Overexpression of the HMG-CoA Reductase Gene Leads to Enhanced Artemisinin Biosynthesis in Transgenic *Artemisia annua* Plants

Authors Samina Aquil[1,3], Amjad Masood Husaini[1], Malik Zainul Abdin[1], Gulam Muhammad Rather[2]

Affiliations
[1] Centre for Transgenic Plant Development, Department of Biotechnology, Jamia Hamdard, New Delhi, India
[2] Department of Chemistry, Indian Institute of Technology, New Delhi, India
[3] Current address: Department of Biochemical Engineering and Biotechnology, Indian Institute of Technology, New Delhi, India

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- *Artemisia annua* (Asteraceae)
- transgenic
- *HMGR*
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Abstract

An effective and affordable treatment against malaria is still a challenge for medicine. Most contemporary drugs either are too expensive to produce or are not effective against resistant strains of the malaria parasite *Plasmodium falciparum*. The plant *Artemisia annua* L. is the source of artemisinin, an effective drug against malaria for which no resistant strains of the bacterium have been reported. However, the artemisinin content of *A. annua* is very low, which makes its production expensive. Here we report the use of transgenic technology to increase the artemisinin content of *A. annua*. We report the production of transgenic plants of *A. annua* into which we transferred 3-hydroxy-3-methylglutaryl CoA reductase (*HMGR*) gene from *Catharanthus roseus* (L.) G. Don using *Agrobacterium*-mediated gene transfer technology. Transgene integration and copy number were assessed by PCR and Southern hybridization, which confirmed the stable integration of multiple copies of the transgene in 7 different transgenic lines of *A. annua*. The leaf tissue of three of the *A. annua* transgenic lines possessed significantly higher HMGR activity compared with wild-type controls, and this activity was associated exclusively with microsomal membranes. The artemisinin content of the shoots of one of the transgenic lines depicted an increase of 22.5% artemisinin content compared with wild-type control *A. annua* plants.

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Correspondence
Dr. Amjad Masood Husaini
Division of Plant Breeding and Genetics
Sher-e-Kashmir University of Agricultural Sciences & Technology of Kashmir
Shalimar
J&K 191121
India
dr.amjadhusaini@hotmail.com

Correspondence
Dr. Malik Zainul Abdin
Centre for Transgenic Plant Development
Department of Biotechnology
Jamia Hamdard
New Delhi
India
mzabdin@rediffmail.com

Introduction

Artemisia annua L. (Asteraceae) is an annual herb native to China that grows naturally as a part of steppe vegetation in northern parts of China at an elevation of 1000–1500 m above sea level [1]. In 1798, a decoction of *A. annua* was suggested in the *Wenbing Tiaobian* as a treatment for malaria [2]. The isolation and characterization of artemisinin from *A. annua* is considered one of the most promising discoveries in recent research in medicinal plants. There are no reports of artemisinin-resistant *P. falciparum*; artemisinin and its derivatives are effective against multidrug-resistant *P. falciparum* strains prevalent in Southeast Asia and Africa [3]. The artemisinin derivative artesunate is a fast-acting drug in terms of parasite clearance compared with the traditional drug quinine and can reduce mortality in severe cases of malaria [4]. Artemisinin acts rapidly on the asexual stages of *P. falciparum*, the most malignant form of malaria. Because of the emerging resistance of *P. falciparum* to conventional anti-malarial drugs (quinine and chloroquine), artesunate plus amodiaquine and artemether–lumefantrine are the main artemisinin-based combination therapy (ACT) candidates for the treatment of drug-resistant malaria [5]. However, ACTs are very expensive because of the relatively low yields of artemisinin in *A. annua*.

Several methods have been investigated for increasing the artemisinin content of *A. annua*, such as organ culture, hormone medium, and metabolic manipulation. All these methods show potential for future development, but the improvements delivered by these methods so far have not met the demand.

