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Original Article

Rol genes enhance content of artemisinin and other secondary metabolites in Shennong hybrid of *Artemisia annua*

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ABSTRACT

Objective: *Artemisia annua* is the chief source of artemisinin, a potent antimalarial agent, in which other bioactive phytochemicals are also present. Due to low levels of bioactive compounds including artemisinin and flavonoids, it is necessary to increase the level of the secondary metabolites by regulating the expression of *rol* genes in the plant.

Methods: A hybrid variety of *A. annua* (Hyb1209r, Shennong) developed by the Centre for Novel Agricultural Products, University of York, UK, was selected to produce transgenics of *rolB* and *rolC* genes. Genetic transformation was carried out via *Agrobacterium tumefaciens* GV3101 harboring *rolB* and *rolC* genes of *Agrobacterium rhizogenes* cloned separately. HPLC was used for the qualitative and quantitative analysis of flavonoids and artemisinin. Furthermore, thin layer chromatography (TLC) was also used to analyze artemisinin content.

Results: Comparative analysis via HPLC revealed considerable enhancement in the phytochemical content of transgenic *A. annua* plants as compared to the wild type plant. Transgenics of *rolB* gene showed an average increase of 321% in rutin, 97.2% in caffeic acid, and 218.4% in myricetin, respectively. In the case of *rolC gene* transgenics, an average increase of 197.5% in rutin, 76.3% in caffeic acid, and 209.3% in myricetin was observed. Transgenics of *rolB* and *rolC* genes showed a 14.3%–28.6% and 2.8%–12.7% increase in artemisinin content respectively by HPLC analysis. TLC analysis showed that an average 142.2% and 110.2% enhancement in artemisinin for *rolB* and *rolC* transgenics respectively, compared with the wild type. An enhanced production of total flavonoids (average 30.2% and 25.5% increase in *rolB* and *rolC* transgenics, respectively) and total phenolics (average 34.3% and 25.8% increase in *rolB* and *rolC* transgenics, respectively) was observed as a result of transformation. Transformed *A. annua* plants showed improved free radical scavenging activity (average 46.5% and 29.1% increase in *rolB* and *rolC* transgenics, respectively) and total reducing power (average 32.7% and 26.4% increase in *rolB* and *rolC* transgenics, respectively) compared with untransformed plant.

Conclusion: *rolB* and *rolC* genes were effective for developing *A. annua* plants with an enhanced level of phytochemicals.

1. Introduction

Artemisia annua L. is an aromatic annual herb native to China, where it has been used in traditional medicine for centuries as a remedy for fever and malaria. Malaria is a serious global health problem leading to over one million deaths annually (Enserink, 2008). Out of the four malaria causing parasites, *Plasmodium falciparum* is the main cause of illness and death. Artemisinin, a sesquiterpene-lactone produced mostly in *A. annua* is a highly potent antimalarial agent and part of the Artemisinin Based Combination Therapy (ACT). ACT has been recommended by the World Health Organization as the most efficient means to treat malaria and reduce its transmission rate (Mutabingwa, 2005). Nevertheless, low production of artemisinin in *A. annua* is a severe restriction to meet the worldwide demand for ACT. Thus, considerable effort has been dedicated to increase the production of artemisinin content in the whole plant of *A. annua* (Xiao, Lv, Tan, Z, & Z, 2017). Some progress has been made by the Centre for Novel Agricultural Products (CNAP), University of York, UK under the "Artemisia research project whose key objective is to tackle malaria with fast track plant breeding and to ensure the

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availability of artemisinin with lower production costs for ACT. Out of 268 hybrids were selected for field trials (Townsend et al., 2013). Two were identified on the basis of artemisinin content, reliable field performance, and seed productivity. These hybrids have been unconfined commercially, and are named Hyb1209r (Shennong, artemisinin 1.5%) and Hyb8001r (Zenith, artemisinin 1.4%) (Townsend et al., 2013). Phytochemical analysis has also revealed the presence of other valuable compounds in *A. annua* such as phenolics, flavonoids, steroids, purines, coumarins, lipids, monoterpenoids, triterpenoids, and aliphatic compounds (Cafferata, Gatti, & Mijailosky, 2010; Ferreira, Luthria, Sasaki, & Heyerick, 2010). Hence, besides antimalarial affects, *A. annua* is a source of metabolites with other biological activities such as anti-inflammatory, antitumor, antihypertensive, anticancer, antimicrobial, and anti-oxidant activities (Das, 2012).

Among different approaches, genetic engineering has been the most appropriate option to improve secondary metabolite production by directly modifying the biosynthetic pathways involved in the production of secondary plant compounds (Benedito & Modolo, 2014). In this context, *rol* genes of *A. rhizogenes* are regarded as potent inducers of secondary metabolism in various plants (Bulgakov, 2008). The *rolA* gene, a DNA binding protein is involved in the stimulation of growth. The *rolB* gene is reported to be capable of regulating the signal transduction pathway of auxin due to its tyrosine phosphatase activity (Filippini, Schiavo, Terzi, Costantino, & Trovato, 1994), and the *rolC* gene has cytokinin glucosidase activity (Estruch, Chriqui, Grossmann, Schell, & Spena, 1991). However, each gene appears to have its own effect on plant morphology and stimulates the production of different secondary metabolites.

