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Mahmuda Umme Rayhan
Seoul National University Agriculture and Life Sciences Library: Seoul National University College of Agriculture and Life Sciences

Tofazzal Islam (tofazzalislam@bsmrau.edu.bd)
Bangabandhu Sheikh Mujibur Rahman Agricultural University https://orcid.org/0000-0002-7613-0261

Soo-un Kim
Seoul National University Agriculture and Life Sciences Library: Seoul National University College of Agriculture and Life Sciences

Research Article

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Posted Date: April 20th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1560430/v1

AaCYPdwf, a new growth regulatory cytochrome p450 gene from *Artemissia annua*

Mahmuda Umme Rayhan[1, 2], Tofazzal Islam[2*], Soo-un Kim[1]

[1]Department of Applied Life Chemistry, College of Agriculture and Life Sciences, Seoul National University, Seoul 151-912, Republic of Korea.

[2]Institute of Biotechnology and Genetic Engineering (IBGE), Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur 1706, Bangladesh

[*]Corresponding author's E-mail: tofazzalislam@bsmrau.edu.bd

Abstract

Artemisia annua L. produces artemisinin (qinghaosu), which is inhibitory against malarial parasite, *Plasmodium falciparum*. Genetic engineering is thought to be one of the most reasonable approaches to enhance the production of artemisinin by *A. annua* plant. This study aimed to clone and investigate a growth regulatory cytochrome p450 gene from *A. annua*. We cloned a cDNA coding growth regulatory gene from *A. annua*. The gene had high homology with the steroid 23-alpha-hydroxylase produced-gene of *A. thaliana, Oryza sativa* and *Zinnia elegans*. The full-length cDNA was 1708 bp long, containing an open reading frame of 1443 bp that encodes 480 amino acids. We transformed *A. annua* plants with the full-length cDNA driven by 35S promoter through *Agrobacterium*-mediated transformation. To identify the function of the gene, we applied RNAi gene silencing technique. The plants transformed with the full-length cDNA were normal in growth but the plants transformed with RNAi showed extreme dwarfism. The dwarfism in the gene silenced plants was rescued by the application of brassinolide and gibberellin hormones indicating that the mutants were deficient in the synthesis of a growth regulatory gene which we named

AaCYPdwf. Phenotypic characteristics of mutant and phylogenetic analysis clustered the *AaCYPdwf* with other brassinolide producing genes. Our results suggest that *AaCYPdwf* is a new gene which likely involved in regulation of brassinosteroid biosynthesis in *A. annua* plants.

Keywords: Artemisia annua, cytochrome P450, overexpression, RNAi.

1. Introduction

Malaria is one of the most destructive diseases, which affects millions of people across Asia and Africa every year. According to a report of the World Health Organization (2019) [1], there were 228 million cases of malaria infection in 2018. Majority of the malarial cases and deaths are associated with the *Plasmodium falciparum* [2]. Malaria caused by *P. falciparum* can have devastating effects like anemia, cerebral complications (from coma to convulsions), hypoglycemia and glomerulo-nephritis [3–5]. It is particularly more serious in non-immune people including children, tourists and pregnant women. For such a serious disease, there are few remedies in the form of herbs that contain important chemical compounds [3,5–8]. The current available effective therapies for malaria include the combination of artemisinin with other drugs. Artemisinin is a sesquiterpene lactone present in the aerial parts of an Asian plant, *Artemisia annua* L [9]. It was initially isolated by the Chinese scientist, Tu Youyou in 1972 from Artemisia herb and used as an antimalarial drug against *Plasmodium* [10]. For that great discovery, Tu Youyou received the Nobel Prize in Physiology or Medicine in 2015 [11,12].

The natural product, artemisinin has many biological activities including antimalaria, anticancer and anti-inflammatory [13–16]. The artemisinin and its derivatives also possess anthelmintic,

fungicidal and antiviral properties [17]. Although it has been found to be a useful medicine, the content of artemisinin in the aerial parts of *A. annua* is only 0.01–1.2%, while the annual world demand of this life saving compound is around 119 metric tons (MT) [18]. It is not sufficient to fulfill the rising demand of artemisinin while total synthesis of artemisinin is too costly [19]. It is not possible to chemically synthesize a large amount of artemisinin on a commercial basis, due to high cost and strong toxicity[10]. Conventional breeding of high artemisinin yielding plants have also not been successful [19]. The content of artemisinin in the *A. annua* plant was tried to increase through metabolic engineering [20]. In this regard, two different approaches were used, one is overexpression of the enzyme genes involved in the biosynthetic pathway of artemisinin[21–23] , and the other one is to block enzyme genes that are in competition with the artemisinin pathway from expression [20]. A number of studies have been done to elaborate the biosynthetic pathway of artemisinin and its expression in different laboratory subjects like *Escherichia coli* and Baker's yeast [24,25]. In one strategy, yeast is exploited for the production of artemisinin acid by expression of the biosynthetic pathway of artemisinin [25–27]. This semisynthetic method is also expensive because of the cost of growth media and maintenance of yeast culture in bioreactors [27]. On the other hand, Paddon et al. [26] developed a genetically modified strain of *Saccharomyces cerevisiae* by expressing the artemisinin pathway genes in this yeast. The artemisinin production rate achieved was around 25 g/L of the fermentation media. However, there are a number of disadvantages such as these include the costly media, and complication of isolation and purification of artemisinin from the fermenting media[26].

Overexpression of a key enzyme of the growth regulatory hormone can enhance the growth as well as production of dry matter or biomass production of the plant [28]. By genetic engineering, the

high-yielding transgenic *A. annua* plant could be a tool for more artemisinin production. It is thought to be one of the most reasonable approaches to enhance the production of artemisinin. Gibberellin, brassinosteroid and auxin are the main growth promoting plant hormones [29]. Among them brassinolide significantly promotes biomass production, growth [28] and helps in regulating many biological process[30–32]. Discovery of genes that involved in regulation of the biosynthesis of brassinosteroid hormones is important for genetic engineering of the *A. annua* for higher growth and dry matter production to obtain the higher artemisinin in this medicinal plant. In biosynthesis pathway of brassinolide (BR), oxidation and hydroxylation are the crucial steps [33]. A number of cytochrome p450 genes like *CYP85A*, *CYP90A*, *CYP90B*, *CYP90C*, *CYP90D* and *CYP724* are known to involve in oxidation and hydroxylation of BR biosynthetic pathway [34]. This study aimed to clone and investigate the characteristics of a putative cytochrome p450 gene from *A. annua,* which involves in the biosynthesis of brassinolide.

2. Materials and methods

2.1. Plant materials

Seeds of *A. annua* were collected from *A. annua* stock maintained in Gangwha-do Island (no specific permissions were required), Korea. Seeds were surface sterilized by soaking in 5% NaOCl for 20 min after immersing in 70% (v/v) ethanol for 2 minutes. They were then rinsed in sterile distilled water several times until the pH was 7 or lower. They were finally germinated under sterile conditions in 90×20 mm Petri dishes containing MS-germination medium. One quarter Murashige and Skoog salts (Duchefa, Haarlem, Netherlands) were supplemented with 2% (w/v) sucrose and solidified with 0.8% (w/v) agar. Germinated plants were grown in an experimental

greenhouse maintained at 16 h light (using Hg-and Na-vapour lamps) and 8 h dark condition at 22℃ and a relative humidity of 40%.

2.2. Strains and culture media

Bacterial strains used in this experiment were *Escheriahia coli* DH10B and EHA105 for plasmid propagation and transformation. *E. coli* was cultured on Lurita-Bertani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl). LB medium was prepared with 1.5% (w/v) Bacto-agar. Different selection mediums were used for plant transformation (Supplementary Table 1).

2.3. Plasmids and primers

pGEM® –T Easy System vector (Promega, Madison, WI, USA) was used for the subcloning. pBI121 and pFG5941 plant expression vectors (Clontech, Seoul, Korea) were used for plant transformation. Oligonucleotides used for DNA sequencing were synthesized by Bioneer. Co. (Daejeon, Korea) for the subsequent polymerase chain reaction (PCR) and DNA sequencing. The sequences of primers are listed in Supplementary Table 2.

2.4. Enzymes and chemicals

Reverse transcriptase, dNTPs, and Taq DNA polymerase were purchased from Invitrogen (Carlsbad, CA). Restriction enzymes, plasmid DNA purification kit and PCR purification kit were from Promega (Madison, WI, USA) and TaKaRa (Kyoto, Japan). Other chemicals were purchased from Sigma-Aldrich (Milwaukee, WI, USA), Fluka (Buchs, Switzerland), and Merck (Darmstadt, Germany).

2.5. Equipment used

The thermal cycler used for polymerase chain reaction was T gradient (Biometra, Gottingen, Germany). The centrifugation was carried out with Avanti^{TM} 30 centrifuge using F1202 and C0650 rotors (Beckman, Palo Alto, CA).

2.6. RNA extraction, cDNA synthesis and cloning of full-length gene

Total RNA was extracted from the leaves of *A. annua* by the method of acid: phenol: guanidinium. First-strand cDNA was reverse-transcribed by the SuperScript^{TM} II RT reaction followed the manual of the GeneRace^{TM} Kit (Invitrogen). All the primers for rapid amplification of cDNA ends by PCR are shown in the supplementary Table 2. The specific primers for amplification of this gene were designed by primer 3. PCR was conducted in a 50 µL total volume containing 100 ng cDNA of *A. annua* 0.8 µM each primer, and 1unit Taq DNA polymerase (Promega, Madison, WI, USA) in a reaction buffer composed of 10 mM Tris-HCl, pH 9.0, 50 mM KCl, 2 mM MgCl2, and 0.2 mM each deoxyribose nucleotide. Reaction mixture was preheated at 94℃ for 5 min, followed by 40 cycles of denaturing at 94℃ for 1 min, annealing at 52.5 ℃ for 1 min, and polymerization at 72℃ for 2 min. The reaction was completed by an additional 10 min extension at 72℃. The PCR product was purified and cloned into the pGEM®-T Easy vector (Promega) for sequencing.