The artemisinin biosynthesis pathway is beginning to be understood. Several key enzymes involved have been discovered including 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR), farnesyl diphosphate synthase, and sesquiterpene synthase [6]. The synthesis of sesquiterpene, which determines the direction of car-

bon flow, has been reported to correlate with the activity of HMGR [7]. A recent study accomplished an increase in the artemisinin content of *A. annua* by suppressing the expression of squalene synthase, a key enzyme of the sterol pathway (a competitive pathway of the artemisinin biosynthetic pathway) through RNAi, indicating that the pathway can be manipulated to obtain higher production of artemisinin [8]. The overexpression of HMGR thus appears promising for obtaining a constant high production of artemisinin. In this paper we report the transformation of *A. annua* with the *HMGR* gene from *Catharanthus roseus* (L.) G. Don for enhanced production of artemisinin.

Materials and Methods

Chemicals and plant material

All chemicals and plant hormones were purchased from Sigma Chemicals unless otherwise specified. The plants of *Catharanthus roseus* (L.) G. Don and *Artemisia annua* were collected from the Herbal Garden of Jamia Hamdard University, New Delhi, India, and were identified by Dr. Javed Ahmed, Department of Botany, Jamia Hamdard University. The voucher specimens of *C. roseus* (L.) G. Don (U 30160) and *A. annua* (U 880) are deposited in the Herbarium of the Department of Botany at Jamia Hamdard University.

Gene isolation and transformation

Genomic DNA was isolated from the leaves of *C. roseus* (L.) G. Don according to methods described elsewhere [9]. The gene-specific primers for the *HMGR* gene were designed using the published c-DNA sequence of *C. roseus* L. HMGR [10]. The sequences of PCR primers were as follows: 5′GGGGATCCATGGACTCTCGCCGGC-GATC (forward primer) and 5′GGGTCGACTCATCACTCTCTAACT-GAGAG (reverse primer). The *HMGR* gene was amplified from the purified DNA of *C. roseus* (L.) G. Don by polymerase chain reaction (PCR). PCR was performed in a reaction mixture (50 µL) consisting of 1 × reaction buffer, 0.2 mM dNTPs, 20 pmol of each primer DNA, 0.5 µg template DNA, and 1 unit of *Taq* DNA polymerase (MBI Fermentas). The reaction mixture was heated at 94 °C for 4 min for melting of template DNA followed by 32 cycles at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. At the end of 32 cycles, an additional final extension at 72 °C for 5 min was carried out to extend any premature synthesis of DNA. Following agarose gel electrophoresis, the desired DNA fragment of 3 kb was cut and purified using a QIAquick gel extraction kit (Qiagen). The isolated *HMG-CoA* reductase gene was cloned into the plant transformation vector pBinAR (provided by Prof. P.A. Kumar, NRCPB, IARI, New Delhi, India), containing kanamycin resistance gene (*npt*II) as a selectable marker (**Fig. 1**). The recombinant binary vector pBinAR was mobilized into *Agrobacterium tumefaciens* strain LBA 4404 using the freeze-thaw method [11]. Genetically engineered *A. tumefaciens* was maintained on yeast extract–mannitol (YEM) medium (Himedia) containing 1.5% agar, 50 µg/mL kanamycin, and 10 µg/mL rifampicin.

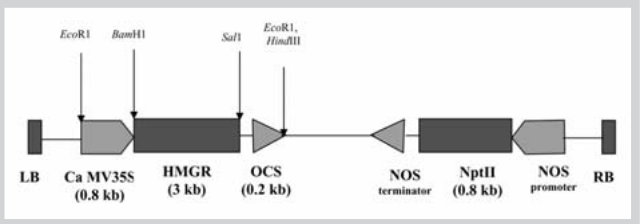

Fig. 1 The *HMG-CoA* reductase gene construct. The gene is cloned under the control of the CaMV 35S promoter in the binary vector Bin AR, containing the *npt*II gene as selection marker.