Here we evaluated the effect of transformation of *A. annua* (Hyb1209r, Shennong) with *rol* genes (*rolB* and *rolC*) on the content of artemisinin and other important phytochemicals using HPLC methods. As these genes have been proven to be effective in increasing the flavonoids and artemisinin concentration in other species of *Artemisia* (Dilshad et al., 2016; Dilshad, Cusido, Estrada, Bonfill, & Mirza, 2015a). The effect of transgenes on phenolic and flavonoid content and anti-oxidant activities, and the relationship between the expression of transgenes and secondary metabolite concentration were also investigated through different biological assays.

2. Materials and methods

2.1. Transformation and molecular analysis to confirm transgene integration and expression

Seeds of *A. annua* Hyb1209r (Shennong) were obtained from East West Seeds International Company (Thailand), seed germination and transformation was carried out by an earlier reported method (Dilshad et al., 2015a; Dilshad, Cusido, Palazon, Estrada, Bonfill, & Mirza, 2015b). PCR analysis for transgenes integration and their expression by reverse transcriptase was carried out as reported previously (Dilshad et al., 2015a, b).

2.2. Quantitative analysis

2.2.1. Determination of total phenolic content (TPC)

The TPC was evaluated by following a previously described method (Ul-Haq et al., 2012). Plant extracts were tested at 4 mg/mL dimethyl sulfoxide (DMSO). The results were expressed as gallic acid equivalents. DMSO was run as negative control.

2.2.2. Determination of total flavonoid content (TFC)

The TFC in test samples was assessed by applying the aluminum chloride colorimetric method (Kiani, Safdar, Mannan, & Mirza, 2012; Ul-Haq et al., 2012) with slight modifications. Plant extracts were tested at the concentration of 4 mg/mL DMSO. Quercetin and DMSO were employed as positive and negative controls, respectively. TFC was expressed as quercetin equivalents.

2.2.3. Polyphenols quantification by HPLC–DAD

HPLC-DAD analysis was carried out to quantify polyphenols in transformed and wild type *A. annua* plant extracts, and HPLC-DAD as well as TLC analysis were performed to quantify artemisinin.

Dry extracts of all samples were dissolved in methanol to obtain a concentration of 10 000 µg/mL. Stock solutions (1000 µg/mL) of standards including rutin, catechines, myricetin, apigenin, gallic acid, caffeic acid, kaempferol, and quercetin were prepared in methanol. Using these stock solutions, serial dilutions of 10, 20, 50, 100, 150, and 200 µg/mL were prepared for the standard calibration curve.

Chromatographic analysis was carried out using the HPLC system with C_{18} analytical column attached to a diode array detector (DAD; Agilent Technologies, Germany), Agilent Chem Station Rev. B.02-01-SR1 (260), and an Agilent 1200 series binary gradient pump. Injection volume was 20 µL and the flow rate was maintained at 1 mL/min. Samples were filtered via 0.45 µm membrane filters before injection into the column. Mobile phase A contained methanol-acetonitrile-acetic acid-water with a ratio of (10:5:1:85) and mobile phase B contained methanol-acetonitrile-acetic acid with a ratio of (60:40:1). In gradient method volume of B was 0%–50% in 0–20 min, then 50%–100% in 20–25 min followed by 100% in 25–30 min. Absorptions of the samples were recorded at different wavelengths: 257 nm (rutin), 279 nm (catechin, gallic acid), 325 nm (apigenin, caffeic acid), and 368 nm (kaempferol, myricetin, quercetin). HPLC analysis was performed at ambient temperature. For compound identification, UV absorption spectra and retention time of plant extracts were compared with standards. Before starting the next analysis, the column was always reconditioned for 10 min.

2.2.4. Artemisinin quantification through HPLC and TLC (Thin layer chromatography)

The samples for artemisinin quantification were prepared at a concentration of 10 mg/mL in acetonitrile. One mg of standard reference artemisinin (Sigma-Aldrich, UK) was also dissolved in 1 mL of acetonitrile.

Artemisinin was analyzed by the isocratic method (Kiani et al., 2012) with some modifications. Simply, the mobile phase was prepared by mixing acetonitrile/H_2O (1:1). The mobile phase was run from 0 to 30 min isocratically and the flow rate was 1 mL/min. Injection volume was 20 µL and detection wavelength for artemisinin was 210 nm. After each complete run, the column was washed for 20 min. Artemisinin was quantified by the given formula:

Artemisinin (µg/mL) = (Concentration of standard/Area of standard) × Area of sample

For TLC analysis, plant samples were prepared at the concentration of 100 mg/mL in the acetonitrile. Standard artemisinin at the concentration of 1 mg/mL was run as a positive control. Plant samples along with standard artemisinin were analyzed on 3.5 mm reverse phase TLC plate and quantification was done using JUST TLC software. The mobile phase for this analysis consisted of methanol and acetonitrile in the ratio of 6:4.

