene from Artemisia annua L. with sesquiterpene synthases from angiosperms (Fig. 16). The 7 different
types of domain are found in ADS protein (ACL15394) (Table 5). The highly conserved region, aspartate rich motif (DDxxD), is the characteristic of all terpene synthases (Wallaart et al., 2001). This motif is involved in the coordination of the substrate bound divalent metal ion (Mg$^{2+}$ and Mn$^{2+}$) (Starks et al., 1997). Two basic residues Arg$^{264}$ and Arg$^{441}$ from 5-epi-aristolochene synthase from Nicotiana tabacum (Starks et al., 1997) corresponding to Arg$^{249}$ and Arg$^{427}$ in ADS protein are brought close to each other by loop movement (Fig. 17). The deletion of 13 amino acid residues from ADS protein has shown no influence on its catalytic properties, as they may not constitute the essential structure of protein. The presence of a new motif SlwD, a casein kinase II phosphorylation domain site at position 104-107 in ADS protein, not found in other terpene synthase, is a unique finding of our study. The same motif in HIV coat protein gp120 has been reported by Wallace et al. (1995). It has been shown that the activity of enzymes of terpenoid biosynthetic pathway in higher eukaryotes is regulated by phosphorylation/dephosphorylation. Enzymes whose activity is regulated by phosphorylation differ both with respect to the spatial relationship between their active and regulatory sites and the mechanisms by which phosphorylation modulates activity. Therefore, this new site present in ADS protein may be part of regulatory region of the enzyme and the DDxxD may be the part of active site for binding of the substrate.

5.1.1 Molecular Modeling and Docking

The three-dimensional (3D) structure of protein is of major importance in providing insights into their molecular functions. The result showed that the putative ADS protein contains 72 % of α-helices, 23 % β-turns and 26 % of random coils (Fig. 18). Penetrating through most part of the secondary structure, α-helices and random coils are the most abundant structural elements of ADS3963, while β turns are intermittently distributed in protein. The
total energy values of the predicted 3D model of ADS protein were calculated as 93 % before energy minimization and 90 % after energy minimization for Ramachandran plot (Fig. 19). The refined model of ADS, analyzed by VADAR for the evaluation of the Ramachandran plot quality, was found to be satisfactory based on expected values representing those numbers which would be expected for highly refined X-Ray and NMR protein structure (Table 4). The description of reaction regulation in enzymes responsible for activating and catalyzing small molecules requires identification of ligand movement into the binding site and out of the enzyme through specific channels and docking sites.

Recent studies have revealed that the core sequences of many proteins were nearly optimized for stability by natural evolution. Surface residues, by contrast, were not so optimized, presumably because protein function is mediated through surface interactions with other molecules. Here, we sought to determine the extent to which the sequences of protein ligand-binding and enzyme active sites could be predicted by optimization of scoring functions based on protein ligand-binding affinity rather than structural stability (Willard et al., 2003). In an attempt to find the possible binding sites of FPP on Amorpha-4, 11-diene synthase (ADS), PASS was performed. The output of PASS contains center coordinates for 17 binding sites. Docking of FPP was performed with respect to all the 17 binding sites of the enzyme (Table 6). Lower energy corresponds to better binding therefore; initial six of these binding sites (Table 7) were studied for the interaction with FPP (Fig. 20 & 21). Based on the docking studies the following stretches are Ala 321-Ala324-Lys-398; Ala 234-Val237-Phe283-Thr286-Tyr-287; Ser94-Met95-Trp141-Trp430-Asn434; Ser94-Arg96-Glu104-Leu107-Lys142-Lys431; Lys137-Arg143-Ile147-Ala150-Gln151-Leu478 and Ser218-Gly219-Tyr224-Arg228-Cys352-Met356-Aln450 may constitute to substrate binding. These results may have implications for
understanding the role of amorpha-4, 11-diene synthase in cyclization of FPP.

5.2 Genetic Transformation of *Artemisia annua* L.

*Artemisia annua* L. is very important medicinal plant used to extract anti-malarial compound, artemisinin. Extensive efforts are currently being directed towards the development of novel transgenic *A. annua* L. varieties over-expressing genes of various enzymes of isoprenoid biosynthetic pathway in order to increase artemisinin biosynthesis and accumulation. As there are now increasing evidences that mevalonate synthesised through mevalonate pathway is utilized in the biosynthesis of many other isoprenoids (Arsenault et al., 2010), there could be a strong competition among these pathways for available mevalonate. It, therefore, becomes a limiting factor for the artemisinin biosynthesis.