Cocultivation and plant transformation

The *in vitro* cultures of *A. annua* maintained at 25 °C under a 16-h photoperiod with 50–60 $\mu Em^{-2} \cdot s^{-1}$ light intensity served as the source of explants. The leaf discs from 8-week-old *in vitro* cultures of *A. annua* were used for cocultivation with *A. tumefaciens*. Twenty milliliters of liquid YEM medium containing 50 µg/mL kanamycin, 10 µg/mL rifampicin, and 100 µM acetosyringone (ACROS Organics) was inoculated with bacteria and incubated at 28 °C on a water bath shaker at 180 rpm overnight (16–18 h) and used in the late log phase at OD A600 of 1.0. The bacteria were centrifuged at 1500 rpm for 2 min and the pellet was resuspended in 20 mL of MS liquid medium [12]. Leaf explants were immersed in a petri plate containing the *Agrobacterium* suspension under gentle shaking at room temperature for 20 min. Explants were blot dried on sterile filter paper and cultured on callus induction medium containing MS salts + B_5 vitamins + BA (13 µM) + NAA (0.5 µM) + GA_3 (0.3 µM) + sucrose (3%) and agar (0.8%) for cocultivation. Twenty-five explants were cultured per plate. All explants were cocultivated for a period of 48 h in the dark at 25 °C. In control experiments, explants were placed on callus induction medium without cocultivation with *A. tumefaciens*.

Selection and plant regeneration

After cocultivation, the leaf explants were washed with milliQ water twice (5 min each) and then with autoclaved milliQ water containing cefotaxime (500 µg/mL) for 15 min. The explants were then blotted dry and cultured on callus induction selection medium (CISM) containing MS salts + B_5 vitamins + BA (13 µM) + NAA (0.5 µM) + GA_3 (0.3 µM) + kanamycin (50 mg/L) + cefotaxime (500 mg/L) + sucrose (3%) and agar (0.8%). After 4 weeks the explants were subcultured on the same medium for 4 more weeks. Callus was subcultured on shoot regeneration selection medium (SRSM) containing MS salts + B_5 vitamins + amino acids [asparagine (39.93 mg/L), glutamine (102.3 mg/L), cysteine HCl (5.26 mg/L), and arginine (52.26 mg/L)] and kinetin (1.0 mg/L) + kanamycin (50 mg/L) + sucrose (3%) and agar (0.8%). Subculturing was done at 4-week intervals. The shoots excised from proliferating shoot cultures were elongated on SRSM minus kanamycin. The elongated shoots were then rooted on medium containing MS salts + B_5 vitamins + NAA (0.5 mg/L) + sucrose (3%) and agar (0.8%) and evaluated for percentage rooting after 5 weeks.

PCR and southern blot analysis of putative transformants

Genomic DNA was isolated from *in vitro* developed plantlets of control and transgenic *A. annua* plants [9]. Integration of the transgene into the plant genome was screened by PCR using an *npt*II-specific primer pair. The sequences of PCR primers were as follows: 5′CAATCGGCTGCTCTGATGCCG (forward primer) and 5′ AGGCGATAGAAGGCGATGCGC (reverse primer). The PCR fragments were then fractioned on 0.8% agarose gel. For Southern blot analysis, genomic DNA (10 µg) was digested with *Sal*I, resolved on a 0.7% agarose gel, and blotted on a nylon membrane (Roche Diagnostics). For hybridization of DNA, a probe was prepared using the 3-kb *HMGR* gene obtained by complete restric-

tion enzyme digestion of pBinAR with *Bam*HI and *Sal*I enzymes and subsequent gel extraction (MinElute; Qiagen). The probe was labeled with digoxigenin using the DIG High Prime DNA labeling and detection starter kit (Roche Diagnostics), and the blot was probed with the labeled *HMGR* gene at 44 °C. The filter was washed and the probe detected following the manufacturer's instructions.

