COMPARISON OF THE EXPRESSION LEVEL OF TWO SESQUITERPENE CYCLASES GENES IN THE TRANSFORMED HAIRY ROOTS OF *Artemisia annua* L. PLANT

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As early as 500 BC, ancient Chinese scripts reported the use of the annual herb, *Artemisia annua*, for treatment of "fever" (Klayman, 1985). Much later, Qinghaosu, artemisinin, a sesquiterpene lactone, was isolated in pure form from the aerial parts of *Artemisia annua* L. (annual wormwood) plants, and its structure was determined in 1979 (Klayman, 1985). Artemisinin is currently the best therapeutic against both drug-resistant and cerebral malaria-causing strains of *Plasmodium falciparum*. It has since become popular throughout Southeast Asia and Africa, where malaria has become resistant to nearly all anti-malarial drugs, including chloroquine, quinine, mefloquine, and Fansidar (Newton *et al.*, 1999).

Artemisinin is an important therapeutic that, along with its derivatives, is a proven treatment for a number of diseases besides malaria (Dhingra *et al.*, 2000), other parasites like schistosomiasis (Utzinger *et al.*, 2001; Borrmann *et al.*, 2001), and more recently cancer and Hepatitis B (Romero *et al.*, 2005). Artemisinin has also been shown to be effective against a variety of cancer cell lines including breast cancer, human leukemia, colon, and small-cell lung carcinomas (Efferth *et al.*, 2001; Singh and Lai 2001). Furthermore, artemisinin may be especially effective in treating drug resistant cancers (Sadava *et al.*, 2002; Efferth *et al.*, 2002). However, the drug is in short supply as its complex structure still requires that it be extracted from plants. Although others are working on a synthetic trioxolane (Vennerstrom *et al.*, 2004) and bacterial produced artemisinin precursors (Martin *et al.*, 2003) that may replace artemisinin as an inexpensive therapeutic, *A. annua* plants are still the only current source of the drug.

In the whole plant artemisinin has been reported to accumulate in leaves, small green stems, buds, flowers and seeds (Liersch *et al.*, 1986; Ferreira *et al.*, 1995) with highest levels in leaves and

flowers. Neither artemisinin nor its precursors, however, were detected in roots (Charles *et al.*, 1990). Artemisinin content in full bloom flowers was 4-5 times higher than in leaves (Ferreira *et al.*, 1995). Both leaves and flowers of *A. annua* have trichomes. Duke *et al.* (1994) showed that artemisinin is sequestered in the glandular trichomes of *A. annua*, and that glandless types produce no artemisinin (Ferreira *et al.*, 1995). Some researchers reported that artemisinin content is highest just prior to flowering (Liersch *et al.*, 1986; Woerdenbag *et al.*, 1994) while others found an artemisinin peak at full flowering stage (Morales *et al.*, 1993, Pras *et al.*, 1991; Singh *et al.*, 1988).

The concentration of artemisinin in *A. annua* is very low, in the range of 0.01-0.8 % (Van Geldre *et al.*, 2000), although some strains may be as high as 1.5% (J. Simon, personal communication). Although chemical synthesis is possible, it is complicated and economically not viable due to very poor yields (Abdin *et al.*, 2003). Thus, the plant remains the only commercial source of the drug, and the relatively low yield of artemisinin in *A. annua* is a serious limitation to the commercialization of the drug (Laughlin, 1995; Bertea *et al.*, 2005). The enhanced production of artemisinin either in cell/tissue culture or in whole plants of *A. annua* is therefore, highly desirable and should be achievable by a better understanding of the biosynthetic pathway and its regulation by both exogenous and endogenous factors. Furthermore, once the pathway genes and their regulatory controls have been elucidated, metabolic engineering can be employed to overexpress gene(s) coding for enzyme(s) associated with the rate limiting step(s) of artemisinin biosynthesis or to inhibit the enzyme(s) of other pathways competing for its precursors. In the biosynthesis of artemisinin, very little is known about enzymes responsible for the production of the different intermediates. At one of the important branchpoints farnesylpyrophosphate (FPP) is cyclised by the action of a sesquiterpene cyclase to amorpha 4,11-diene (ADS), which leads sequentially to artemisinic acid, dihydroartemisinic acid and artemisinin by a series of oxidation steps and nonenzymatic conversions (Back *et al.*, 1996; Bouwmeester *et al.*, 1999) and (Fig. 1). Thus, the cyclase reaction establishes an important stereochemical framework upon which all other chemical modifications take place (Back *et al.*, 1996). Several publications report on the fact that this cyclisation step is a putative regulatory point, probably rate limiting (Voegeli *et al.*, 1988). The accumulation of artemisinic acid and dihydroartemisinic acid in absence of any intermediates en route from FPP also supports this hypothesis (Bouwmeester *et al.*, 1999). Farnesyl diphosphate synthase, which produces FPP, has already been cloned from *A. annua* (Matsushita *et al.*, 1996), but this enzymatic reaction is probably not rate limiting for the production of artemisinin. In this study we compare the expression level of the two key genes of AN biosynthesis (ADS and EPS)