Keeping in view these findings, we have attempted to over-express not only *hmgr* encoding HMGR, the rate limiting enzyme of mevalonate pathway, but also *ads*, the gene encoding ADS, the enzyme catalyzing the first rate limiting step in artemisinin biosynthesis, linking it to the mevalonate pathway (Koobkokkruad., 2008; Wallaart et al., 2000; Bouwmeester et al., 1999). We believed that by over-expressing both *hmgr* and *ads*, it would be possible to enhance the cellular mevalonate pool and also divert more mevalonate towards artemisinin biosynthesis. We therefore, transformed *A. annua* L. plants with *Agrobacterium tumefaciens* strain EHA105 harboring pCambia containing *hmgr* and *ads* driven by ubiquitin and 35S promoters, respectively. We used genomic sequence encoding of these enzymes, as the intron play significant role in gene expression (Trapp and Croteu, 2001). Genetic transformation of *Artemisia annua* L. using different genes of isoprenoid pathway has already been reported by many workers (Zhang et al., 2009; Han et al., 2005, 2006; Sa et al., 2001; Chen et
al., 2000; Ghosh et al., 1997; Vergauwe et al., 1996). The transformation frequencies and artemisinin levels in these studies however, varied because of differences in *Agrobacterium* strains, type of vectors and the chemotypes of *A. annua* L. used.

5.2.1 Regeneration of *Artemisia annua* L.

The first step in the development of a transgenic plant through metabolic engineering for enhanced production of a secondary metabolite is to develop an efficient regeneration protocol. We, therefore, first developed a protocol for high frequency shoot regeneration from leaf explants of *Artemisia annua* L. plants using pre-standardized shoot-induction medium (SIM) with combination of 0.05 mg/L NAA and 1.5 mg/L 6-BAP (Nafis et al., 2010), we achieved 88% shoot-induction frequency from leaf explants of *A. annua* L. plants.

5.2.2 Factors Influencing Genetic Transformation of *A. annua* L.

5.2.2.1 Pre-culture of Explants

Pre-culture of explants is a critical factor to achieve high frequency of *Agrobacterium* mediated transformation, as it makes the cells of explants competent enough to withstand the injury caused by bacterial infection. Observation on the effect of bacterial concentration on transformation efficiency of pre-cultured explants was carried out. In this study, 5-6 days old pre-cultured leaf explants produced higher number of transformed shoots. Pre-culturing of *A. annua* L. explants prior to inoculation and cocultivation with *Agrobacterium* in earlier studies have also been found to improve the transformation frequencies in some medicinal plants (Bae et al., 2005; Han et al., 2005).

5.2.2.2 *Agrobacterium tumefaciens* Strain and Vector

The advantages of *Agrobacterium* mediated gene transfer over other methods include the high efficiency of transformation and less
chances of silencing of transgene expression. Binary disarmed vectors are commonly used as plant expression vectors for genetic transformation of plants. Type of the *Agrobacterium tumefaciens* strain and vector used for transformation in co-cultivation experiments play a very important role in the transformation system. Vergauwe et al. (1996) have found that shoot formation, after infection of explants with the EHA101 (pEHA101) (pTJK136) strain of *Agrobacterium* was very low (3%) on selection medium when compared with other studies (Han et al., 2005). The chromosomes in *Agrobacterium* and activating potency of genes in virulence region are important internal factors influencing the infecting ability of *A. tumefaciens* (Wang et al., 1998). Han et al. (2005) used EHA105 and LBA4404 strain of *Agrobacterium tumefaciens* in their transformation experiments and found that the former was superior to the later. These two strains not only have different chromosome background, but also different Vir-helper plasmids with different levels of activating potencies. We have, therefore, used EHA105 in our study with a transformation efficiency of 3.5% achieved by us.