HMGR extraction

Crude enzyme extracts were isolated, following the procedure of Russell [13] with modifications, from leaf tissue of 5-month-old *A. annua* plantlets, maintained under *in vitro* conditions inside a culture room [temperature: 26 ± 1 °C; light intensity, 44.85 µE · $m^{-2} \cdot s^{-1}$; photoperiod: 16/8 h (day/night)]. All steps were carried out at 0–4 °C unless noted otherwise. Leaf tissues (1.0 g) were frozen in liquid nitrogen and ground with a mortar and pestle after the addition of 6 mL of a homogenizing buffer containing 10 mM Tris-HCl (pH 7.0), 0.35 M sucrose, 30 mM EDTA, 10 mM β-mercaptoethanol, 0.1 % bovine serum albumin (BSA), and 3.5 % (w/v) polyvinylpolypyrrolidone. The homogenate was squeezed through a layer of Miracloth (Calbiochem) and the filtrate was centrifuged in a swinging bucket rotor at 500 *g* for 3 min to remove cell debris and nuclei. The supernatant was then centrifuged in a swinging bucket rotor at 3500 *g* for 3 min to pellet plastids. The plastids were ruptured by suspending them in a medium containing 10 mM MOPS buffer (pH 7.5), 10 mM KCl, 25 mM EDTA, and 10 mM dithiothreitol (DTT). The rupture medium was used at a ratio of 2 mL per gram of original tissue. Plastidic membranes were then collected by centrifugation, suspended in a medium containing 0.25 M K-phosphate buffer (pH 7.8) and 25 mM DTT at a ratio of 30 µL per gram original tissue, and assayed for plastid HMGR activity. The supernatant of the 3500 *g* centrifugation was then centrifuged at 12 000 *g* for 15 min to pellet mitochondria and the remaining chloroplasts to avoid contamination of the microsomal fraction. The supernatant of this centrifugation was then centrifuged at 105 000 *g* for 1 h to pellet microsomal membranes, which were suspended in 0.2 M K-phosphate buffer (pH 6.9) and 25 mM DTT at a ratio of 0.15 mL suspension medium per gram original tissue.

Protein assay

Protein content of the membrane preparations was measured by using the Pierce Coomassie protein assay reagent, which employs the Bradford [14] method for protein determination, with BSA as a standard.

HMGR assay

HMGR activity was determined by means of the radioassay method described by Russell [13] with modifications. The enzyme assay mixture was prepared on ice in 1.5-mL microcentrifuge tubes. The mixture consisted of 1 µL 0.2 M DTT, 1 µL 160 µM nicotinamide adenine dinucleotide phosphate (NADPH), 5 µL enzyme extract (microsomal, pH 6.9, or plastidic, pH 7.8), 7 µL 0.3 % BSA solution, and 6 µL *R,S*-[3-^{14}C]HMG-CoA (NEN Research Products) with specific activity of 2176 Bq/nmol. The reaction mixture was incubated for 30 min in a water bath at 30 °C. The reaction was stopped by adding 2 µL of 6 M HCl and 2 µL of 1 M mevalonate solution. The mevalonate solution was prepared by mixing 2 M mevalonate lactone and 0.1 M potassium hydroxide at a ratio of 1 : 1 (v/v). After it was mixed, the reaction mixture remained at room temperature for 1 h for mevalonate lactonization to be completed. The reactants were centrifuged at 10 000 *g* for 3 min to pellet membrane fragments. The supernatant was then analyzed by thin-layer chromatography (TLC) analysis by applying 10 µL of supernatant to Whatman LK50F silica gel plates (20 × 20 cm; Whatman). The plates were developed in 50 mL of diethyl ether–acetone (3 : 1 v/v) for 15 min and analyzed by X-ray autoradiography. Radioactive spots corresponding to the substrate HMG-CoA (R_f = 0.0) and the product mevalonate (MVA, R_f = 0.9) were scraped and quantified by liquid scintillation spectrometry. The identity of the product was confirmed by cochromatography with radiolabeled mevalonolactone (RS-[2-^{14}C], specific activity 1.85 TBq · mol^{-1}; NEN Research Products). Specific activity of HMGR was calculated after correcting for counting efficiency and recovery of MVA formed per milligram of protein per hour. Each treatment was duplicated and each experiment was performed twice.