2.3. Determination of anti-oxidant potential

2.3.1. Free radical scavenging activity

Free radical scavenging activity of all extracts at three different concentrations of 500, 250, and 125 µg/mL was evaluated by measuring the discoloration of purple-colored 2,2, diphenyl-1-picrylhydrazyl (DPPH; 9.6 mg/100 mL methanol) solution, following

the standard method (Bibi, Ullah, Mannan, & Mirza, 2011). The assay was performed in triplicate and the positive control employed was ascorbic acid. The negative control was the reagent solution without a sample.

2.3.2. Determination of total reducing power by potassium ferricyanide colorimetric assay

The reducing power of the test samples was determined by following the previously reported protocol (Ul-Haq et al., 2012). Plant extracts were used at the concentration of 4 mg/mL DMSO. Ascorbic acid was employed as the reference standard. The reducing power results were expressed as ascorbic acid equivalents after triplicate analysis.

2.4. Statistical analysis

All experiments were performed in triplicate. Results of phytochemical analysis and anti-oxidant assays were presented as mean ± standard deviation and their statistical significance was determined by the *t*-test ($^{*}P < 0.05$; $^{**}P < 0.01$; or $^{***}P < 0.001$). IC_{50} values were calculated by Table Curve 2D Ver.4 software. The data obtained for HPLC analysis of polyphenols and DPPH free radical assay were analyzed statistically by two way ANOVA analysis using GraphPad Prism Ver.5.0 software.

3. Results and discussion

3.1. Transformation and characterization of transgenic lines

A. tumefaciens strain GV3101 containing *rolB* and *rolC,* was used to transform *A. annua* nodal explants. One hundred explants were infected for each gene in the transformation experiment. Each shoot from a single explant was subcultured every two weeks on fresh selection medium gradually reducing the quantity of cefotaxime. After three months, putative transgenic plants were subjected to PCR analysis, which showed 779 bp long amplified product for *rolB* and 540 bp for *rolC*, whereas no amplified product was detected in the genome of the wild type plant (Fig. 1A and B). Transformation efficiency was found to be 32% for *rolB* and 28% for *rolC* transformed plants on the basis of plants presenting PCR positive results with respect to a total number of co-cultivated explants. Three transformants of *rolB* and three of *rolC* were analyzed. Reverse-transcriptase PCR analysis verified transgene expression at the transcriptional level in all transgenic shoots, although expression varied among the lines (Fig. 1C and D). Clear morphological differences were observed between transformed and control plants. Transgenic plants showed stunted growth and were shorter than control plants. Leaves of transformed plants were very narrow and small, while broad leaves were observed in control plants. Stems of transgenic plants were hard in texture, in comparison with soft texture in control plants. More branching was observed in transformed plants (Fig. 2). However, this did not influence their overall growth rate. The *rol* genes could be a possible cause of morphological changes because they have been documented to cause hormonal imbalance. For example, increased tyrosine phosphatase activity of *rolB* causes enhanced sensitivity toward auxin in transformed cells, which can result in the altered phenotype of *rolB* transformants (Maurel et al., 1994). The *rolC* gene in transgenic plants causes an imbalance in cytokinin/auxin ratio in support of cytokinins (Schmülling, Fladung, Grossmann, & Schell, 1993). The decreased height of transgenic plants is consistent with the experiential phenotype of transgenic plants harboring *rolC*, illustrated mainly by reduction of plant height and lessening of apical dominance (Estruch et al., 1991; Schmülling et al., 1993). Shorter internodes and lanceolate leaves with wrinkly boundaries have been observed in *rolC* transformed plants in previous studies (Oono, Kanaya, & Uchimiya, 1990; Schmülling, Schell, & Spena, 1988). The decreased plant height and narrow leaves of transformed plants in the current study are contradictory to the increased height and broad leaves observed in an *A. annua* plant transformed with combined *rol ABC* genes (Bushra Hafeez, 2012), possibly due to a combined effect of *rol* genes.