5.2.2.3 Genotype of *A. annua* L. Plant and Type of Explants Used in Co-Cultivation

*A. tumefaciens* has differential ability to infect different species of plants and in some cases, even has different ability to infect different genotypes of the same species. Generally speaking, the specificity of genotype is related to the cell physiological conditions, which include metabolic status of the cell after wounding, endogenous levels of hormones, and structure of cell wall etc. (Wang et al., 1998). Among various explants such as leaves, stem segments, and root segments, the leaves were more often used in transformation experiments involving *A. annua* L. plants (Ghosh et al., 1997; Vergauwe et al., 1996). It is because the leaves have the highest shoot induction frequency (Vergauwe et al., 1996). Also, A.
*Agrobacterium tumefaciens* has the strongest ability to infect leaves (Ghosh et al., 1997). When the cotyledons or hypocotyls from 8-day-old seedlings were used as explants, the transformation efficiency declined and was found very low. On the other hand, when the seeds were used as explants, the transformed seeds were able to germinate but unable to root on the selection medium (Vergauwe et al., 1998). Most investigators hence, preferred leaf discs in their transformation experiments (Zhang et al., 2009; Chen et al., 2000). In this study, we have used leaflets of *A. annua* L. instead of leaf discs as leaf discs after co-cultivation were unable to survive on selection medium due to mechanical and biological injuries in magnitude as compared to leaflets. The shoot induction was therefore, achieved in our study from wounded regions of leaflets after their co-cultivation with *A. tumefaciens* strain EHA105. The second reason for this observation could be higher number of cells for bacterial infections.

### 5.2.2.4 *Agrobacterium* Density, Infection Time and Co-Cultivation Period

The transformation experiments were performed to optimize the effectiveness of *Agrobacterium* density, infection time and co-cultivation period. In this study, the infection time of one hour, inoculum density of 0.6 OD<sub>600</sub> nm and the co-cultivation period of 2 days dark at 25-28 °C (MS basal) gave the higher transformation efficiency. If the optical density of bacterial culture is greater than 0.6 at OD<sub>600</sub> nm and co-cultivation period is beyond 2 days, then these results in bacterial overgrowth and leaching of bacteria from leaf explants leading to low transformation efficiency (Fig. 25).

### 5.2.2.5 Antibiotics

To develop a selective growth system for genetically transformed *A. annua* L., the effects of hygromycin at various concentrations on leaf explants of *A. annua* L. plants were studied during shoot
production. This experiment was conducted by us, because higher levels of antibiotics in the shoot induction medium may put the explant in stress and so the shoot induction frequency is reduced and the growth of shoot is also retarded (Ling et al., 1998) in previous study. Vergauwe et al. (1996) used cefotaxime as decontaminating antibiotic and reported that it results in retardation of callus formation and inhibition of the shoot inducing capacity in transformed Artemisia annua L. Contrary to this, the results of our study have indicated that 500mg/L cefotaxime in shoot induction medium was found to be beneficial and resulted into maximum number of shoot formation (81.25%) in transformed leaflets of A. annua L. Our findings are also supported by Han et al. (2005), where they reported that use of cefotaxime (500mg/L) in the transformed shoots resulted into increased shoot regeneration in this plants.

After co-cultivation with transformed Agrobacterium tumefaciens strain EHA105, these leaf explants were transferred to the shoot induction selection medium containing 20 mg/L hygromycin (SISM) for 4-5 weeks. Most of the untransformed leaf explants were died. Some of the explants however, developed hygromycin resistant shoots during this period. These hygromycin resistant shoots were further elongated in the medium devoid of hygromycin for 3-4 weeks. The elongated shoots were thereafter, transferred to the root induction medium (MS+0.5mg/L NAA). The roots appeared within 2 weeks after transferring the shoots on rooting medium. A total of 240 explants were co-cultivated with A. tumefaciens EHA105, out of which only 12 produced shoots on SISM with an overall 5% transformation frequency. Out of 240 leaf explants from A. annua L. transformed, only 12 hygromycin resistant shoots were obtained. When PCR analysis of the DNA samples from these shoots was performed using hptII gene specific primers, only 8 shoots were found to contain the 500bp transgene, hptII amplicons (Fig. 30). Thus, the overall transformation efficiency achieved in our study
was 3.33%, as against the earlier study carried out by Han et al. (2005) where they reported 4-10% transformation frequency in fascicled shoots of A. annua L. The low transformation frequency obtained in our study could be due to the use of different A. tumefaciens strains, expression vector, the chemotype of A. annua L. plant and the number of transgenes. However, the transformation efficiency becomes irrelevant to artemisinin biosynthesis as clonal multiplication of a transformed line can easily be achieved.

5.3 Molecular Analysis of Transgenic Plants

5.3.1 PCR Analysis
The transgenic status of hygromycin resistant plants was first confirmed by PCR analysis. For this analysis, we used hptII gene specific primers, as it is a transgene and an integral part of the expression cassette (Fig. 9). Out of 12 putative transgenic plants, 8 showed 0.5 kb amplification products when amplified with the hptII gene specific primers (Fig. 30). No amplification was, however, detected in the non transgenic lines of A. annua L., as the hptII is the bacterial gene.