2.7. Bioinformatics analysis

Open reading frame (ORFs) was determined using NCBI online tools (http://www.ncbi. nlm.nih.gov/gorf.html). The physical and chemical parameters, such as molecular mass (MW), theoretical p*I* and stability of the deduced amino acids were predicted by ProtParam software online (http;//web.expasy.org/protparam/). Gene was analyzed by ExPASy website to check the hydrophobicity.

2.8. Multiple sequence alignment

The protein sequence was compared with *ent*-kaurenoic acid hydroxylase and steroid 23-alpha-hydroxylase produced gene of *Arabidopsis thaliana*, *Pisum sativum*, *Cucurbita maxima*, *Hordeum vulgare*, *Ginkgo biloba, Oryza sativa* and *Zinnia elegans*. The amino acid sequences of respective gene were used as query to search the respective genome in NCBI database (http://www.ncbi.nlm.nih.gov/) with the BLASTP program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins). Based on domain composition analyses by using the Pfam (http://pfam.sanger.ac.uk/) and NCBI CDD (http://www.ncbi.nlm.nih.gov/cdd) programs.

2.9. Phylogenetic tree analysis

Phylogenetic analysis was performed to understand the relationship between protein sequence of GA and BR. To gain insight into the phylogenetic relationships and functional associations of p450 genes, monocots and dicot plants (*A. thaliana, P. sativum, C. maxima, H. vulgare, G. biloba, O. sativa and Z. elegans*) were used to construct a phylogenetic tree based on maximum-likelihood (ML) methods using MEGA 5.0 [35].

2.10. Recombinant plasmid construction

For the over expression of the gene a full-length cDNA clone was constructed into pBI121binary vector. A full-length cDNA clone of this gene is 1708 bp long and carried an open reading frame of 480 amino acids. Primer ORF -F and ORF -R were designed from the ORF of this gene including 95 upstream region. The purified DNA was sub clone into pGEMT-easy vector. To generate the RNAi constructs for gene suppression 499 bp fragments of sense and antisense gene sequences are generated by PCR from the gene into pFGC vector as described in Fig. 1. At first

for the construction of sense sequence pFGC vector was digested by AscI and SwaI enzymes and ligate the sense fragment using RNAi -F and RNAi -R which were designed from 95 bp upstream of ORF regions of the gene. Afterwards, antisense sequence was ligated into pFGC sense ligated vector. For this pFGC vector was digested by BamHI and XbaI enzymes later on ligated with antisense fragment of the gene.

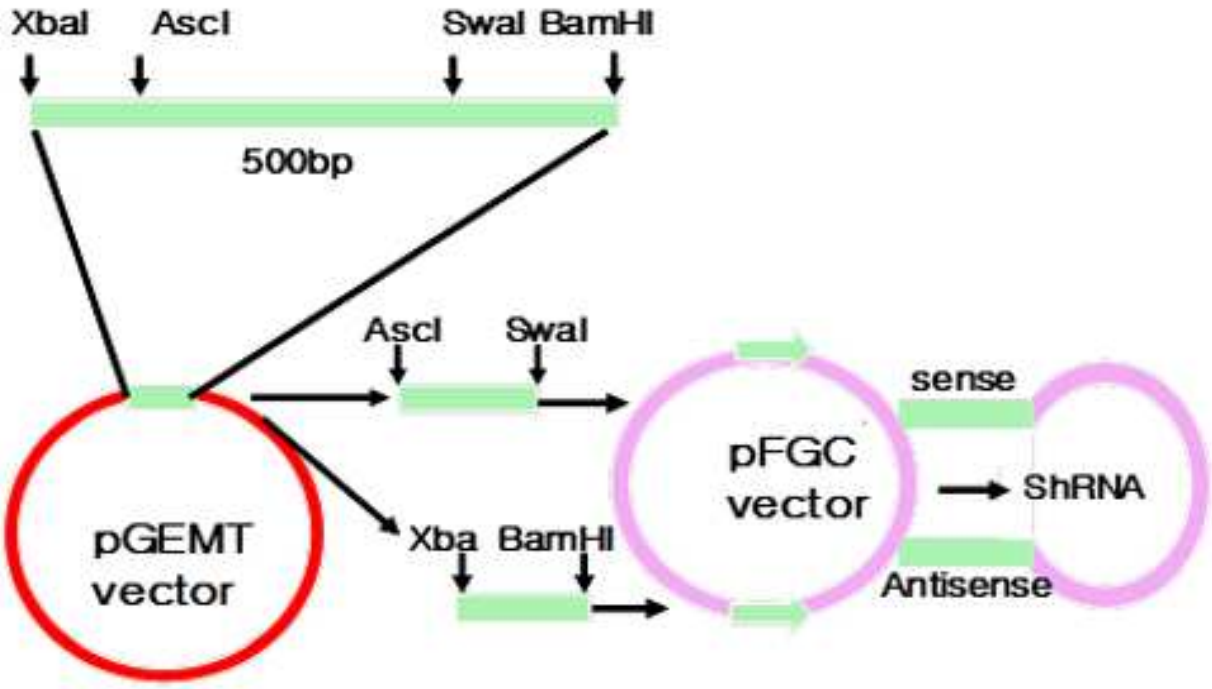


Fig. 1. Schematic diagram of construction of RNAi into pFGC5941 vector.

2.11. Introduction of constructed vectors into *E. coli*

Constructed pBI121 and pFGC vectors were mobilized into *A. tumifaciens* strain EHA105 using the liquid nitrogen Freeze-thaw method according of An et al. [36] with minor modifications. Transformed monoclones were selected on the LB agar plate containing kanamycin (100mg/L).

2.12. Plant transformation

Fresh explants of *A. annua* were transformed by *A. tumifacience* and co-cultivated on MS medium (Supplementary Table 1) in the dark for 3 days. Subsequently, the explants were transferred to shoot-inducing medium Ms-shoot (Supplementary Table 1) with 500 mg/L Cefo to destroy the Agrobacterium. The uninfected explants on MS- shoot medium were used as controls. Seven days later, the explants were transferred to MS-shoot medium with 400 mg/L Cefo. The infected explants then transferred to fresh medium weekly for the first month. One month later, the shoots were transferred to MS-shoot medium with 250 mg/L Cefo and sub-cultured every 2 weeks. One month later the shoots were transferred to MS-root medium (Supplementary Table 1).

2.13. Extraction of genomic DNA and PCR analysis of transgenic plants

Transgenic plants were screened through PCR with gDNA isolated from the transgenic lines for the presence of the expected sequence. For this genomic DNA was extracted from the leaves of transgenic lines for the presence of the expected sequence. Genomic DNA was extracted from the leaves of transgenic plants using genomic DNA extraction kit provided by Promega (Madison, WI, USA). Supplementary Table 3. depicts the primer name and annealing temperature used for each plant gDNA.

2.14. NMR analysis

Three control plants and three overexpressed plants were sampled for this purpose. NMR spectra were recorded on JNM LA 400 spectrometer (JEOL, Japan) operating at 400 MHz. Samples were dissolved in $CDCl_3$. Chemical shift values were given in δ (ppm) with respect to TMS.

2.15. Spray application of brassinolide and gibberellic acid on plants

For BR epibrassinolide (>90%) was sprayed on RNAi mutant plants at 0.1 µM with 0.01% Tween 20 daily for 7 days. Side by side we also apply the exogenous GA to show the interaction. GA was sprayed in the form of gibberellin GA_3 (TLC, >90%) on RNAi mutant plants at the dose of 25 ppm with 0.01% Tween 20 daily for 7 days. Control plants were treated with 0.01% Tween 20 only.

2.16. Experiment design and statistical analysis

For in vitro culture 135 plant explants were taken per sample. Three identical and independent experiments were performed and data were pooled from these experiments. All experiments were repeated three times and data were combined from all experiments for each parameter (45 explants per replicate). Transformation efficiency has been expressed as percentage of number of PCR-positive plants to the initial number of explants. Statistical analysis was done with SPSS 20.0 (IBM SPSS Statistics, Chicago, USA). Student's t test was employed to determine if there were significant differences between the control and each of the other plant types at $p < 0.05$.

3. Results

3.1. Full-length cloning of *AaCYPdwf* from *A. annua*

After searching the different growth regulatory gene from NCBI database, several primers were designed. Among them, one pair of primer amplify the gene in *A. annua*. Finally, a full-length

putative cytochrome P450 gene was cloned using 5′ and 3′ RACE PCR. A 1411bp 5′end and 855bp 3′end was obtained for the gene listed in Table 1. This full-length gene contained 1443bp ORF encoding 480 amino acids, which was deposited in NCBI Genbank with an accession number of DQ363133.

Table 1. Information of *AaCYPdwf* unigene from RACE-PCR fragment.

Gene name	Unigene length (bp)	5′-RACE fragment (bp)	3′-RACE fragment (bp)	Full length of ORF (bp)
AaCYPdwf	541	1411	855	1443

3.2. Bioinformatics analysis

The proteomics analysis of this unigene are computed by the online Pxpasy's ProtParam tool. Primary structure analysis revealed that the molecular mass of the putative cytochrome P450 protein was 54.81 kDa with a theoretical p*I* = 9.14. The instability index of this protein was 34.59, which indicated that it is a stable protein. The grand average of hydropathicity (GRAVY) value was close to zero that means the gene *AaCYPdwf* has both hydrophilicity and lipotropy. The cDNA-coding DQ363133 was analyzed by ExPASy website to check the hydrophobicity, which showed hydrophobicity in N-terminal region of the translated protein. It indicates that gene *AaCYPdwf* anchored to the ER membrane as a typical signature of P450 [37].