in the transformed hairy roots as a source of artemisinin production.

MATERIALS AND METHODS

Plant materials

A. annua L. Plants were grown in an experimental greenhouse using Hg- and Na vapor lamps 16 h/day and at a temperature of 22°C and a relative humidity of 40%.

Establishment of hairy root cultures

Agrobacterium rhizogenes ATCC 15834 was grown on YMB medium (in 1g, K_2HPO_4, 0.5, $MgSO_4$,-H_2O, 2.0, NaCl, 0.1, mannitol, 10.0, yeast extract, 0.4, and agar, 15.0), pH 7.0 at 25-28°C. Fresh (2-3 d) cultures, subcultured directly from the original stock, were used. An aliquot of 0.4 ml of 200 pmol acetosyringone (Sigma Chemical) solution was added to 10 ml of bacterial stock suspended in 30 ml of YMB to induce virulence. Prior to infection, the bacterial suspension was incubated on a shaker (100 RPM) at 25-28°C for 48-72 hours. Artemisia annua seeds were surface sterilized by swirling in 10% (v/v) Clorox for 15 min, and then in 70% ethanol (v/v) for 5 min, followed by three rinses with sterile distilled water, each for 5 min. The seeds were germinated on 0.8% (w/v) agar at 25°C under 16 hours light for 5-7 days. Germinated sterile seeds were transferred to hormone-free White's media (White, 1963) supplemented with 3% (w/v) sucrose and 2.0 g I-' gelrite and grown at room temperature in Plant Cons with a 16 hour photoperiod. Hairy root cultures were established by infecting the stems of 4-5 week old sterile seedlings in Plant Cons with A. rhizogenes via injection of 25 µl of bacterial suspension into a shoot tip or leaf stalk with prior wounding. Hairy roots were formed at the site of infection after 10 days. Hairy roots were excised from the plantlets and cultured on solid White's medium with 30% (w/v) sucrose and 250 g 1-l carbenicillin and incubated at 25°C in darkness. Roots growing up off the gelrite surface were excised and incubated on fresh medium with carbenicillin. After 2 weeks, the roots were transferred to 50 ml fresh liquid White's medium as shown in Fig. (2) with carbenicillin and incubated at 25°C on a rotary shaker (100 RPM) for two more weeks with subsequent subculture to Gamborg's B5 medium (Gamborg *et al.*, 1968) with 3% (w/v) sucrose.

Isolation of nucleic acids

Genomic DNA was isolated from plant leaves according to the extraction procedure from Stacey and Isaac (1994). Plasmid DNA samples were prepared by an alkaline lysis method with the Wizard DNA Purification System (Promega) according to the manufacturer's instructions. Total RNA was isolated with the Trizol reagent according the procedures of Life Technologies.

Oligonucleotide primer design

Two sesquiterpene cyclases (SQC), previously identified as key genes in two separate sesquiterpenoid paths in *A. annua,* are EPS, epicedrol synthase and

ADS, amorpha-4, ll-diene synthase that catalyze the first committed step in the biosynthesis of Artemisinin (Van Geldre *et al.*, 2000). Forward and reverse primers were designed to amplify both ADS and EPS fragments from the untranslated region (3 'UTR) that shows no homology with the two genes. Forward primer of ADS (5' ATG AGG AGT ATG CCC AAA CC 3') and its reverse primer (5' TGC GTC TGA TTT ATT ATT GCC 3'), forwrad primer of EPS (5' CTA GCG AGC AAC AAG CCT ACG 3') and its reverse primer (5' CTC GAA GAT GGA AAC ATC ATC G 3') were thus selected with respective annealing temperatures of 55°C and 57°C calculated with [2°*(A+T)+4°*(C+G)].