5.3.2 Southern Analysis
The PCR-positive transgenic A. annua L. lines were further tested using Southern blot for the integration of hptII gene and its copy number in their genome. Genomic DNA samples from eight putative transgenic and non-transgenic plants were digested with EcoRI. The digested genome samples from these plants resolved on agarose gel and blotted on polystyrene membrane. The blots were the hybridized with DIG-labeled hptII gene probe employing the method as described in DIG kit. All the transgenic plants showed different number and sizes of hybridized bands confirming the integration of transgenes at different loci through independent transformation events (Fig. 31). The transgenic plants TR1, TR2, TR4, TR5, TR7 and TR9 had single copies of hmgr and ads, while TR3 and TR8
contained multiple copies (Fig. 31). However, non-trangenic plant DNA did not show any hybridization signal, as expected due to the use of DNA probe of hptII a bacterial gene in this study.

It is known that Agrobacterium mediated transformation often results in insertion of single or multiple copies of the transgenes at different low (Gheysen et al., 1991). The transgenic plants containing single copy of transgenes are however, desirable as multiple copies of T-DNA adversely influence the stability and expression of the introduced gene (Starn et al., 1997). Multiple-copy genotype could result from insertions at different loci along the chromosomes or at the same locus. The origin of multiplicity at a single locus remains unknown, although copy organization is either in tandem or as inverted repeats at a given locus (Delroles and Gardner, 1988). The low stability and expression of transgenes in transgenic plants with multiple copies as observed by us and in earlier studies could be due to gene silencing either through DNA methylation or co-suppression (Tang et al., 2006; Hobbs et al., 1993; van der Krol et al., 1990).

5.3.3 Analysis of expression of hmgr and ads
To confirm the expression of HMG-CoA reductase and amorpha 4, 11-diene synthase genes at mRNA level, RT-PCR analyses were performed using total RNA samples isolated from the leaves of transgenic and non-transgenic of A. annua L plants and hmgr as well as ads specific primers. The results showed that the transgenes were expressed differentially in transgenic A. annua L. plants (Fig. 32). Further, no amplification was observed in non-transgenic lines with hmgr specific primers, but amplification with ads specific primer was observed in both transgenic and non-transgenic plants (Fig. 32A & B). The TR4, TR5 and TR7 transgenic lines had shown higher expression levels of both hmgr and ads genes, when compared with other transgenic plants (TR3 and TR8) with multiple
copies of transgenes. The low expression levels of the transgenes in the later could be due to the gene silencing either through DNA methylation or co-suppression (Tang et al., 2006 Hobbs et al., 1993; van der Krol et al., 1990). The expression levels of transgenes in TR1, TR2, TR4, TR5, TR7 and TR9 also varied, which could be due to the positional effect of transgenes (Spiker and Thompson, 1996; Meyer, 1995).

5.4 Influence of Transgenes *hmgr* and *ads* on Growth of *A. annua* L. Plants
To observe the impact of *hmgr* and *ads* transgenes on the biomass accumulation (in terms of dry weight) was observed in transgenic and non-transgenic *A. annua* L. plants after second, fourth and six week of culture. In our study, we have observed that the chlorophyll and soluble protein contents of all transgenic plants of *A. annua* L. was higher as compared to non-transgenic plants. The transgenic lines with higher expression of *hmgr* and exhibiting increased activity of HMGR enzyme have also found to be higher chlorophyll and soluble protein contents and accumulated more biomass when compared with other transgenic and non-transgenic *A. annua* L. plants (Table 10, 11 &12). This increased in soluble protein, chlorophyll and biomass could be due to the expression of *hmgr* only as *ads* over-expressed in transgenic *A. annua* L. lines is highly specific for artemisinin biosynthesis and these compound has unique role in the growth of plants excepts secondary functions (Zhang et al., 2010; Ma et al., 2009; Wu et al., 2006).