3.2. Total phenolic and flavonoid content

The total phenolic and flavonoid content of transformed and untransformed plants of *A. annua* are presented in terms of gallic acid equivalent (GAE) and quercetin equivalent (QE), respectively. In the wild type plant extract, 30.9 mg GAE/g DW (dry weight) of the plant was quantified, and the increased phenolic content was observed in transformed plants compared to the wild type. The *rolB* transgenic lines showed an average 35.7% increase in phenolic content, which was the highest in TB3 (42.51 mg GAE/g DW) followed by TB1 (42.4 mg GAE/g DW) and TB2 (39.7 mg GAE/g DW). In *rolC* transgenics, an average increase of 25.8% was observed, with the highest phenolic content in TC2 (40.18 mg GAE/g DW) followed by TC1 (38.87 mg GAE/g DW) and TC3 (37.77 mg GAE/g DW)

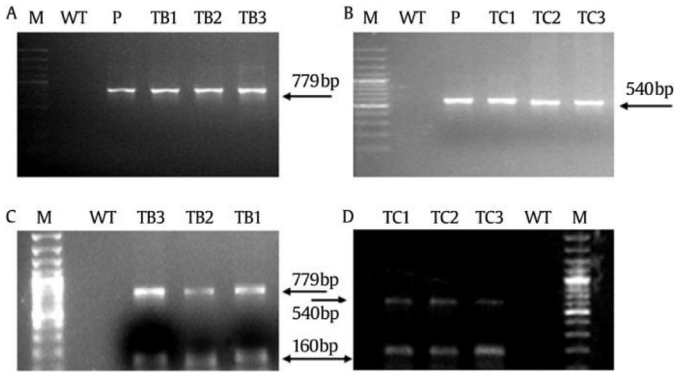

Fig. 1. Conventional PCR amplified products of *rol* B (A) and *rol* C (B), and relative expression of *rol* B (C) and *rol* C (D) by semi quantitative reverse transcriptase-PCR (amplification of β-actin 160 bp as internal control).
Note: TB1–TB3 represent *rolB* transgenic lines and TC1–TC3 represent *rolC* transgenic lines. WT indicates wild type plant, same as below.

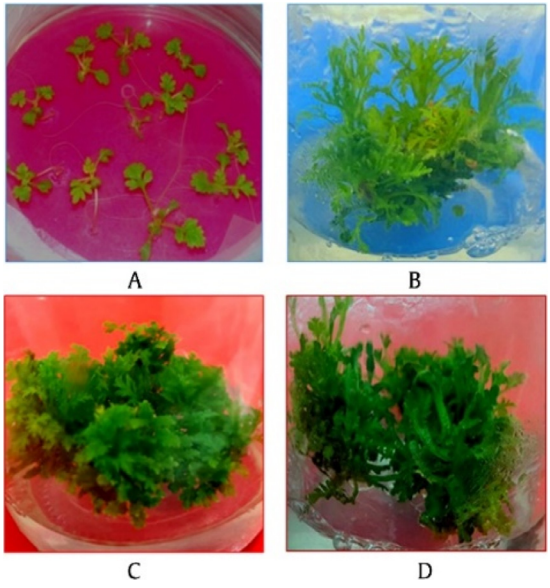

Fig. 2. Tissue culturing of *A. annua* plant: *in vitro* seedlings growth of wild type (A), regeneration of wild type (B), transformant of *rol B* (C), and transformant of *rol C* (D).

(Fig. 3A). The results of total flavonoid content were synchronous with those of the total phenolic content. A total of 9.12 mg QE/g DW was found in the wild type plant extract. In *rolB* transgenic lines, an average enhancement of 30.3% in TFC was observed. The highest flavonoid content was found in the transgenic line TB3 (12.4 mg QE/g DW), followed by TB1 (12.02 mg QE/g DW) and TB2 (11.2 mg QE/g DW). In *rolC* transformants, line TC2 showed the highest flavonoid content (11.95 mg QE/g DW), followed by TC1 (11.4 mg QE/g DW) and TC3 (10.9 mg QE/g DW) (Fig. 3B). The individual *rol* genes of *A. rhizogenes* are potential inducers of secondary metabolism in transformed plant cells and boost the biosynthesis of secondary metabolites (Bulgakov, 2008). Our recent report also revealed that the *rolB* transformation of another hybrid of *A. annua* (Hyb8001r, Zenith) led to a 1.5-fold increase in total phenolics and flavonoid content and the *rolC* transformants showed a 1.4-fold enhancement (Dilshad et al., 2016). Similarly, the transformation of combined *rolABC* genes in *A. dubia* resulted in an average increase of 57.8% and 60.1% in TFC and TPC, respectively (Kiani et al., 2015). Our results are also in accordance with a previous study, where the transformation of tomato with *rolB* led to an 11%–58% increase in TPC (Arshad et al., 2014). Plant phenolics have anti-oxidant properties and play a major role in fighting oxidative stress, cytotoxicity, and cell death by chelating trace elements or quenching free radicals, thereby strengthening the anti-oxidant defenses (Kumar et al., 2013). Likewise, in addition to the roles of flavonoids in plant defense, their function as anti-oxidant compounds has also been revealed (Kubo et al., 1999). Therefore, the increase in flavonoid and phenolic content after the introduction of *rol* genes might contribute significantly toward enhancing the anti-oxidant potential of the transformed *A. annua* plant.