Polymerase chain reactions

The primers were subsequently used in a Hot Start polymerase chain reaction (PCR) on genomic DNA from *A. annua*. (10 mM Tris–HCl (pH 8.3), 50 mM KCl, 200 mM dNTP, 1.2 U Taq polymerase and 20 pmol of each primer in a total volume of 50 ml; concentrations of MgCl2 ranged from 1.5 to 4.5 mM). Cycling conditions were set as follows: 94°C for 1 min, 55°C for 1 min and 72°C for 2 min for a total of 40 cycles. This PCR resulted in two products of 350 (ADS) and 563 (EPS) as detected by agarose gel electrophoresis and ethidium bromide staining. Those products were sequenced after TA-cloning in pGem-T-Easy Vector System (Promega) according to the manufacturer's instructions. Recombinant plasmids were isolated after amplification in *E. coli* JM109 and analysed for insert by blue/white screening.

RNA analysis with RT-PCR

In order to isolate corresponding cDNA sequences, Dnase-treated RNA isolated from leaves and hairy root cultures harvested in different intervals of culture ages (5, 10 and 14 days culture age) was denatured for 10 min at 70°C and directly chilled on ice then subsequently transcribed to cDNA with AMV Reverse Transcriptase and oligo dT primer during 60 min at 42°C. This reaction could be used immediately for PCR with 20 pmol of both specific primers and 2.5 U Taq in 10 mM Tris–HCl (pH 8.3), 50 mM KCl and 200 mM dNTP. The reaction conditions were 94°C during 1 min, 55°C during 1 min and 72°C during 2 min for a total of 40 cycles. The experiment was repeated using an indigenous constitutively expressed control (β actin) as shown in Fig. (5).

RESULTS AND DISCUSSION

Isolation and cloning of both ADS and EPS genes

Two sesquiterpene cyclases (SQC), previously identified as key genes in two separate sesquiterpenoid pathways in *A. annua,* are EPS, epicedrol synthase and ADS, amorpha-4, ll-diene synthase that catalyze the first committed step in the biosynthesis of Artemisinin (Van Geldre *et al.*, 2000). Forward and reverse primers were designed to amplify both ADS and EPS fragments from the untranslated re-

gion (3 'UTR) that shows no homology with the two genes. The ADS fragment of interest had 350 base pairs (bp) while the EPS fragment had 563 base pairs (bp) as shown in Fig. (3). Both ADS and EPS fragments were successfully amplified from the total genome of *Artemisia annua* by Polymerase Chain Reaction (PCR). The 2 PCR products were sequenced then cloned by TA-cloning in pGem-T-Easy Vector System (Promega) according to the manufacturer's instructions. Recombinant plasmids were isolated after amplification in *E. coli* JM109 and analyzed for insert by restriction digestion using *ECOR*I that flanking the (EPS) insert and *Hind*III and *XHO*I that flanking the ADS insert as shown in Fig. (4 a,b).

Agrobacterium rhizogenes mediated transformation of A. annua explants.

Prior to transformation of the different strains of seeds, we analyzed whole plants for their relative contents of AN. Preliminary data indicated that the YU strain had the highest level of artemisinin and so most of our transformation efforts were directed at this strain. Transformation efficiency was highest when sterile plantlets were wounded prior to injection of the bacterial suspension into the stems. Each individual hairy root emanating from a wound was subcultured onto plates, disinfected from residual Agrobacteria and subcultured through several passages on plates prior to initiation of liquid cultures. Although many roots were isolated, only 12 axenic clones were reliably subcultured into sterile liquid media for RNA analysis (Fig. 5). The 12 clones were grown in liquid B5 medium for 22 days and analyzed for RNA at days 5, 10, and 14 (Fig. 6). Although other in vitro cultures of A. annua, including shoots, callus, and cell suspensions have been reported, only recent work by Woerdenbag *et al.* (1993b) with shoot cultures has shown significant levels of AN production. The highest reported levels of AN are found in whole plants, mainly Chinese (Luo *et al.*, 1980) and Vietnamese strains (van Phiet *et al.*, 1992; Woerdenbag *et al.*, 1993 a&b). These data suggest that whole plants may be the best means of production. However, production of AN via whole plants requires weeks or months to obtain high yields, whereas hairy root cultures show potentially higher production rates in a few days (Weathers *et al.*, 1994). The purification of AN and the other AX compounds from leafy tissues is relatively complex because of the presence of oils and pigments which are lacking in roots. Thus, roots should provide a cleaner extraction source which has been substantiated in preliminary experiments, that's why we used in this study hairy root cultures to detect the expressions of the 2 key genes in Artemisinin biosynthesis.