3.3. Regeneration of transgenic plants

The number of explants producing Km- and Basta-resistant calli or shoots, respectively for over expressed and pBI121-only plants and for RNAi-inserted and pFGC-only plants were recorded after 8 weeks of selection (Fig. 2). Initially, there were three plates for each transformant category with 45 explants per plate. Three weeks after infection, the Km- and Basta-resistant

explants were selected and cultured separately. After 8 weeks of infection, small shoots were separated and cultured on MS root medium. These shoots induced root within 1-2 weeks and the rooted shoots were transferred into soil. The frequency of regeneration ability was almost similar (Table 2, Fig. 3). The pBI121, pFGC, RNAi and control plants showed same frequency of antibiotic resistant callus, shoot and root. The overall performance of overexpressed plant was lower than the others. Transformation efficiency of pBI121 and pFGC plants were higher than the over expressed and RNAi plants. Extraction of gDNAs from all RNAi plants were not possible due to very low amounts of leaf samples.

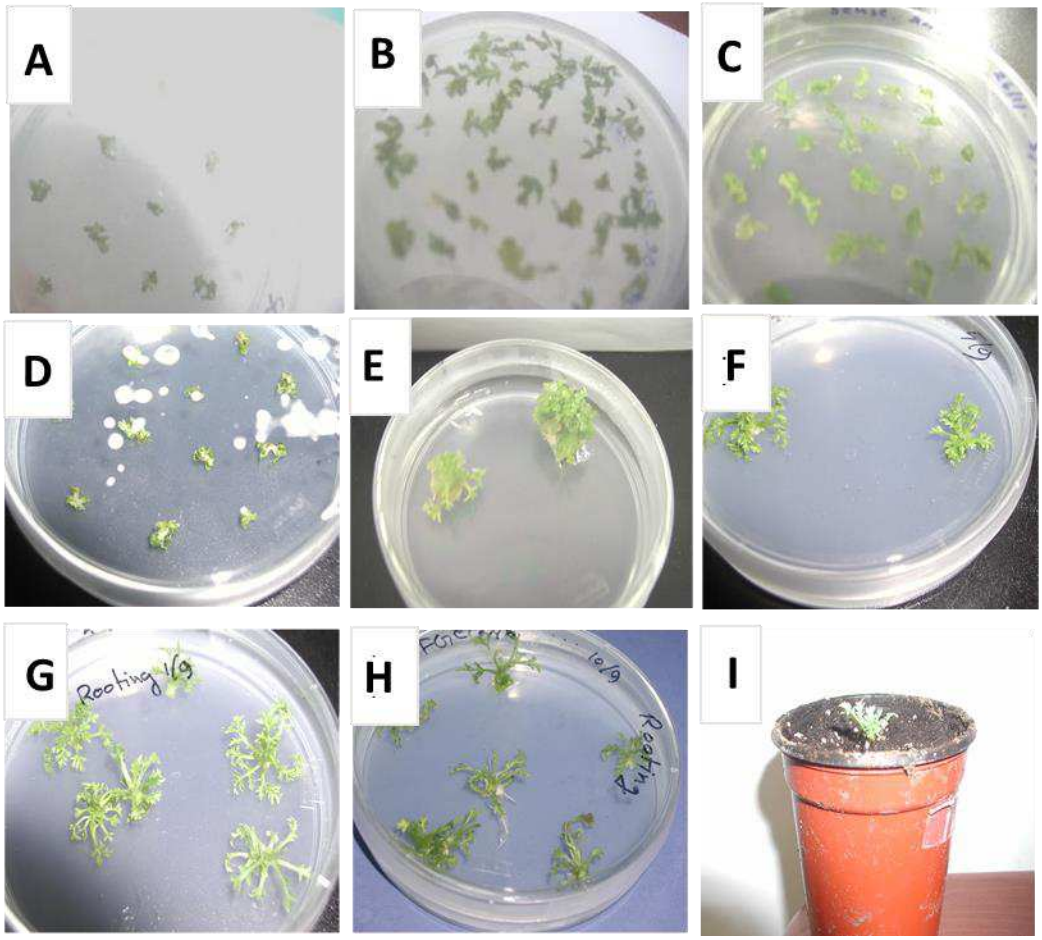


Fig. 2. Regeneration of transgenic plants. (A) After infection of plant explants kept 3 days in dark, (B) Plant explants place on MS shoot with 500 mg/L Cefo, (C) Antibiotic resistant plant explants maintained in MS shoot medium with 300 mg/L Cefo, (D) Plant explants turned into callus form, (E) Combined shoot lets, (F) individual shoot on MS shoot medium, (G) Shoot on MS root medium, (H) Root formation, (I) Small plant transfer into soil.

Table 2. Response of regeneration ability in different steps and transformation efficiency of transgenic plants.

Plant	Frequency of Antibiotic resistant callus (mean ±SE)	Frequency of Antibiotic resistant shoot (mean ±SE)	Frequency of Root induced shoots (mean ±SE)	Frequency of PCR positive plants (mean ±SE)	Average number of regenerated plantlets per plant (mean ±SE)	Transformation efficiency (%)
pBI121	12.33±0.67	5.67±0.33	3±0.58	1.33±0.33	1.33±0.33	3.3
pFGC	12±0.58	5.33±0.33	3.33±0.33	1.33±0.33	1.33±0.33	3.3
Over expressed	11.67±0.67	4.67±0.33	2.67±0.33	1±0.58	1±0.58	2.5
RNAi	12±0.58	7±0.58	4.67±1.45	1±0.58[a]	1±0.58	2.5
Control	12.33±0.67	7±0.58	5.33±0.33	1.33±0.33	1.33±0.33	

a. RNAi transgenic plant samples was too small to extraction of gDNA

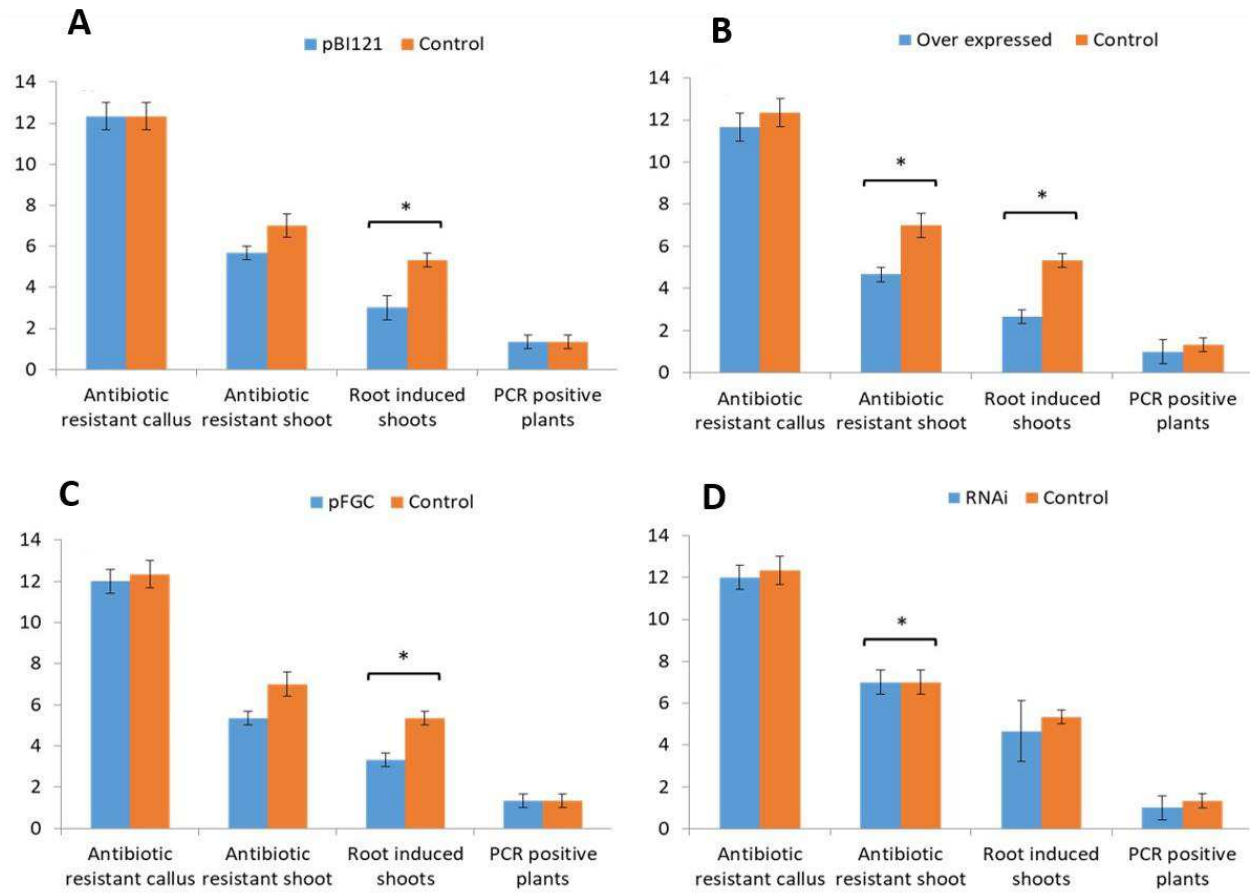


Fig. 3. Response of regeneration ability in different steps of transgenic plants. (a) pBI121 plants, (b) Overexpressed plants, (c) pFGC plants, (d) RNAi plants. Data is represented as Mean ± SE. The asterisk (*) indicates the significant difference at $P < 0.05$.