3.3. Quantification of polyphenols and artemisinin

HPLC was used for the quantification of polyphenols and artemisinin in transformed and untransformed plants, as it is the preeminent method for chemical profiling of plant extracts (Springfield et al., 2005) and TLC was used for the quantification of artemisinin. For polyphenol quantification, the reverse phase HPLC profile of methanolic extracts of transformed and control plants were compared with the absorption spectrum and retention time of standard compounds (rutin, catechins, myricetin, apigenin, gallic acid, caffeic acid, kaempferol, and quercetin). Rutin, caffeic acid, and myricetin were detected in transformed and control plants. The amount of these polyphenols was higher in transgenic lines compared with control, while catechins, apigenin, gallic acid, kaempferol, and quercetin were not present in either the transformed or untransformed plant extracts. In the wild type plant extract rutin (0.73 mg/g DW), caffeic acid (0.68 mg/g DW), and myricetin (19.9 mg/g DW) were detected (Fig. 4A). Enhanced levels of these polyphenols were observed in all *rolB* and *rolC* transgenic lines. In *rolB* transgenics, average increases were of 321% in rutin, 97.2% in caffeic acid, and 218.4% in myricetin; and in *rolC* transgenics, 197.5% in rutin, 76.3% in caffeic acid, and 209.3% in myricetin. Increased levels of rutin, caffeic acid, and myricetin could be attributed to the presence of *rol* genes. Transformation of another hybrid of *A. annua* (Hyb8001r, Zenith) with *rolB* resulted in increases in rutin and caffeic acid (3-fold), quercetin (4-fold), and isoquercetin (6-fold); *rolC* transformation led to a 3-fold increase in rutin and quercetin, 2.6-fold increase in caffeic acid, and 5-fold increase in isoquercetin (Dilshad et al., 2016). Statistical analysis was performed by ANOVA and the effect of transgenic lines and polyphenols was found to be highly significant (Table 1). Similar changes were observed in the flavonoid content of *Artemisia carvifolia* when transformed with *rolB* and *rolC* genes (Dilshad et al., 2016). A 100-fold increase in resveratrol production was observed in *rolB*-transformed *Vitis amurensis* (Kiselev et al., 2007). It has been reported that *rolC* alone has a stimulatory effect on the production of tropane alkaloids and ginsenosides (Kiselev et al., 2007; Bulgakov et al., 1998). Similarly, it has been reported that transformation of *A. dubia* by *rol ABC* genes resulted in an increased level of caffeic acid in transformed shoots (0.08%) compared to control shoots (0.01%) (Kiani et al., 2015). All the detected phytochemicals are very important on the basis of their reported medicinal properties; For instance, rutin shows strong anti-oxidant, antidiabetic, anticancerous, and anti-adepogenic activities (Chua, 2013). Caffeic acid has strong anti-oxidant properties and also acts as an immune and inflammatory reducer (Kim & Lee, 2004; Jafri et al., 2014). Myricetin is also a strong anti-oxidant and has various pharmacological applications such as anti-inflammatory, anti-carcinogenic, and anti-atherosclerotic activity and it is also effective against retroviral infections (Ong & Khoo, 1997).

For artemisinin quantification in wild type and transformed plant extracts, UV spectra and retention time of artemisinin were used as a reference. All the transgenic lines showed a considerable increase in their artemisinin content; Among *rolB* transgenic lines, TB3 exhibited the maximum increase of 28.6% (4.94 mg/g DW), while lines TB1 and TB2 showed an increase of 22.3% (4.7 mg/g DW) and 14.3% (4.39 mg/g DW), respectively. In the *rolC* transgenic lines, the highest relative increase in artemisinin (12.7%) was observed in TC2 (4.33 mg/g DW) followed by TC1 (4.31 mg/g DW; 12.2%) and TC3 (3.95 mg/g DW; 2.8%) compared with the control (3.84 mg/g DW) (Fig. 4B).

An increase in artemisinin was also observed through TLC analysis, which was on average 142.2% in the *rolB* transgenic lines and 110.2% in the *rolC* transgenic lines relative to the wild type. The TLC fingerprint was shown in Fig. 5A and numerical values