Detection of sesquiterpene cyclase transcripts

Reverse Transcription polymerase chain reaction (RT-PCR) was done to compare the expression of both ADS and EPS genes at different time intervals of hairy roots cultures. mRNA was isolated from 5, 10 and 14 days old cultures. By

using oligo dT as an initial primer in the RT- PCR, it was detected that EPS is highly expressed and more abundant than ADS in leaves and hairy roots (Fig. 5 a&b). The experiment was repeated using an indigenous constitutively expressed control (β actin) as shown in Fig. (5c).The data also reveals a positive correlation between culture age and the expression of both ADS and EPS genes (Fig. 6). Semi-quantitative RT-PCR was also done to test the quantitative expression of ADS (Fig. 7) using different no. of PCR cycles 20, 25, 30 and 35). We found that there is direct relationship between the expression level of ADS and the no. of cycles as shown in Fig. (7).

The first committed step of artemisinin biosynthesis is the cyclization of farnesyl diphosphate (FDP) by a sesquiterpene synthase (ADS) to produce the characteristic 15 carbon ring system (Bertea *et al.*, 2005). Although the complete biosynthetic pathway for artemisinin has not yet been established, artemisinic acid is now considered to be the biogenic precursor of artemisinin (Wallaart *et al.*, 1999a, b; Sy *et al.*, 2001; Brown and Sy, 2004). Artemisinic acid and amorpha-4,11-diene are structurally closely related, which made the latter the more likely candidate for the cyclization product. Detection and partial purification of amorpha-4,11-diene synthase from the plant was first reported by Bouwmeester *et al.* (1999). The low level of the volatile amorpha-4,11-diene in the plant and the high amorphadiene synthase activity were considered to be strong evidence that amorpha-4,11-diene is an intermediate in the biosynthesis of artemisinin. We aimed in this study to test the expression level of both amorpha-4,11-diene synthase (ADS) and Epicedrol synthase (EPS) in transformed hairy root culture systems while we know that in the whole plant artemisinin has been reported to accumulate in leaves, small green stems, buds, flowers and seeds (Liersch *et al.*, 1986; Ferreira *et al.*, 1995) with highest levels in leaves and flowers. Neither artemisinin nor its precursors, however, were detected in roots (Charles *et al.*, 1990), that's explain why we found an abundant expression of EPS than ADS in the transformed hairy root cultures since EPS is not related to Artemisinin biosynhesis as indicated in Fig. (2). Additionally, we found direct relationship between cultures ages and the expression level of EPS as shown in Fig. (6). This would mean a big step forward in the overproduction of artemisinin through genetic manipulation.

SUMMARY

Artemisia annua L. is the only source of artemisinin, a new promising antimalarial drug. The relatively low yield of artemisinin in *A. annua* is a serious limitation to the commercialization of the drug. The enhanced production of artemisinin either in cell/tissue culture or in whole plants of *A. annua* is therefore, highly desirable and should be achievable by a better understanding of the biosynthetic pathway and its regulation by both exogenous and endogenous factors. Our efforts are focused on the overproduction

of this valuable medicine by genetic engineered *A. annua* plants. Two sesquiterpene cyclases (SQC), previously identified as key genes in two separate sesquiterpenoid paths in *Artemisia annua,* are EPS, epicedrol synthase and ADS, amorpha-4, 11-diene synthase that catalyze the first committed step in the biosynthesis of artemisinin (AN). Forward and reverse primers were designed to amplify both ADS and EPS fragments from the untranslated region (3 'UTR) that shows no homology with the two genes. The ADS fragment of interest had 350 base pairs (bp) while the EPS fragment had 463 bp. Both ADS and EPS fragments were successfully amplified from the total genome of *A. annua* by Polymerase Chain Reaction (PCR). Reverse Transcription Polymerase Chain Reaction (RT-PCR) was done on transformed hairy roots of *A. annua* L. to compare the expression of both ADS and EPS genes under conditions were the production of AN were thought to be either high or low. By using oligo dT as an initial primer in the RT-PCR, it was detected that EPS is highly expressed in different transformed hairy root cultures than ADS, this was expected from previous literatures and from the AN level produces by transformed hairy roots.

REFERENCES

Abdin, M. Z., M. Israr, R. U. Rehman and S. K. Jain (2003). Artemisinin, a novel antimalarial drug: biochemical and molecular approach for enhanced production. Planta Med., 69: 289-299.