3.4. PCR analysis of gDNA of transgenic plants

All regenerated transgenic plants were analyzed by PCR (Figs. 4-7). The transgenic lines had a band which was absent in the gDNA of untransformed control plants. The PCR product bands obtained from the transgenic plants gDNAs were longer than the original lengths (Table 3) that confirmed the integration of the transgene in the genome [38].

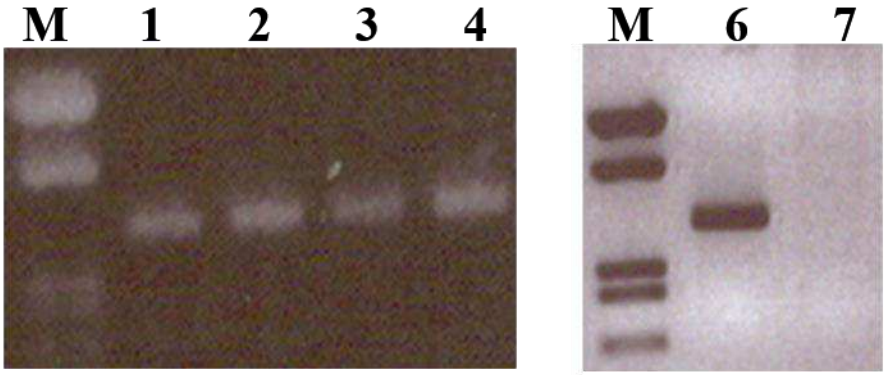


Fig. 4. Agarose gel electrophoresis of the PCR analysis of pBI121 plants. The templates for PCRs were: 1-4, Transgenic plant genomic DNA; 7, untransformed plant genomic DNA as negative control; 6, pBI121 vector as positive control. M is the molecular weight marker [*Hin*dIII/*N*coI digest of pUC 19].

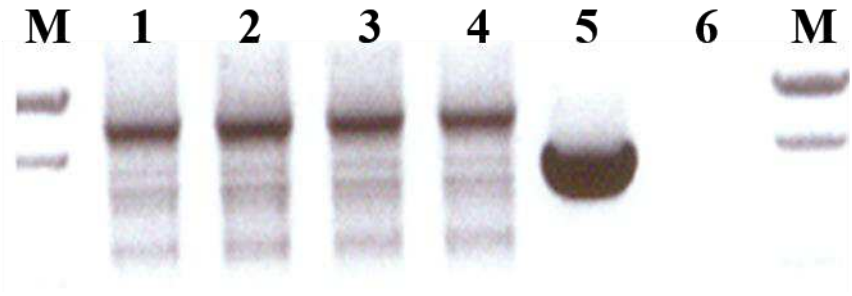


Fig. 5. Agarose gel electrophoresis of the PCR analysis of pFGC plants. The templates for PCRs were: 1-4, transgenic plants genomic DNA; 5, pFGC vector as a positive control; 6, untransformed plant genomic DNA as negative control; M is the molecular weight marker [HindIII/NcoI digest of pUC 19].

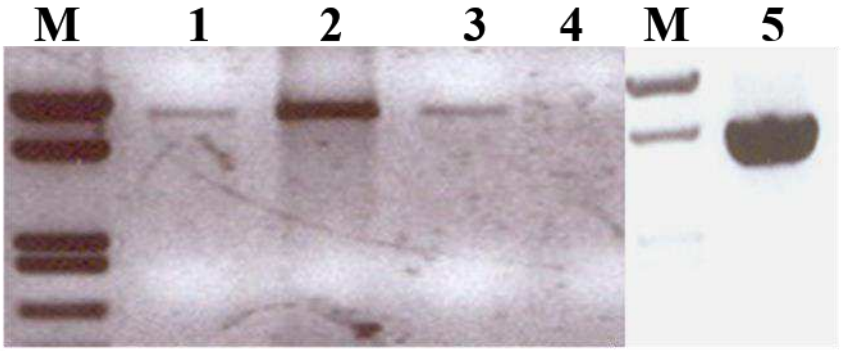


Fig. 6. Agarose gel electrophoresis of the PCR analysis of over expressed plants. The templates for PCRs were: 1- 3, transgenic plants genomic DNA; 4, untransformed plant genomic DNA as negative control; 5, constructed pBI121 vector as positive control. M is the molecular weight marker [HindIII/NcoI digest of pUC 19].

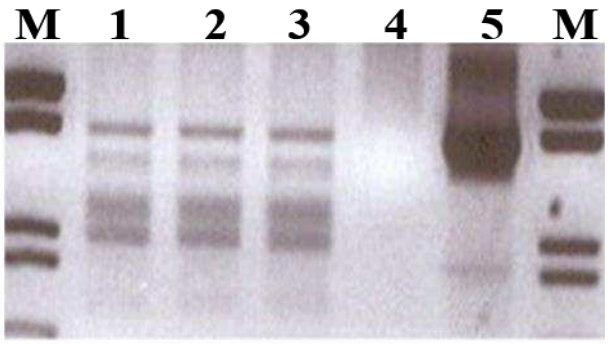


Fig. 7. Agarose gel electrophoresis of the PCR analysis of RNAi plants. The templates for PCRs were 1-3, transgenic plant genomic DNA; 4, untransformed plant genomic DNA as negative control; 5, pFGC vector as positive control. M is the molecular weight marker [*Hin*dIII/*N*coI digest of pUC 19].

289 **Table 3. PCR products bands of transgenic plants gDNAs.**

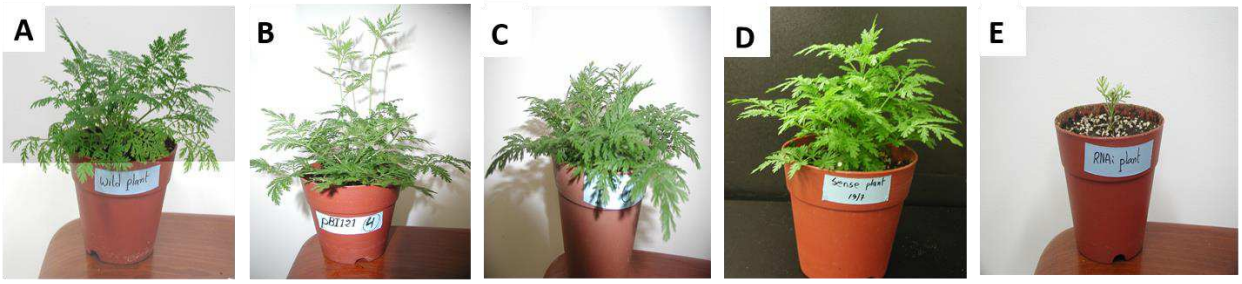
Plants gDNA	Endogenous length (bp)	Original length (bp)
pBI121 vector	1100	800
pFGC vector	1900	1366
Overexpressed	2200	1443
RNAi	1449	1366

290

291

292 ### 3.5. Analysis of overexpressed and RNAi mutant plants

293 The overexpressing transgenic plants were morphologically similar to the control and pBI121
294 vector plants (Fig. 8). On the other hand, RNAi mutant plants showed extremely dwarf phenotypes
295 than control and empty pFGC vector plants and display darker green dwarfed stature with epinastic
296 round leaves, short petioles and reduced apical dominance (Fig. 8). These results indicate that the
297 RNAi mutants are brassinolide deficient dwarf plants [39].



298

299 **Fig. 8. Fourteen weeks old transgenic plants.** (a) Control (Wild) plant, (b) pBI121 vector plant,
300 (c) pFGC vector plant, (d) overexpressed plant, (e) RNAi plant

301

302 ### 3.6. Effects of GA and BR on RNAi mutant plants

303 Four-month-old RNAi mutant plants were treated with GA and BR. It was found that GA and BR
304 promoted the growth of the plants, whereas mutant plant with mock treatment showed no

development and the condition of the plant deteriorated. Completely dead plants were confirmed at seven-leaf stage (Fig. 9). These results suggest that *AaCYPdwf,* a P450 gene, plays a key role in GA or BR biosynthesis pathway in *A. annua* plants.

The growth rate of GA-treated plant was higher than that of BR-treated plant (Fig. 9). There was a rapid growth phenomenon in GA-treated plants. The growth pattern remained the same from the initial stage to the final stage (45th day). On the other hand, BR-treated plants showed stagnant growth up to 20 days, and then retained rapid growth phenomena.