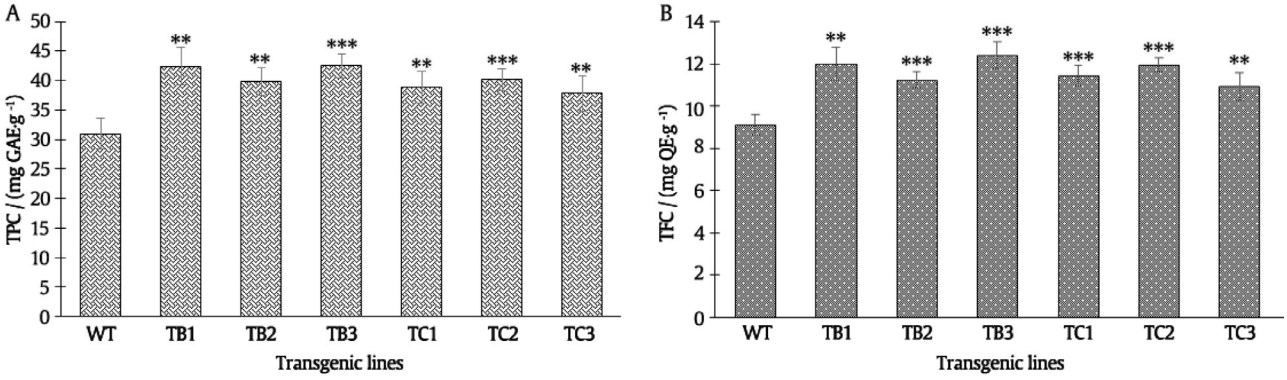

Fig. 3. Comparative analysis of total phenolic content (A) and total flavonoid content (B) of transformed and wild type plants of *A. annua*. (***$P < 0.001$ and **$P < 0.01$ *vs* WT group).

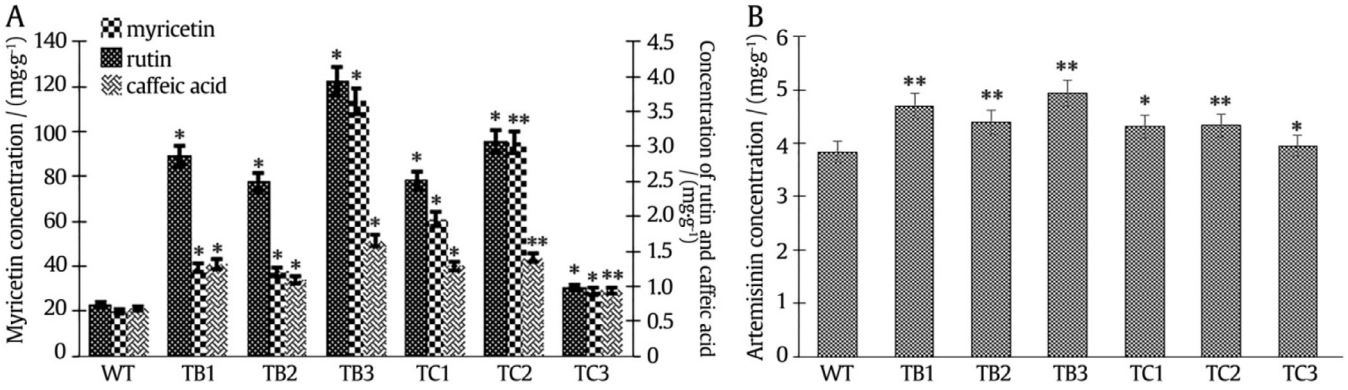

Fig. 4. HPLC analysis for quantification of some important polyphenols (A) and artemisinin (B) in transformed and wild type plants of *A. annua*. ($^{**}P < 0.01$ and $^{*}P < 0.05$ *vs* WT group).

Table 1
Analysis of variance for HPLC analysis of polyphenols.

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F* value	*P* value
Transgenic lines	6.0	8407	1401	560.18	< 0.0001
Polyphenol standards	2.0	40790	20390	8152.97	< 0.0001
Interaction (AXB)	12.0	14150	1179	471.44	< 0.0001
Residual (error)	42.0	105.1	2.501		
Total	62.0	63450			

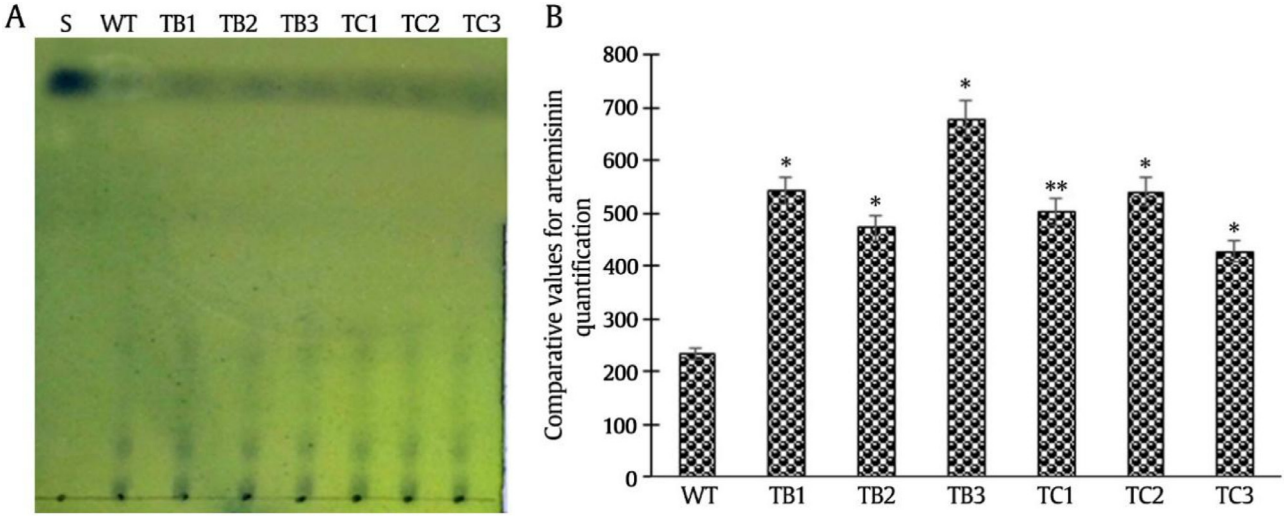

Fig. 5. (A) Comparative analysis of TLC fingerprints (*S* represents standard artemisinin) and (B) comparative artemisinin quantification in transformed and wild type plants of *A. annua*. ($^{**}P < 0.01$ and $^{*}P < 0.05$ *vs* WT group).