Back, K. and J. Chappell (1996). Identifying functional domains within terpene cyclases using a domain-swapping strategy. Proc. Natl. Acad. Sci., 93: 6841-6845.

Bertea, C. M., J. R. Freije, H. Van der Woude, F. W. Verstappen, L. Perk, V. Marquez, J. W. De Kraker., M. A. Posthumus, B. J. M. Jansen, A. E. De Groot, M.C.R Franssen and H. J. Bouwmeester (2005). Identification of intermediates and enzymes involved in the early steps of artemisinin biosynthesis in Artemisia annua. Planta Med., 71: 40-47.

Borrmann, S., N. Szlezak., J. F. Faucher, P. B. Matsiegui, R. Neubauer, R. K. Biner, B. Lell and P. G. Kremsner (2001). Artesunate and praziquantel for the treatment of *Schistosoma haematobium* infections: a doubleblind, randomized, placebo-controlled study. J. Infect. Dis., 184: 1363-1366.

Bouwmeester, H. J. T. E. Wallaart, M. H. Janssen, B. van Loo, B. J. Jansen, M. A. Posthumus, C. O. Schmidt, J. W. De Kraker, W. A. Knig and M. C. Franssen (1999). Amorpha-4, 11-diene synthase catalyzes the first probable step in artemisinin biosynthesis. Phytochemistry, 52: 843-854

Charles, D. J., J. E. Simon, K. V. Wood and P. Heinstein (1990). Germplasm variation in artemisinin content of *Artemisia annua* using an alternative method of artemisinin analysis from crude plant extracts. J. Nat. Prod., 53: 157-160.

Dhingra, V., V. M Rao and L. Narasu (2000). Current status of artemisinin and its derivatives as antimalarial drugs. Life Sci., 66: 279-300.

Duke, M. V., R. N. Paul, H. N. Elsohly, G. Sturtz and S. O. Duke (1994). Localization of artemisinin and artemisitene in foliar tissues of glanded and glandless biotypes of *Artemisia annua* L. Int. J. Plant Sci., 155: 365-372.

Efferth, T., H. Dunstan, A. Sauerbrey, H. Miyachi and C. R. Chitambar (2001). The anti-malarial artesunate is also active against cancer. Int. J. Oncol., 18: 767-773.

Efferth, T., A. Olbrich and R. Bauer (2002). mRNA expression profiles for the response of human tumor cell lines to the anti-malarial drugs artesunate, arteether, and artemether. Biochem. Pharmacol., 64: 617-623.

Ferreira, J. F. S., J. E. Simon and J. Janick (1995). Relationship of artemisinin content of tissue cultured, greenhouse and field grown plants of *Artemisia anuua*. Planta Med., 61: 351-355.

Klayman, D. L. (1985). Qinghaosu (artemisinin): an anti-malarial drug from China. Science, 228: 1049-1055.

Laughlin, J. C. (1995). The influence of distribution of anti-malarial constituents in Artemisia annua L. on time and method of harvest. Acta Hort., 390: 67-73.

Liersch, R., H. Soicke., C. Stehr and H. U. Tullner (1986). Formation of artemisinin in Artemisia annua during one vegetation period. Planta Med., 52: 387-390.

Liu, Y., H. C. Ye and G. F. Li (2002). Cloning, E. coli expression and molecular analysis of a novel sesquiterpene synthase gene from *Artemisia annua*. Acta Bot. Sin., 44: 1450-1455.

Martin, V. J., D. J. Pitera, S. T. Wither, J. D. Newman and J. D. Keasling (2003). Engineering a mevalonate pathway in Escherichia coli for production of terpenoids. Nat. Biotechnol., 21: 796-802.

Matsushita, Y., W. K. Kang and B. V. Charlwood (1996). Cloning and analysis of a cDNA encoding farnesyl diphosphate synthase from Artemisia annua. Gene, 172: 207-209.

Morales, D. J. and J. E. Charles (1993). Seasonal accumulation of artemisinin in *Artemisia annua* L., Acta. Hort., 344: 416-420.

Newton, P., and N. White (1999). New development in treatment and prevention. Annu. Rev. Med., 50: 179-192.

Pras, N., J. Visser, S., Batterman, H. Woerdenbag, T. Malingre and C. Lugt (1991). Phytochem. Anal., 2: 80-83.

Romero, M. R., T. Efferth, M. A. Serrano, B. Castano, R. I. Macias, O. Briz and J. J. Marin (2005). Effect of artemisinin/ artesunate as inhibitors of hepatitis B virus production in an '*in vitro*' system. Antiviral Res., 68: 75-83.