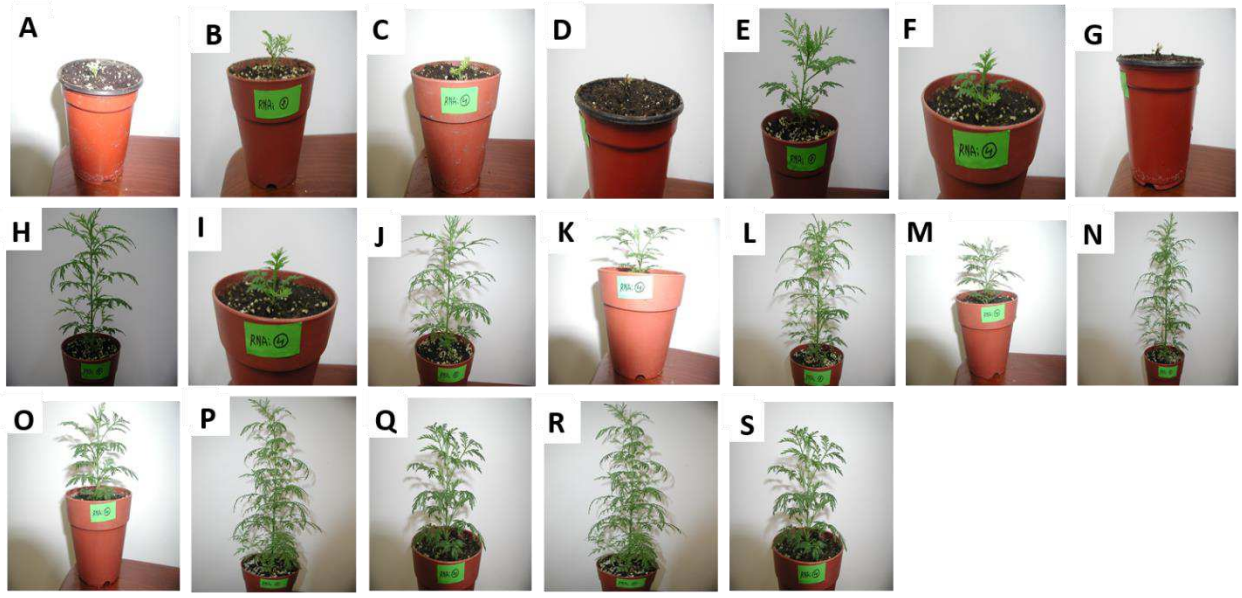


Fig. 9. Effects of GA and BR on plants. (a) Control plant, (b) GA-sprayed plant, (c) BR-sprayed plant, (d) 12 days of control plant, (e) 12 days of GA-sprayed plant, (f) 12 days of BR-sprayed pl ant, (g) 17 days of control plant, (h) 17days of GA-sprayed plant, (i) 17 days of BR-sprayed plant , (j) 20 days of GA-spayed plant, (k) 20 gays of BR-sprayed plant, (l) 25 days of GA-sprayed pla nt, (m) 25 days of BR-sprayed plant, (n) 30 days of GA-sprayed plant, (o) 30 days of BR-sprayed

plant, (p) 37 days of GA-sprayed plant, (q) 37 days of BR-sprayed plant, (r) 45 days of GA-spray ed plant, (s) 45 days of BR-sprayed plant.

3.7. Multiple sequence alignment and phylogenetic tree

The cloned P450 gene *AaCYPdwf* showed 30-31% identity with ent-kaurenoic acid hydroxylase-produced protein. On the other hand, it showed 30-32% identity with steroid 23-alpha-hydroxylase produced protein (Fig. 10). Hypothetical phylogenic tree of gibberellic acid (GA)- and brassinosteroid (BR)-produced protein families were created. These results demonstrated that the cloned new gene is more similar to a BR-producing than a GA-producing gene (Fig. 11). Taken together of these results, the full-length cDNA was named as *AaCYPdwf*.

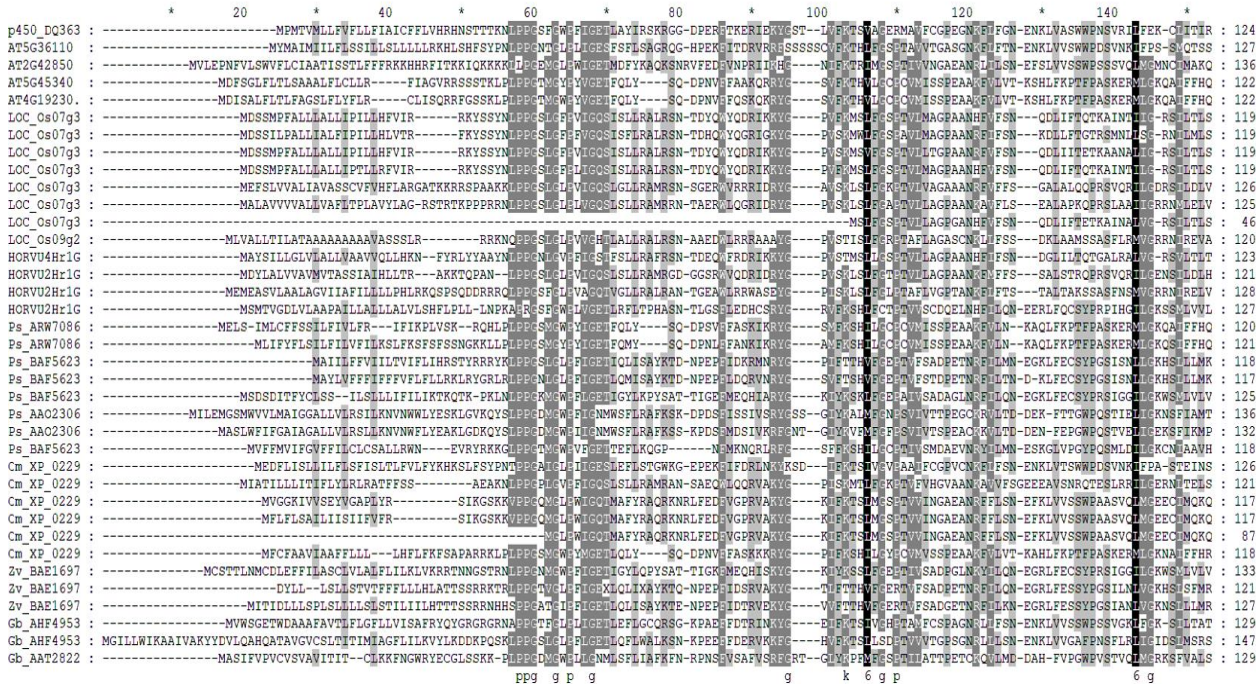


Fig. 10. Multiple sequence alignment of cloned P450, ent-kaurenoic acid hydroxylase and st eroid 23-alpha-hydroxylase protein sequence.

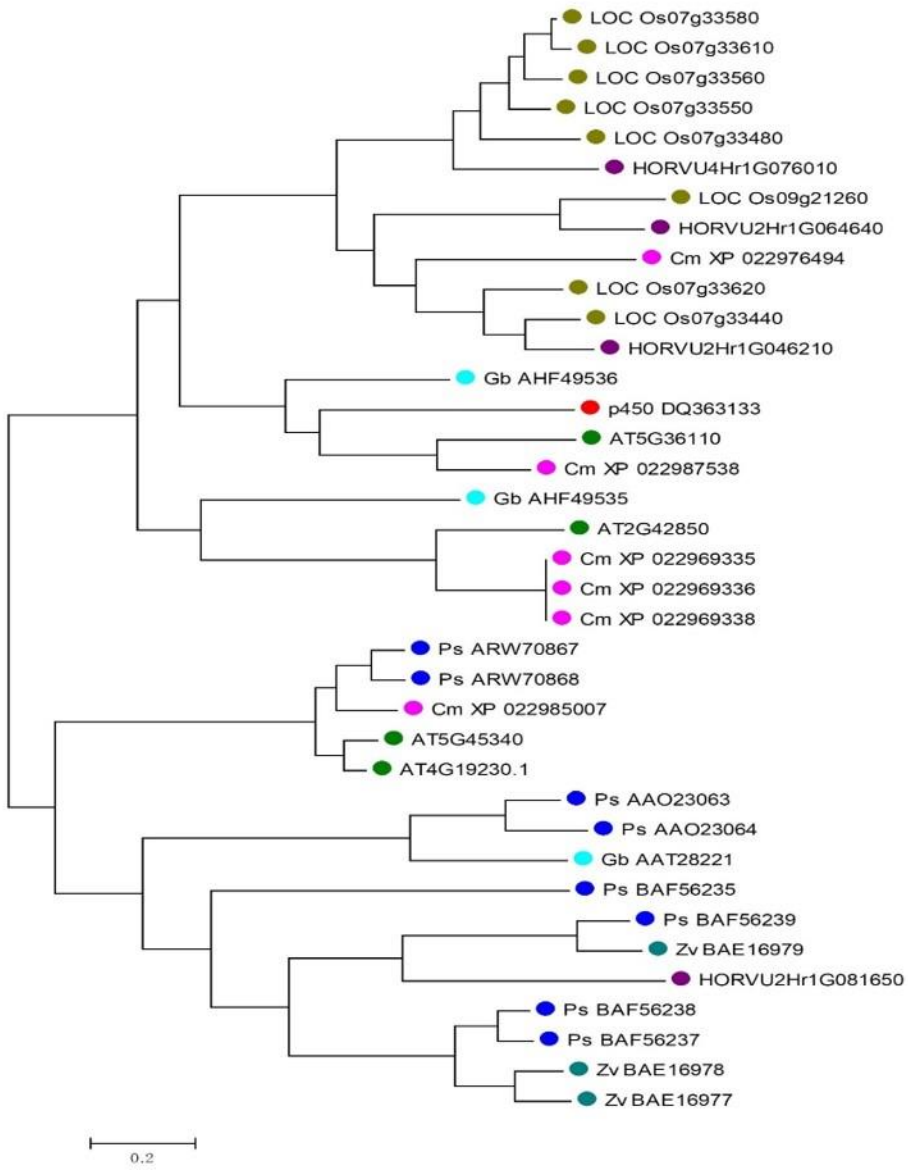


Fig. 11. Hypothetical phylogenetic tree derived from seven selected GA and BR produced protein sequence. Our P450 shown with red filled circle and other reference sequences from different plants are marked with different color circle.