after comparative quantification of particular spots through TLC software were shown in Fig. 5B.

Previously, it is reported that *rol* genes (*rolA, rolB*, and *rolC*) when expressed individually or combined result in increased plant secondary metabolism by transcriptional activation of defense genes through an unknown mechanism (Shkryl et al., 2008). The increase in the level of artemisinin can be clearly correlated with the introduction of *rol* genes. We previously reported the increase of 3–7 fold and 2.3–6 fold in the artemisinin content in *rolB* and *rolC* transgenics of *A. carvifolia*, respectively (Dilshad et al., 2015a). In the other hybrid of *A. annua* (Hyb8001r, Zenith) evaluated recently, the increase in artemisinin in *rolB* transgenics was 2–9 fold and 4 fold in *rolC* transgenics (Dilshad et al., 2015b). Our findings were also supported by another study that *rolABC* genes in transgenic *A. dubia* roots led to a 10-fold increase in artemisinin compared with control roots (Kiani et al., 2012).

It has also been reported that *rolB* transformed shoots of tomato showed an 18%–62% increase in lycopene content compared with control shoots (Arshad et al., 2014). It has been suggested that flavonoids could act as artemisinin synergists and facilitate artemisinin interaction with heme by converting Fe^{+3} to Fe^{+2} and thus providing more effective treatment for malaria in combination with artemisinin (Liu et al., 1992). Myricetin is reported to promote the inhibition of the parasitic enzyme thioredoxin reductase and therefore may work in synergism with artemisinin against *Plasmodium falciparum* (Bilia et al., 2008). In our study, the highest increase in myricetin (469%) relative to the control was observed in *rolB* transgenic shoots of TB3. Thus, enhanced levels of myricetin

Table 2
Analysis of variance for DPPH radical scavenging activity.

Source of variation	Degrees of freedom	Sum of squares	Mean square	*F* value	*P* value
Transgenic lines	6.0	2100	350	250.59	< 0.0001
Concentrations /(μg ·mL^{-1})	2.0	14280	7139	5111.92	< 0.0001
Interaction (AXB)	12.0	747.4	62.29	44.60	< 0.0001
Residual (error)	42.0	58.65	1.396		
Total	62.0	17180			

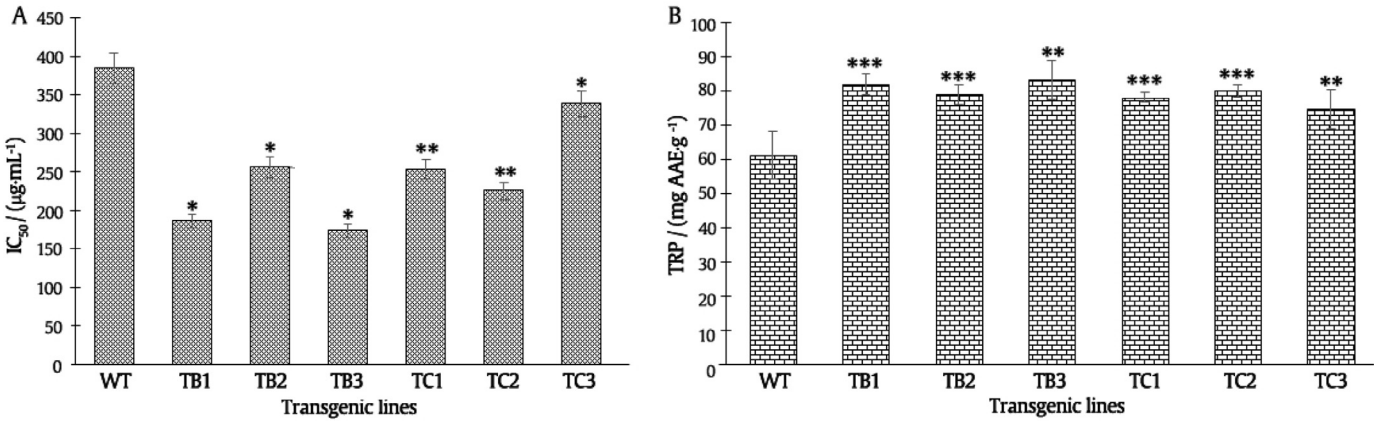

Fig. 6. Comparative analysis of DPPH free radical scavenging activity (A) and total reducing power (B) of transformed and wild type plants of *A. annua*. ($^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$ *vs* WT group).

and other flavonoids due to the introduction of *rol* genes may be helpful for artemisinin in the fight against malaria.