5.5 HMG-CoA Reductase, Amorpha-4, 11-Diene Synthase Activities, Mevalonate and Artemisinin Contents
The mevalonate, synthesized by the catalytic action of HMG-Co A reductase, is at the beginning of isoprenoid biosynthesis pathway
and serves as the common precursor for the production of a number of natural products e.g. sesquiterpenoids, triterpenoids, sterols, carotenoids and phytoalexins. The results obtained suggest a strong relationship between the enzyme activity and the contents of mevalonate, artemisinic acid and artemisinin, which further confirm that major carbon supply for artemisinin biosynthesis, comes from cytosolic mevalonate pathway rather than plastidial Rohmer (MEP/DXP) pathway. Low HMG CoA reductase activity and contents of mevalonate, artemisinic acid and artemisinin at rosette stage might be due to low enzyme and substrate availability in early stage of plant growth and development. There are several studies, showing a strong correlation between HMG-CoA reductase activity and biosynthesis of isoprenoid compounds (Chappell et al., 1995; Narita and Gruissem, 1989; Stermer and Bostock, 1987; Chappell and Nable, 1987; Bach, 1986), as HMG CoA reductase regulates the carbon flux from primary to secondary metabolism leading to synthesis of isoprenoids Some molecular evidences support the developmental increase in HMG-CoA reductase and concomitant increase in isoprenoid compounds. The HMG-CoA reductase is reported to be encoded by specific small gene families. The members of these gene families are differentially expressed in specified organs during developmental or in response to environmental factors, and distinct HMG-CoA reductase isoforms may be critical in directing the flux of pathway intermediates into specified isoprenoid compounds (Chappell, 1995; Wiessenborn et al., 1995; Choi et al., 1994; Enjuto et al., 1994).

The production of isoprenoids is supposed to be tightly regulated by the activities of rate limiting enzymes and they are thought to play a regulatory role in the biogenesis of isoprenoids. Based on the previous studies, it is proposed that the mevalonate pathway operating in cytosol mainly provides the required carbon for the synthesis of artemisinin as well as other isoprenoids (Maujiram et
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al., 2010; Towler and Weathers, 2007; Akhila et al., 1987; Kudakasseril et al., 1987). To understand the role of HMGR in artemisinin biosynthesis, we carried out a series of feeding experiments and also used transgenic approach to modulate its activity. Based on the results of these studies, we showed that its activity is co-related with cellular MVA pool and artemisinin accumulation in *A. annua* L. plants (Maujiram et al., 2010; Nafis et al., 2010; Aquil et al., 2009). The increased HMGR activities through over-expression of *hmgr* in transgenic plants have also been reported to enhance the level of many isoprenoid compounds such as, lycopine, phytoalexins, phytosterols etc. (Ayora-Talavera, 2002; Argollo et al., 2000; Chappell et al., 1995).

As there are now increasing evidences that mevalonate synthesised through mevalonate pathway is utilized in the biosynthesis of many other isoprenoids (Arsenault et al., 2010), there may be a strong competition among these pathways for available mevalonate. It, therefore, becomes a limiting factor for the artemisinin biosynthesis. Keeping in view these findings, we have attempted to over-express not only *hmgr* encoding HMGR, the rate limiting enzyme of mevalonate pathway; but also *ads*, the gene encoding ADS, the enzyme catalyzing the first rate limiting step in artemisinin biosynthesis, linking it to the mevalonate pathway (Koobkokkruad et al., 2008; Wallaart et al., 2001; Bouwmeester et al. 1999). We believed that by over-expressing both *hmgr* and *ads*, it would be possible to enhance the cellular mevalonate pool and also divert more mevalonate towards artemisinin biosynthesis. It is interesting to note that the transgenic lines over-expressing *hmgr* and *ads* were also found to have higher HMGR and ADS (*Fig. 33 & 34*) as well as higher artemisinin contents (*Fig. 36*). One of the transgenic lines, TR4 showed 7.65 fold increases in the artemisinin content, 71.83% HMG-CoA reductase and 61.76% ADS activities, when compared with the non-transgenic *A. annua* L. plants and in polyhouse
condition the higher HMG-CoA reductase activity (35.57%), mevalonate content (37.09%) and amorpha-4,11-diene synthase activity (38.81%) (Table 13, 14 & 15) as well as artemisinin content (74.13%) and yield (70.39%) were observed as compared to wild type plants. The higher biosynthesis and accumulation of artemisinin in the transgenic *Artemisia annua* L. strain reported in the present study were not achieved in earlier studies (Zhang et al., 2009; Han et al., 2006; Chen et al., 2000; Sa et al., 2001). This is probably due to the over-expression of both *hmgr* and *ads* genes together in transgenic *A. annua* L. plants by us.