3.8. NMR analysis

Artemisinin produced by control and overexpressed transgenic plants were analyzed by ^{1}H NMR. But the RNAi transgenic plants were too miniscule to collect the sample. However, ^{1}H NMR

spectrum did not show any peak related to the H5 signal of artemisinin presumably due to the low concentration in the sample. Therefore, artemisinic acid produced by transgenic plants was measured by ^{1}H NMR (Supplementary Fig. 1). The plant samples prepared for ^{1}H NMR were dissolved in chloroform-D and NMR peaks at δ 6.4, 5.5 and 4.9, corresponding to H12a, H12b, and H5 of artemisinic acid, respectively, were integrated. The integration value of each peak was normalized against chloroform peak at δ 7.2. The values presented in Fig. 12 are the averages of three peak integrations. These results showed that the amount of artemisinic acid produced by control plants was higher than that of the over-expressed plants. The results of ^{1}H NMR study suggest that *A. annua* over-expressing P450 was not able to produce higher levels of artemisinic acid, indicating that the new P450 gene *AaCYPdwf* is not a regulatory switch for artemisinin biosynthesis.

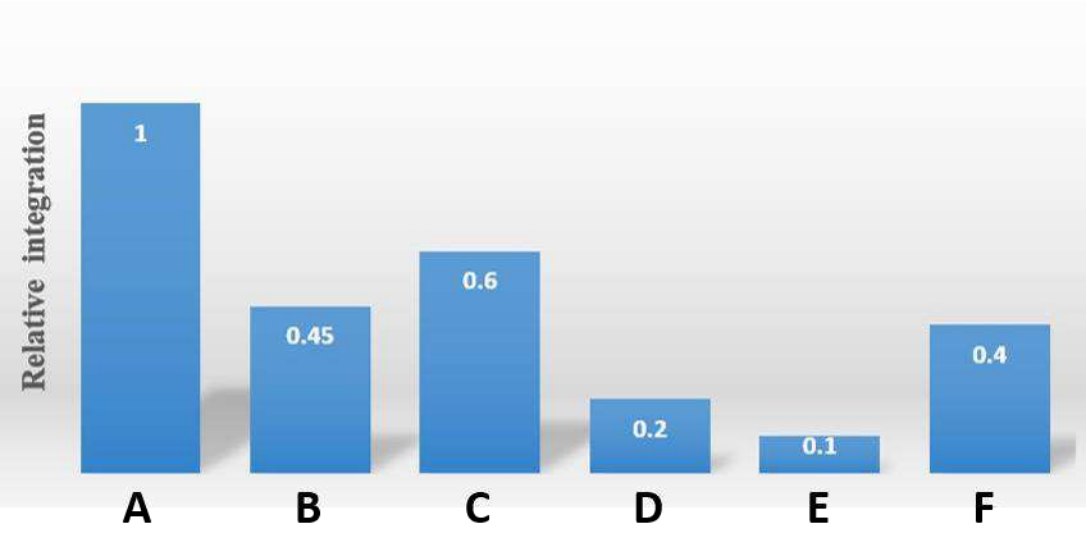


Fig. 12. The content of artemisinic acid in each plant was presented as relative integration. The control plant with the highest content was arbitrarily set at 1.0. (a), (b), (c) control and (d), (e), (f) overexpressed plant samples.

4. Discussion

In this study, we successfully cloned a novel P450 gene *AaCYPdwf* from *A. annua* which is likely to be involved in BR-production in the plant. Silencing of the *AaCYPdwf* gene in *A. annua* resulted extreme dwarf plants which might be linked with lesions in phytohormone synthesis or sensitivity [40–42]. The BR and GA are plant growth regulators that control cell and plant size. Mutations in the genes involved in biosynthesis of BR and GA impair plant growth and cause dwarfism [43]. Early physiological work on wild-type (WT) tissues of different plants showed that GA and BR additively enhance growth, indicating that these two hormones act independently at the cellular level [44]. Therefore, we selected BR and GA for treating the RNAi silenced extremely dwarf mutant plants. Recovering of the dwarf mutant plants by the exogenous application of BR indicating that the novel gene, *AaCYPdwf* is linked to the growth regulation of the plants through BR hormone pathway [45]. There are a number of possibilities, in response to exogenous BR, the transcription levels of this gene might increase [46] and stimulate BR production. The gene might be downstream targets that are involved in the response of the plant cell to BR. Some BAHD family members may be involved in cell-wall biosynthesis and many BR-responsive genes are involved in cellular processes such as cell wall biosynthesis [47–50]. Another reason is that the induction of these genes may indicate a mechanism for BR inactivation like the expression of GA-inactivating oxidases through GA treatment [51]. Additionally, Divi et al. found that exogenous BR increases JA levels in Arabidopsis [52].

In the present study, it revealed that a P450 gene *AaCYPdwf* is involved in biosynthesis of BR. The interference might be in the GA synthesis pathway. The BR showed enhanced growth of the RNAi-treated dwarf plant, but at lower level than that of GA-treated plant, suggesting BR may have some regulatory effects on GA biosynthesis. There are several cross-talks about the

relationship between brassinosteroid, gibberellin and auxin biosynthesis in plants. Several lines of evidence suggest that BR-induced effects in plants might be regulated through auxin-BR interaction and endogenous auxin levels or auxin sensitivity could be changed by BR treatment [53,54]. Furthermore, in Arabidopsis seedlings, the early auxin-inducible genes *IAA5*, *IAA19* and *DR5-GUS* are induced by BR, without increase in IAA levels [55]. Moreover, in case of plants internodes auxin up-regulates GA biosynthesis [56,57]. In Arabidopsis, there are some overlaps in the BR- and auxin-signaling pathways [58], and BRs and auxin have partly similar regulatory functions [59]. This similarity might extend to regulation of the GA pathway [60]. In Arabidopsis, BR treatment increased transcript levels of a key biosynthetic gene GA5 (a GA 20-oxidase gene), which is responsible for the production of GA9 and GA20 [61,62]. Additionally, in certain tissues, BR potentiates GA activity [62], and some plant hormones can affect the concentration, perception and response to other plant hormones [63].

The amino acid sequence of *AaCYPdwf* showed identity to the previously registered deduced amino acid sequence. The plant P450 sequences from the GenBank/EMBL database showed amino acid identities of 52 and 53% with putative cytochrome P450 of the AAC69934 *A. thaliana* family, and cytochrome P450 of the BAD90972 [*Oryza sativa* (japonica cultivar-group)], respectively. The steroid 23-a hydroxylase which showed identity with this gene is an Arabidopsis subfamily CYP85A2. The CYP85A2 subfamily is a cytochrome P450 enzyme that possesses BR C-6 oxidase which converts castasterone to brassinolide represents the final and rate-limiting step in the biosynthesis of brassinosteroids in plant [34]. Considering the phenotypic characteristics, multiple sequence alignment and phylogenetic tree of *AaCYPdwf* gene, we assume that this novel gene is linked with brassinolide biosynthesis in *A. annua* plant. However, a further study in warranted for confirmation of this conclusion.

Interestingly, our overexpressed plants displayed similar phenotypes to control one. The BRs biosynthesis and inactivation are critical components for maintaining the endogenous levels in plants and very important for the regulation of plant growth and development. In case of Arabidopsis and other plant species, several gene transcription, individually or as part of protein complexes [64–67]. Tayengwa et al. [68]also observed the similar phenomena when they overexpressed a flowering time regulator *AHL* gene, the transgenic plants took same time for flowering as like as control. It can be hypothesized that several genes may function as part of complex to regulate expression of a specific gene [69,70].

Several lines of evidence suggest that overexpression of a key regulatory enzyme was found to enhance the production of terpenoid in plants [71,72]. For example, *Panax ginseng* over-expressing *PgSSI* is able to produce high levels of phytosterol and ginsennosides, indicating that *PgSSI* is a key regulatory enzyme for triterpene biosynthesis [71]. Similarly, *Elueutherococcus senticosus* transgenic plants overexpressing squalene synthase (encoding gene *pgSS1*), which is an important enzyme in the production of phytosterols produced 2~2.5- fold phytosterols (β-sitosterol and stigmassterol) than that of wild type plants, suggesting that this gene catalyzes the sterol biosynthesis [72]. Based on these results, it is clear that overexpression of the related enzyme increases the production of terpenoid in the plant. On the other hand, in the current study, overexpression of our cloned P450 *AaCYPdwf* gene had no direct effect on artemisinic acid production. Therefore, this gene seemed no relevancy to the biosynthetic pathway of artemisinin but likely to be involved in biosynthesis of brassinolide in *A. annua*. To elucidate the precise role of *AaCYPdwf,* a further investigation is needed.

5. Conclusion

We cloned a novel cytochrome p450 *AaCYPdwf* gene from *A. annua*. Findings of our studies show that there is no overexpression phenotype of this gene but the absence (silencing) of this gene seemed very critical for plant growth as knockout of this gene resulted extremely dwarf plants. However, exogenous application of brassinolide recovered the dwarfism of the mutants. Taken together, these results suggest that *AaCYPdwf* gene is essential for growth of the plant, which may be linked with the biosynthesis of brassinolide in *A. annua*. However, a further study is needed to elucidate the precise role of *AaCYPdwf* gene in brassinolide biosynthesis and growth of *A. annua*.

Author contributions

Conceptualization: Soo-Un Kim

Data curation: Soo-Un Kim

Formal analysis: Mahmuda Umme Rayhan

Investigation: Mahmuda Umme Rayhan

Resources: Mahmuda Umme Rayhan

Methodology: Mahmuda Umme Rayhan

Project administration: Soo-Un Kim

Software: Mahmuda Umme Rayhan

Supervision: Soo-Un Kim

Funding acquisition: Soo-Un Kim

Visualization: Soo-Un Kim

Validation: Soo-Un Kim

Writing – original draft: Mahmuda Umme Rayhan

Writing – review & editing: Tofazzal Islam, Mahmuda Umme Rayhan

Funding

This work was funded by the Korea Science and Engineering Foundation (KOSEF), South Korea, through a coordinated project to Soo-Un Kim of the Program in Applied Life Chemistry, Department of Agricultural Biotechnology, Seoul National University, Seoul 08826.