Estimation of artemisinin using HPLC

Artemisinin was estimated through HPLC using a method described elsewhere [15]. Because artemisinin lacks any chromatophore for UV detection in HPLC, it was chemically modified (derivatized) to the molecule Q_{260}. Standard artemisinin (0.1 mg) (Sigma-Aldrich) was dissolved in 1 mL MeOH and then treated with 4 mL NaOH (0.2 %) and incubated at 50 °C for 30 min in a water bath shaker. The alkaline reaction mixture (Q_{292}) was cooled to room temperature and neutralized with 5 mL glacial acetic acid (0.1 M in 20 % MeOH) to make a derivative of artemisinin referred to as Q_{260} (λ − 260 nm) (final conc. 10 µg/mL). Artemisinin in sample extracts was also derivatized in the same way. Derivatized artemisinin (Q_{260}) was resolved through a reserved-phase column (125 × 4 mm C_{18} column) with MeOH:10 mM phosphate buffer (pH 7.9, ratio 40 : 60) as the mobile phase at a constant flow rate of 1 mL with the detector set at a wavelength of 260 nm using HPLC equipment (Water Delta 600).

Supporting information

Regeneration of transformants on kanamycin-containing selection medium is provided as Supporting Information.

Results

In order to verify the putative *HMGR* gene that was isolated from the genomic DNA of *Catharanthus roseus* (L.) G. Don, the DNA was sequenced and analyzed for similarity with a cDNA sequence reported earlier [10]. The genomic DNA sequence possesses only a few polymorphic sites in the coding region that affect the amino acid sequence, but the sequence is largely identical to the CDS obtained earlier. The isolated genomic region is 3095 bases long and contains 58.90 % AT and 41.10 % GC bases. The sequence has been submitted to the National Center for Biotechnology Information (NCBI) (Accession No. AY623812).

To transform leaf explants of *A. annua*, the *Agrobacterium*-mediated gene transfer method was employed. All explants retained a healthy green color during the initial 48 h on cocultivation medium. After cocultivation the leaf explants cultured on callus induction selection medium (CISM) developed calluses in 6–8 weeks. Calluses subcultured on shoot regeneration selection medium (SRSM) developed small shoots in 4–5 weeks. These small shoots developed into elongated shoots (approx. 5 cm long) in 3–4 weeks on SRSM devoid of kanamycin. Roots appeared within 15–20 days after transferring them to rooting medium; the rooting percentage was 80 % (**Fig. 1S** as Supporting Information).

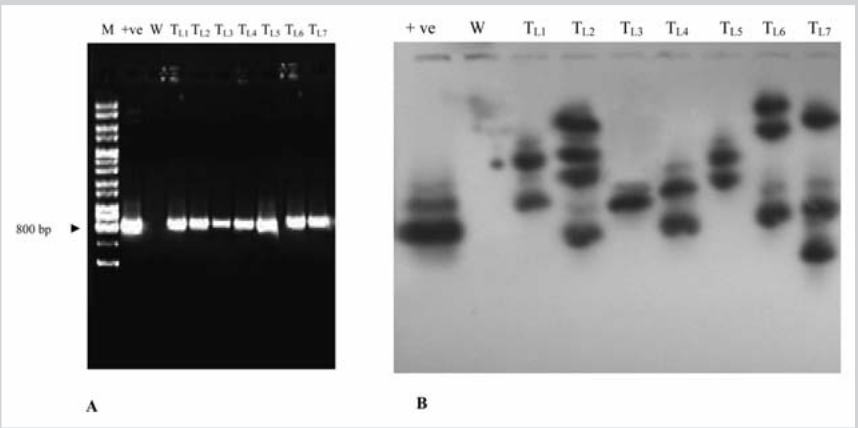

Fig. 2 Molecular analysis of transgenic *Artemisia annua* plants. (**A**) PCR analysis in 7 kanamycin-resistant plants for the presence of the *npt*II gene. Lane 1: DNA molecular weight marker (1 kb); Lane 2: Positive control plasmid DNA; Lane 3: Negative control DNA of a wild-type plant; Lanes 4–10: Putative transgenic plant DNA. (**B**) Southern analysis after genomic DNA was digested with *Sal*I and hybridized with an HMGR-specific gene probe. Lane 1: Plasmid DNA; Lane 2: Negative control (DNA from wild-type plant); Lanes 3–9: DNA sample from transgenic lines.