3.4. Anti-oxidant potential

The anti-oxidant capacity of any sample cannot be completely assessed by a single method (Yıldırım et al., 2000). Therefore, the anti-oxidant potential of transformed and untransformed plants extracts was evaluated by a DPPH free radical scavenging assay and reducing power assay in the present investigation. Through the DPPH assay, it was determined that free radical scavenging activity was higher in all transgenic lines compared with the wild type. The wild type plant showed an IC_{50} value of 384.2 μg/mL; Among the *rolB* transformants, line TB3 showed the maximum relative increase in anti-oxidant activity (54.6%) with an IC_{50} value of 174.2 μg/mL, followed by TB1 ($IC_{50} = 186.3$ μg/mL) and TB2 ($IC_{50} = 255.8$ μg/mL); In *rolC* transformants, TC2 showed the highest increase in anti-oxidant activity (41.3%) with an IC_{50} value of 225.2 μg/mL, followed by TC1 ($IC_{50} = 253.5$ μg/mL) and TC3 ($IC_{50} = 338.2$ μg/mL) (Fig. 6**A).** The lower the IC_{50} value is, the higher the free radical scavenging activity is. Each line showed its individual behavior in scavenging free radicals. The average IC_{50} value for *rolB* and *rolC* transgenics of *A. annua* (Hyb8001r, Zenith) was found to be 130 μg/mL and 152 μg/mL, respectively, compared to 274 μg/mL of the wild type plant (Dilshad et al., 2016). The data was analyzed statistically by ANOVA and the effect of transgenic lines and concentration was found to be highly significant (Table 2). Our findings were consistent with a previous study, where the transformation of *A. dubia* with *rol ABC* gene resulted in a lower IC_{50} value in the transformed shoot (131.6 μg/mL) compared with control shoots (257.2 μg/mL) (Kiani et al., 2015).

The reducing power of methanolic extracts of transgenic and wild type plants was determined in terms of ascorbic acid equivalent (AAE). An increase in the reducing power value was observed in transgenic lines compared with the wild type. In *rolB* transformants, the highest reducing power was shown by TB3 (83.28 mg AAE/g DW) followed by TB1 (81.94 mg AAE/g DW) and TB2 (79.03 mg AAE/g DW), and in *rolC* transfromants, it was shown by TC2 (80.02 mg AAE/g DW) followed by TC1 (78.18 mg AAE/g DW) and TC3 (74.77 mg AAE/g DW) compared to 61.32 mg AAE/g DW of the wild type plant (Fig. 6**B).** Reducing power can be a significant indicator of the potential anti-oxidant activity of plant extracts. A direct relationship between free radical scavenging activity and reducing power has been reported in different plant extracts. The increase in anti-oxidant activity in the transformed plants can be related to the presence of *rolB* and *rolC* genes. Similarly, the transformation of *Rubia cordifolia* calli with the *rolB* gene resulted in improved anti-oxidant activity and suppressed the level of reactive oxygen species (ROS) (Shkryl et al., 2010). Our findings are also in accordance with the report describing the suppression of ROS as a result of higher anti-oxidant activity in *rolC* transformed *R. cordifolia* cells (Bulgakov et al., 2008). The results of anti-oxidant capacity reported here indicate that an increase in anti-oxidant activities of transformed lines is consistent with the relative increase in total phenolic and total flavonoid content.

In this study, a correlation was observed between the enhancement of secondary metabolites and expression of *rol* genes. For example, line TB3 showed the highest increase in all phytochemicals and anti-oxidant activities, which is consistent with the detection of more *rolB* transcripts in this line; The less enhancement found in TB2 and TC3 can be attributed to a less expression of the gene. Our findings are supported by previous work showing a strong correlation between the expression of farnesyl pyrophosphate synthase (FPS) and accumulation of artemisinin in *A. annua* (Banyai et al., 2010). Similar findings were observed in another study where overexpression of *hmgr* and *ads* in transgenic lines resulted in higher artemisinin content in *A. annua* (Alam & Abdin, 2011).

4. Conclusion

The findings suggested that the expression of *rolB* and *rolC* genes could be an effective strategy for enhancing the content of artemisinin and other important phytochemicals in *A. annua*. We also reported the possible role of *rol* genes in improving the anti-oxidant potential of the plant. Accumulation of secondary metabolites and improvement of anti-oxidant activities in all transgenics

correlated with the expression level of *rolB* and *rolC* genes. The expression of the *rolB* gene modulated the secondary metabolite activation to a slightly greater extent than the *rolC* gene. Taken together, these results can contribute to the development of new transgenic varieties of *A. annua* with an enhanced level of phytochemicals and improved pharmacological activities.

Conflict of interest

The authors declare no conflict of interest.

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