Declaration of Competing Interest

The authors declare no conflict of interest

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

Acknowledgements

The authors are thankful to the Korea Science and Engineering Foundation (KOSEF), South Korea, for funding this work. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. We sincerely appreciate Tahsin Islam Sakif of West Virginia University, USA for generous linguistic editing of this manuscript.

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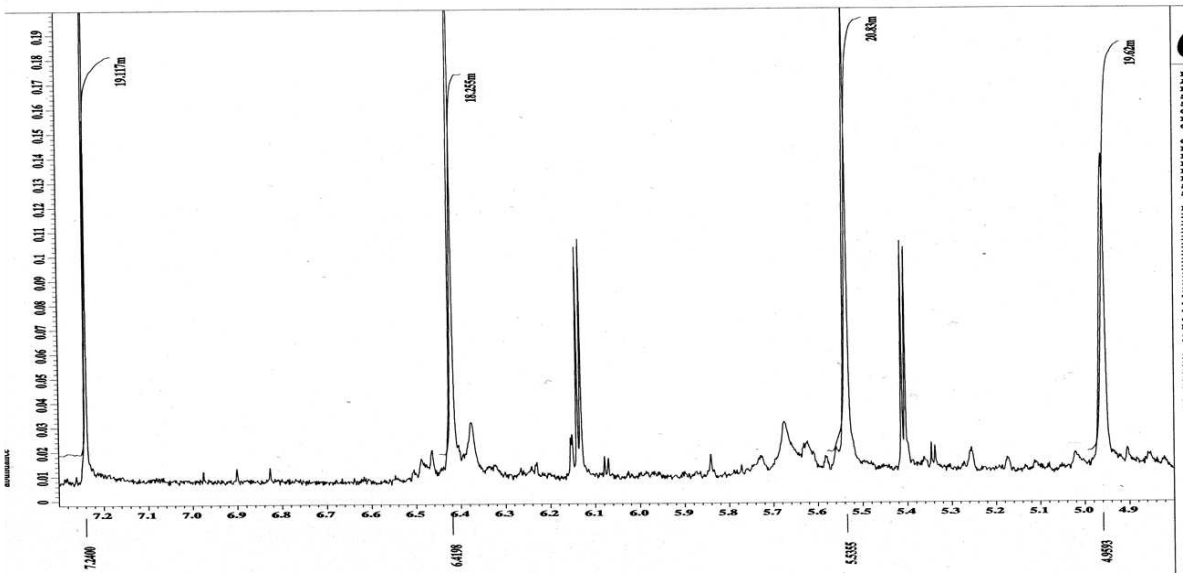
SUPPORTING INFORMATION

Supplementary Figure 1. ^{1}H NMR analysis of artemisinic acid produced by transgenic plants. The plant samples prepared for 1H NMR were dissolved in chloroform-d and NMR peaks at δ 6.4, 5.5 and 4.9, corresponding to H12a, H12b, and H5 of artemisinic acid, respectively, were integrated. The integration value of each peak was normalized against chloroform peak at δ 7.2.

693 Three control plants (a), (b), (c) and three overexpress plants (d), (e) and (f) were used for this
694 work.

a



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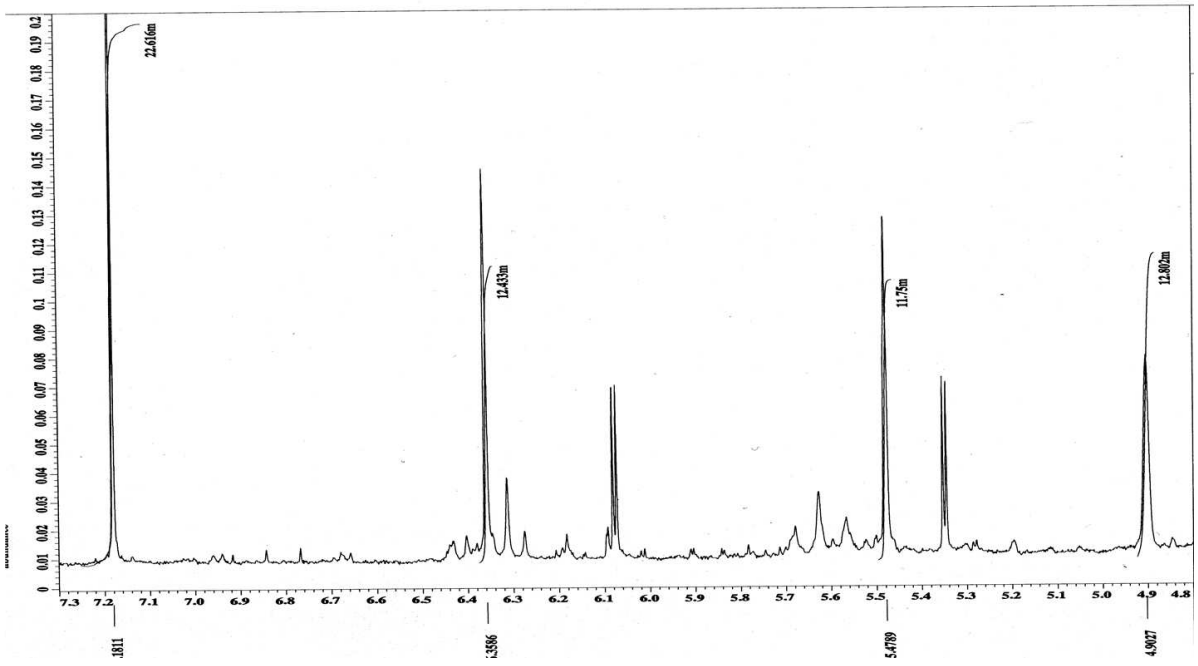
b



696

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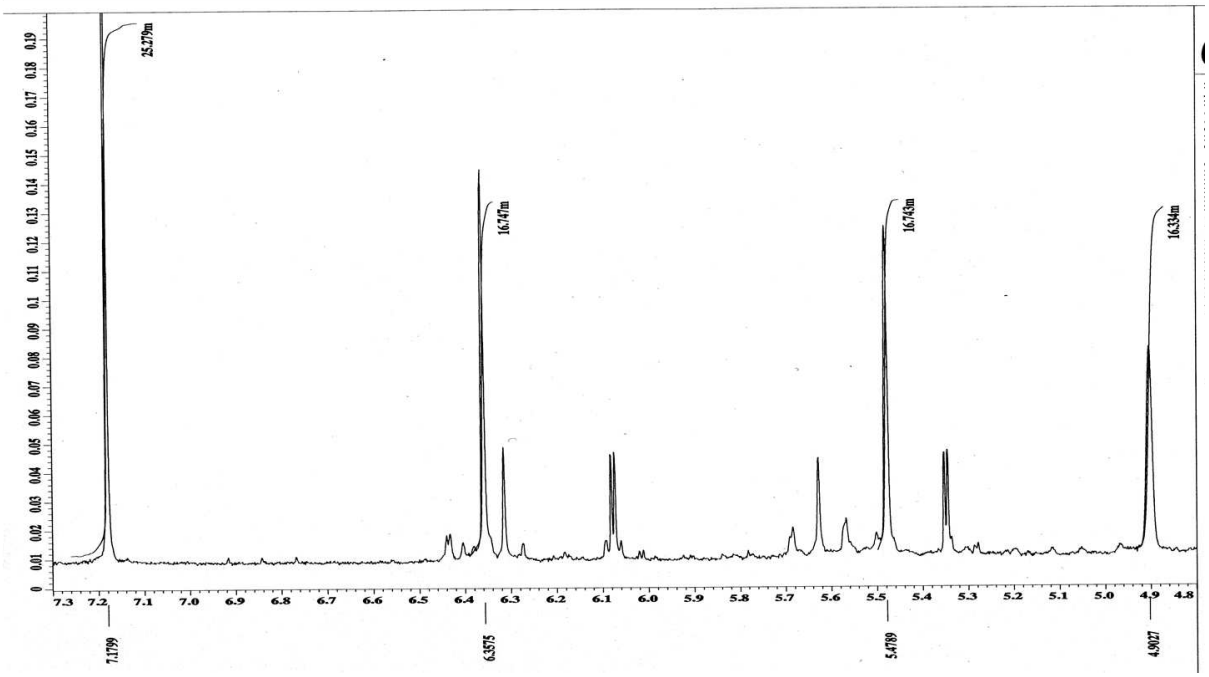
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16.747m
16.743m
16.334m
7.1799
6.3575
5.4789
4.9027