Out of a total of 468 explants, 73 (15.6%) produced calluses on CISM, of which only seven (1.49%) produced shoots on SRSM. On PCR analysis of the genomic DNA of all (seven) putative transformed shoots, the 800-bp *npt*II fragment was successfully amplified, while no amplification was detected in the untransformed control *A. annua* plants (**Fig. 2A**). The effective frequency of transformed shoots (T_0) was therefore 1.49%. In Southern hybridization, a DIG-labeled HMGR probe hybridized to *Sal*I-digested genomic DNA of putative transformed *A. annua* plants and uncut plasmid DNA of pBin AR were used as positive control. No hybridization could be detected in a DNA sample from an untransformed negative control plant. The presence of three to four bands in the *Sal*I-digested genomic DNA in individual lanes indicated the presence of multiple copies of the *HMGR* gene for six of the transgenic lines and at least a single copy in one of the transgenic lines. The bands in the seven transformed plants were of various sizes, indicating that transgene integration occurred at different loci in the genome (**Fig. 2B**).

Each of the transgenic lines was subsequently grown and analyzed for HMGR activity and its potential effect on levels of artemisinin (**Table 1**). These assays indicate that lines T_{L3}, T_{L4}, and T_{L5} possess higher HMGR activity compared with wild-type controls. Our organelle fractionation and enzyme activity assays indicate that this activity is exclusively associated with microsomal membranes. Crude extracts from plastidic membranes and mitochondrial membranes (suspended pellets following centrifugation at 12000 *g*) were incapable of converting radiolabeled HMG-CoA to mevalonate (data not shown).

In order to test whether the higher activity of HMGR would result in an increase in artemisinin levels, the artemisinin levels were calculated using HPLC. The artemisinin peak was detected at 6.42 min (retention time) using 10 mM phosphate buffer in methanol (6 : 4) as solvent system and a 125 × 4 mm C_{18} column. The maximum increase in artemisinin content of 22.5% compared with wild-type (W_T) *A. annua* plants was recorded in transgenic line T_{L3}, followed by 17.7% and 17.1% in T_{L5} and T_{L4} respectively (**Table 2**). The rest of the transgenic lines (T_{L1}, T_{L2}, T_{L6}, and T_{L7}) failed to show any significant increase in artemisinin content.

Discussion

In recent years, there has been remarkable progress in the understanding of the molecular regulation of artemisinin biosynthesis. The genes coding for key enzymes involved in the biosynthesis of artemisinin, such as farnesyl diphosphate synthase (FPS) and amorpha-4,11-diene synthase (AMS), have been cloned from *A. annua* [16–18]. In the present study we attempted to exploit this knowledge to increase the production effectiveness of arte-

Table 1 Expression of microsomal HMGR activity in micro-propagated wild-type and transgenic *Artemisia annua* plants.

HMGR activity [nmol MVA (mg protein)$^{-1}$ · h^{-1}]							
Micro-propagated	**Transgenic**						
	T_{L1}	T_{L2}	T_{L3}	T_{L4}	T_{L5}	T_{L6}	T_{L7}
12.0 ± 1.6	13.1 ± 1.6	13.0 ± 1.2	35.2 ± 1.0	31.1 ± 0.9	33.0 ± 1.2	12.2 ± 1.4	11.2 ± 1.5

Note: Each value represents mean of 3 replicates ± SD

Table 2 Artemisinin content in micro-propagated wild-type and transgenic *Artemisia annua* plants.