Sadava, D., T. Phillips, C. Lin and S. E. Kane (2002). Transferrin overcomes drug resistance to artemisinin in human small-cell lung carcinoma cells. Cancer Lett., 179: 151-156.

Singh, A., R. A. Vishwakarma and A. Husain (1988). Evaluation of *Artemisia annua* satrins for higher artemisinin production. Planta Med., 54: 475-476.

Singh, N. and P. Lai (2001). Selective toxicity of dihydroartemisinin and holotransferrin toward human breast cancer cells. Life Sci., 70: 49-56.

Utzinger, J., S. Xiao, E. K. N'Goran, R. Berquist and M. Tanner (2001). The potential of artemether for the control of schistosomiasis. Int. J. Parasitol., 31: 1549-1562.

Van Geldre, E., I. De Pauw, D. Inze, M. Van Montagu and E. Van den Eeckhout (2000). Cloning and molecular analysis of two new sesquiterpene cyclases from *Artemisia annua* L. Plant Sci., 158: 163-171.

Van Phiet, H., H. Ngoc, I. Ognianou, G. Dien and N. Van Hung (1992). 7th Asian Symp. on Med. Plants, Spices, and other Nat. Prod. (ASOMPS VII) Manila, Phillipines. W05.

Vennerstrom J. L., S. Arbe-Barnes, R. Brun, S. A. Charman, F. C. K. Chiu, J. Chollet, Y. Dong, A. Dorn, D. Hunziker, H. Matile, K. McIntosh, M. Padmanilayam, J. S. Tomas, C. Scheurer, B. Scorneaux, Y. Tang, H. Urwyler, S. Wittlin and W. N. Charman (2004). Identification of an antimalarial synthetic trioxolane drug development candidate. Nature, 430: 900-904.

Vogeli, U. and J. Chappell (1988). Induction of sesquiterpene cyclase and suppression of squalene synthetase activities in plant cell cultures treated with fungal elicitor. Plant Physiol., 88: 1291-1296.

Woerdenbag, H. J., N. Pras, N. G. Chan, B. T. Bang and R. Bos (1994). *Artemisinin* related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetative period in Vietnam. Planta Med., 60: 272-275.

Weathers, P. J., G. Bunk and M. C. McCoy (2005). The effect of phytohormones on growth and artemisinin production in *Artemisia annua* hairy roots. *In Vitro* Cell. Dev. Biol. Plant, 41: 47-53.

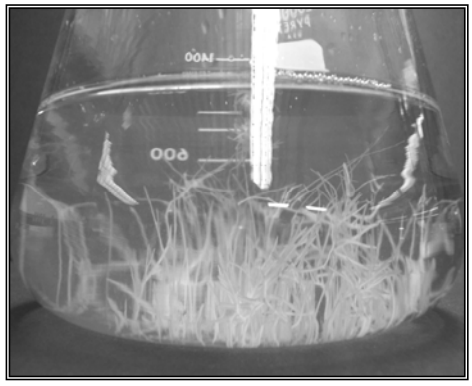

Fig. (1): Transformed hairy root cultures of *A. annua* L by *A. rhizogenes*: Hairy root cultures were established by infecting the stems of 4-5 week old sterile seedlings in Plant Cons with *A. rhizogenes* via injection of 25 µl of bacterial suspension into a shoot tip or leaf stalk with prior wounding. Hairy roots were formed at the site of infection after 10 days.