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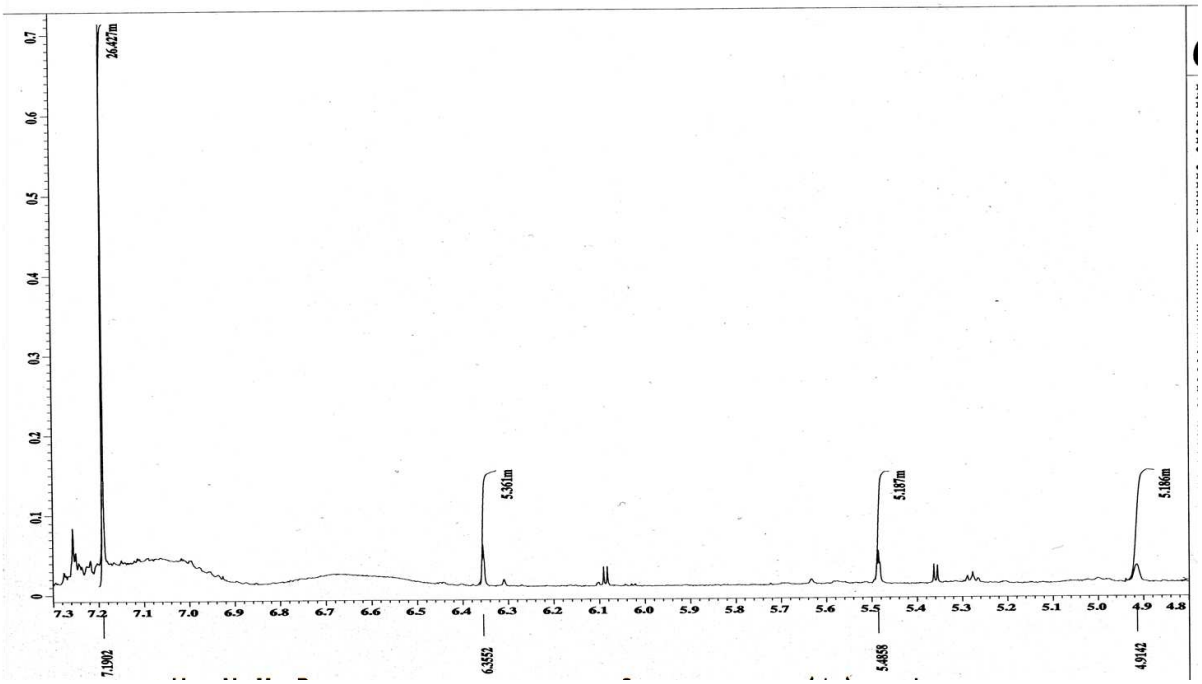
d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5.361m
5.187m
5.186m
7.1902
6.3552
5.4858
4.9142

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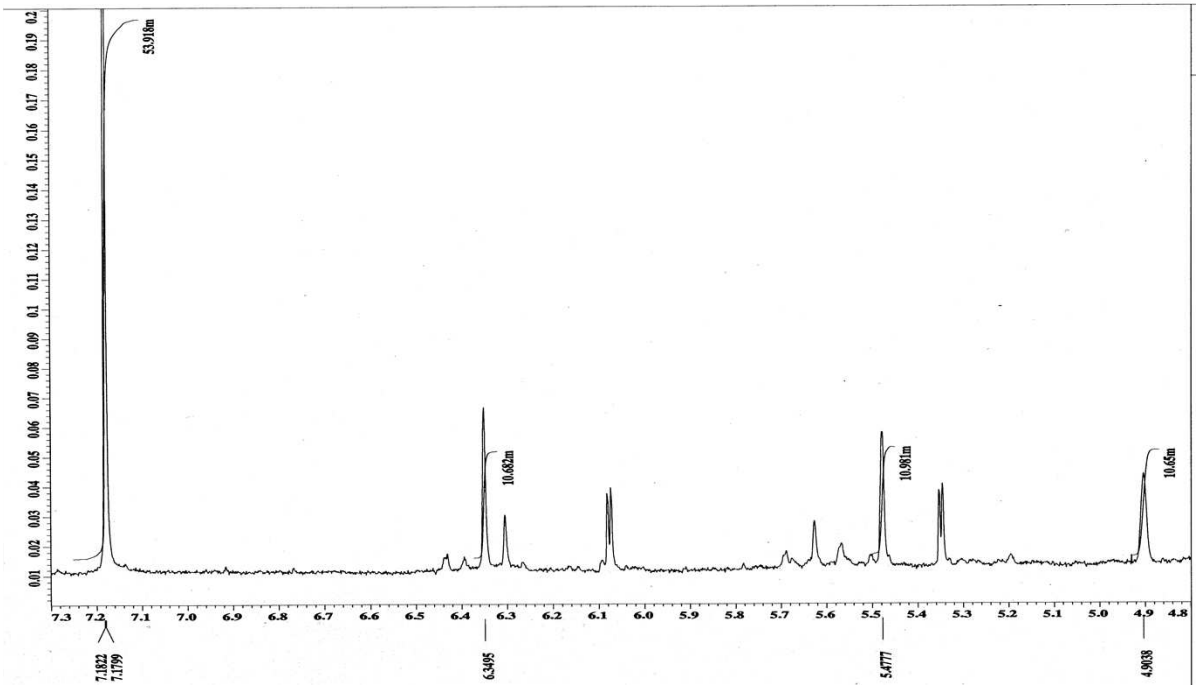
e



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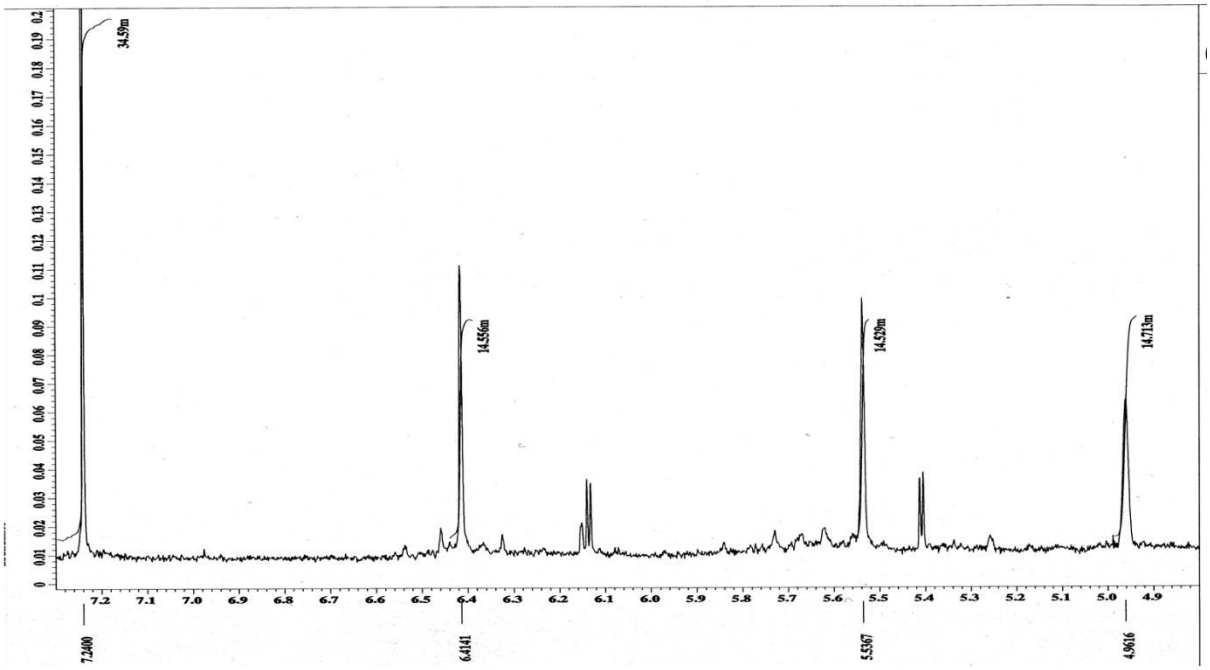
f



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706 **Supplementary Table 1. Culture media for *A. annua* in vitro culture.**

Medium	Composition
MS	0.44% MS salt, 3% (w/v) sucrose, 0.8% agar, pH 5.7
MS shoot 1a	MS + 0.05 mg/L NAA + 0.5 mg/L BAP + 15 mg/L Kanamycin. pH 5.7
MS shoot 1b	MS + 0.05 mg/L NAA + 0.5 mg/L BAP + 50mg/L Basta, pH 5.7
MS root 1a	MS + 0.05 mg/L NAA + 15 mg/L kanamycin, pH 5.7
MS root 1b	MS + 0.05 mg/L NAA + 50 ppm Basta, pH 5.7

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708 **Supplementary Table 2. List of oligonucleotide sequences used in this research.**

Name	Usage	Sequence (5'- 3')
5' RACE primer	RACE PCR	AAG GAT GGA AGA TAA TCT GGA GTG
3' RACE primer	RACE PCR	GAA AGG GGT TGG CCC TGC TCC TCC
ORF -F	Full gene clone	GGATCCATGCCAATGACGGTTATG
ORF -R	Full gene clone	GGATCCTCAAACTTGATGAGGATG
RNAi -F	RNAi PCR	GCTCTAGAGGCGCGCCATTCCAATTCACATAAG
RNAi -R	RNAi PCR	CGGGATCCATTTAAATATCATCTTTCTCAACCA
T7	Sequencing	GTAATACGACTCACTATAGGG
Sp6	Sequencing	ATTTAGGTGACACTATAGAATAC
pBI121-F	Sequencing	CCTCTATATAAGGAAGTTCATT
pBI121-R	Sequencing	ATTTCACGGGTTGGGGTTTCTA
pFGC sense -F	Sequencing	AACAACAACAAACAAACA
pFGC sense -R	Sequencing	TAAGATAAAACGTTGAATGTA
pFGC anti sense -F	Sequencing	GTTTATGTTTTAGTGTTTTCT

pFGC anti sense -R	Sequencing	TATCTCATTAAAGCAGGACTC
35S promoter-F	Transgenic plant sequencing	GGTTAGAGAGGCTTACGCAGG
35S promoter-R	Transgenic plant sequencing	GTCCCCCGTGTTCTCTCCAAATGA
RNAi transgenic-F	Transgenic plant sequencing	AAATGTGTAAGAATTTCTTATG
RNAi transgenic-R	Transgenic plant sequencing	AAAAAAGATGTGAAGAAAACAC

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710 **Supplementary Table 3. PCR of transgenic plants gDNAs.**

Plants gDNA	**Primer name**	**Annealing Temperature (°C)**
pBI121 vector	35S promoter	56.3
pFGC vector	RNAi transgenic	37.8
Overexpressed	ORF	52.5
RNAi	RNAi transgenic	37.8

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