Artemisinin content (µg/g dw)							
Micro-propagated	**Transgenic**						
	T_{L1}	T_{L2}	T_{L3}	T_{L4}	T_{L5}	T_{L6}	T_{L7}
315.1 ± 35.6	320.1 ± 30.2	318.0 ± 34.8	386.0 ± 33.2	369.0 ± 38.3	371.1 ± 31.5	318.0 ± 35.1	302.1 ± 37.5

Note: Each value represents an average of 5 replicates ± SD

misinin from *A. annua.* We accomplished this by the overexpression of an *HMGR* gene under the control of the strong CaMV 35S promoter. The high expression of HMGR in these transgenic plants was associated with higher artemisinin content, which suggests that HMGR overexpression is useful for increasing artemisinin content and can be exploited for obtaining higher artemisinin levels in *A. annua*.

HMGR catalyzes the conversion of HMG-CoA to mevalonate and regulates the flux of carbon from primary to secondary metabolism, leading to the synthesis of these compounds [7, 19–21]. Consistent with this, several studies show a strong correlation between HMGR and the biosynthesis of isoprenoid compounds. In the current study, tissues of transgenic *A. annua* plants contained higher levels of HMGR activity (**Table 2**). Presumably, the overexpression of the *HMGR* gene in a cross-specific environment results in the desired enzymatic reaction occurring at a higher rate. This indicates that the pathway is highly conserved and has scope for further manipulation based on knowledge accumulated from studying different species.

HMGR activity is exclusively associated with microsomal membranes rather than membranes of the plastidic fraction [22, 23].

Because artemisinin is synthesized through the mevalonate pathway, the constitutive expression of HMGR results in the accumulation of artemisinin, probably by channelizing the carbon flux from primary to secondary metabolism. Consistent with this, Chappell and coworkers [7] showed that HMGR was a rate-limiting step for phytosterol biosynthesis in transgenic tobacco plants engineered with a highly expressed *HMGR* gene from hamster.

In the present investigation the artemisinin content of *A. annua* shoot cultures on MS medium was found to be 0.3 mg/g dw. The artemisinin content of the shoots of transgenic line T_{L3} was 22.5% higher than that of the *in vitro* grown non-transgenic plants. The reported artemisinin content of wild-type plants ranges from 0.3 mg/g dw to 0.5 mg/g dw [24], the range in which our results fell. However, in a study that generated transgenic *A. annua* plants containing a reporter gene, the artemisinin content of leaves of wild-type *in vitro* grown plants (1.1 mg/g dw) was much above this range, and the artemisinin content of regenerated transgenic plants was significantly higher (1.7 mg/g dw) than the controls [25]. Because no specific component of the pathway was targeted in the said study, the result could not be explained through current knowledge of the artemisinin biosynthesis pathway.

The seven different transgenic lines that we generated showed variation in the artemisinin levels among themselves. This may be due to position effect or random integration of the transgene at nonspecific sites in the plant genome [26]. In addition to this, a recent study pointed out that plant genomes are made up of homogeneous segments termed isochores, which differ in GC content [27]. Disruption of an isochore by insertion of a transgene with a very different GC content may mark this region for inactivation and methylation [28]. In the present study, four transgenic lines (T_{L1}, T_{L2}, T_{L6}, and T_{L7}) failed to show any significant increase in artemisinin content, which may be due to insertion of these transgenes in isochores of different GC content than the transgene. We obtained the best results in transgenic lines that contained fewer insertions in the genome (**Fig. 2**).

The relatively low rate of effective transformation (1.49%) compared with an earlier report where transgenic frequency of fascicled shoots was reported as 4–10% [29] could be due to differences in the type of *A. tumefaciens* strains and vectors used for transformation [25, 30]. However, this study points to further methods for improving the levels of artemisinin by exploiting a better understanding of the artemisinin biosynthesis pathway. Future experiments in this direction would have to take these observations into consideration.

Overall, the current study shows that plant transgenics could be exploited for the purpose of generating a cheaper drug to cure malaria, which affects millions of people every year around the globe.

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