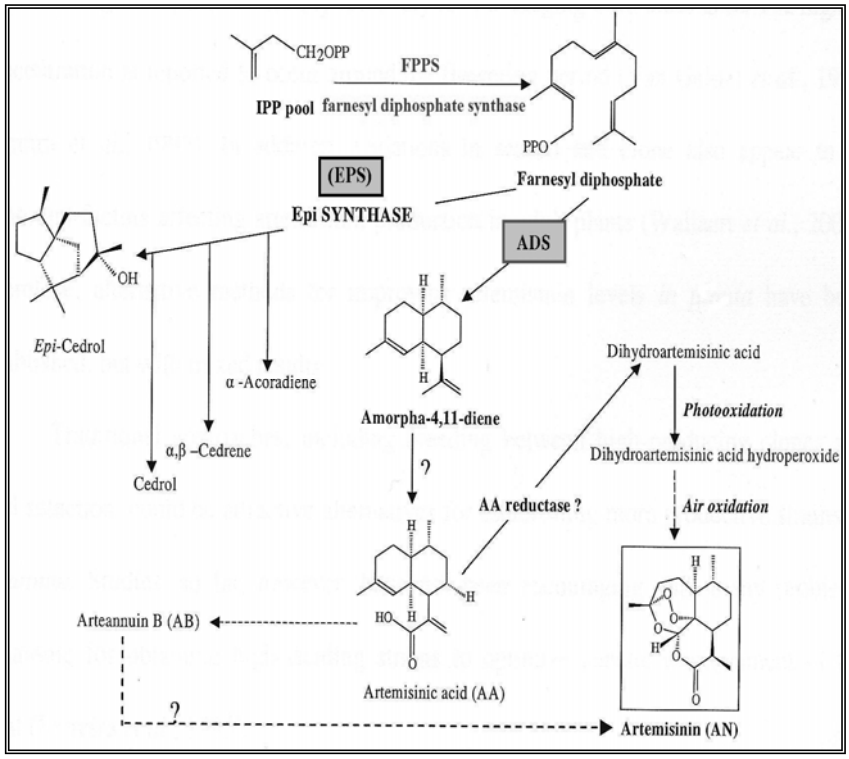

Fig. (2): Proposed biosynthetic pathway for biosynthesis of artemisinin from farnesyl diphosphate adapted from Bertea *et al.* (2005): The first committed step of artemisinin biosynthesis is the cyclization of farnesyl diphosphate (FDP) by a sesquiterpene synthase (Amorphadiene synthase ADS) to produce the characteristic 15 carbon ring system , followed by amorpha-4,11-diene hydroxylation to artemisinic alcohol, oxidation to artemisinic aldehyde, reduction of the C11-C13 double bond to dihydroartemisinic aldehyde and oxidation to dihydroartemisinic acid.

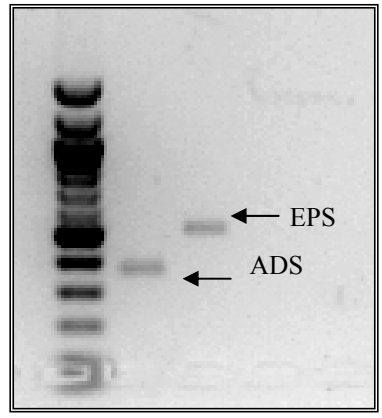

Fig. (3): PCR to amplify both ADS and EPS fragments from the total DNA genome of *A. anua* L: Forward and reverse primers were designed to amplify ADS and EPS fragments from the 3' UTR region. ADS fragment is 350 bp while EPS fragment is 563 bp.

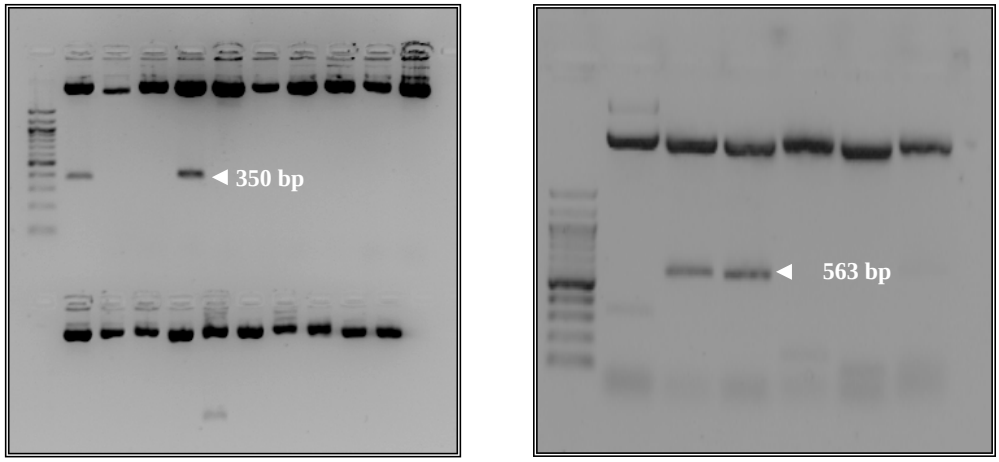

Fig. (4): Screening different ADS and EPS clones by ECORI restriction digestion in case of ADS (350 bp) and *Hind* III & *Xho*I Restriction digestion in case of EPS (563 bp) that flanking the insert (PCR product).

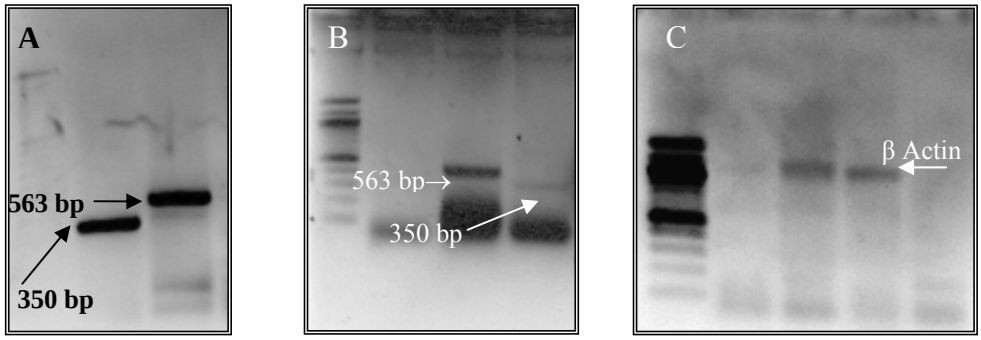

Fig. (5): Reverse Transcription polymerase chain reaction (RT-PCR) was used to detect the expression of both ADS and EPS genes in *A: Leaves* of *A. annua*, B: 14 days transformed hairy root cultures and C: Endogenous control with β actin.

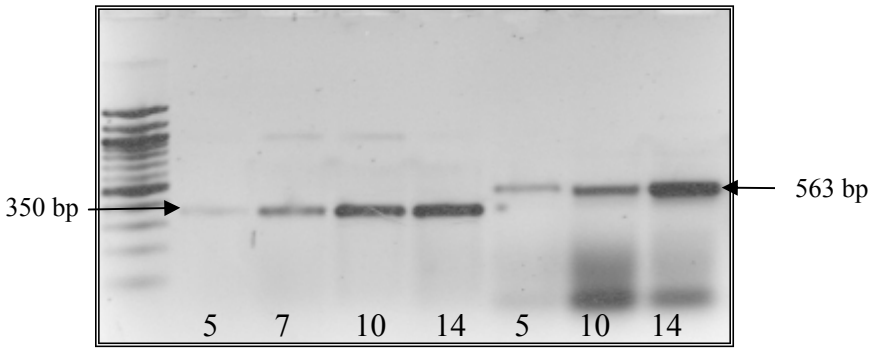

Fig. (6): RT-PCR was done to test the expression of both ADS and EPS genes in different culture ages. Single stranded cDNA was generated from RNA extracted from 5, 7 10 and 14 day-old transformed roots, the corresponding reverse primer and the reverse transcriptase. Double stranded cDNAs were obtained by PCR. The resulting RT-PCR products were analyzed on an agarose electrophoresis gel (1.5%).

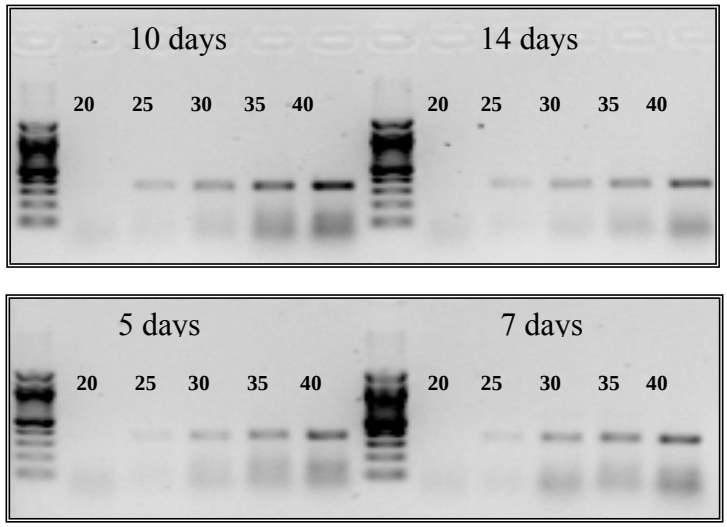

Fig. (7): Semi-Quantitative RT-PCR was done to test the quantitative expression of ADS using different no. of cycles (20, 25, 30, 35 and 40 cycles). Single stranded cDNA was generated from RNA extracted from 5, 7, 10 and 14 day-old transformed roots, the corresponding reverse primer and the reverse transcriptase. Double stranded cDNAs were obtained by PCR. The resulting RT-PCR products were analyzed on an agarose electrophoresis gel (1.5%).