Enhanced artemisinin yield by expression of *rol* genes in *Artemisia annua.* (Malaria J)

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I. ABSTRACT

Despite of many advances in the treatment of malaria, it is still the fifth most prevalent disease worldwide and is one of the major causes of death in the developing countries which accounted for 584,000 deaths in 2013, as estimated by World Health Organization. Artemisinin from *Artemisia annua* is still one of the most effective treatments for malaria. Increasing the artemisinin content of *A. annua* plants by genetic engineering would improve the availability of this much-needed drug. In this regard, a high artemisinin-yielding hybrid of *A. annua* produced by the centre for novel agricultural products of the University of York, UK, was selected (artemisinin maximally 1.4 %). As *rol* genes are potential candidates of biochemical engineering, genetic transformation of *A. annua* with *Agrobacterium tumefaciens* GV3101 harboring vectors with *rol* B and *rol* C genes was carried out with the objective of enhancement of artemisinin content. Transgenic lines produced were analyzed by the LC–MS for quantitative analysis of artemisinin and analogues. These high artemisinin yielding transgenics were also analyzed by real time quantitative PCR to find the molecular dynamics of artemisinin enhancement. Genes of artemisinin biosynthetic pathway were studied including amorphadiene synthase (ADS), cytochrome P450, (CYP71AV1) and aldehyde dehydrogenase 1 (ALDH1). Trichome-specific fatty acyl-CoA reductase 1(TAFR1) is an enzyme involved in both trichome development and sesquiterpenoid biosynthesis and both processes are important for artemisinin biosynthesis. Thus, real time qPCR analysis of the TAFR1 gene was carried out and trichome density was determined. Transgenics of *rol* B gene showed 2-9 increase in artemisinin, 4–12-fold increase in artesunate and 1.2–3-fold increase in dihydroartemisinin. Whereas in the case of *rol* C gene transformants, a fourfold increase in artemisinin, four to ninefold increase in artesunate and one- to twofold increase in dihydroartemisinin concentration was observed. Transformants with the *rol* B gene had higher expression of these genes than *rol* C transformants. TAFR1 was also found to be more expressed in *rol* gene transgenics than wild type *A. annua*, which was also in accordance with the trichome density of the respective plant. Thus it was proved that *rol* B and *rol* C genes are effective in the enhancement of artemisinin content of *A. annua*, *rol* B gene being more active to play part in this enhancement than *rol* C gene.

II. METHODS

Plant Identification: Plant was identified through DNA barcoding by using *psbA-trnH* sequence of chloroplast DNA.

Genetic Transformation: *Agrobacterium tumefaciens* GV3101 harboring vectors with *rol* B and *rol* C gene was used for genet ic transformation of *A.annua*.

PCR: Transgenes integration was confirmed by performing PCR with gene specific primers of *rol* B and *rol* C gene.

Semiquantitative reverse transcriptase PCR analysis: Expression of *rol* Band *rol* C gene was confirmed by semiquantitative reverse transcriptase PCR.

Real time qPCR: Expression analysis of artemisinin biosynthetic pathway genes was carried out by real time qPCR.

Qualitative and quantitative analysis of artemisinin and derivatives by LC-MS: Artemisinin and derivatives were detected and quantified by LC-MS analysis of wild typle *A. annua* and *rol* gene transgenics.

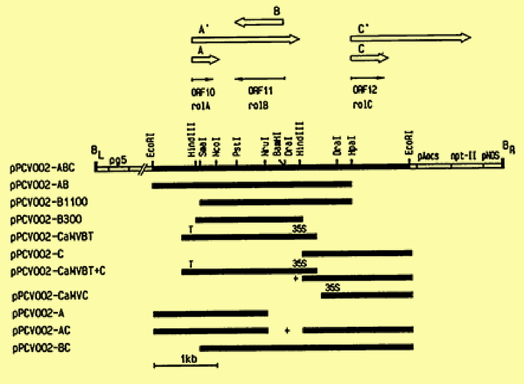
Calculation of trichome density: Number of trichomes of wild type plant and transgenics of *rol* B and *rol* C gene was calculated by using fluorescent microscope with FITC green filters.

III. RESULTS

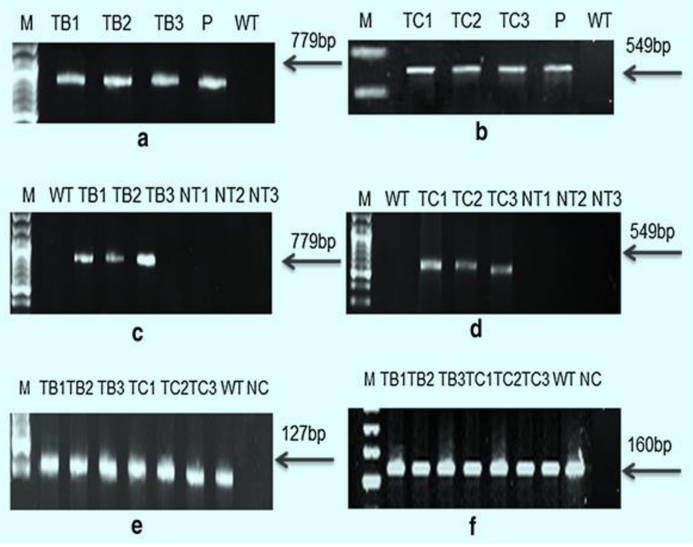
DNA barcoding: *psbA-trnH* sequence of *A. annua*

TGTTATGCATGAACGTAATGCTCATAATTTCCCTCTAGAC
TTAGCTGCTATTGAAGCTCCATCTACAAATGGATAAGAC
TTTGGTCTGATTGTATAGGAGTAGTTTTTGAACTAAAAA
AGGAGCAATAGCTTTCCTCTTGTTTTATCAAGAGGGCGT
TATTGCTCCTTTTTTTATTTAGTACTATTTGGCTTACACA
GTTTCTTTAAAAATATTTTATAGTTTGGTTCGATTCGCGT
GTTTTCTCTTTGTATTCATATTCATTTATATTATAGGTTTG
TATATTCTATTCCAAATTTTTTATGAAGTTTGATTTCCAAT
TCAATTTCAAACCAAAATATATAAAAATTGCATTTTTGCT
TATTTATTACTTTGATAAAAAAGAAATAATATGCTCTTTTT
TATGTTGAGGTAAAAATATAGATAATACTAGATAGATATAT
AGTAGAGGGGCGGATGTAGCCAAGTGGATCAAGGCAGT
GGATTGTGAATC

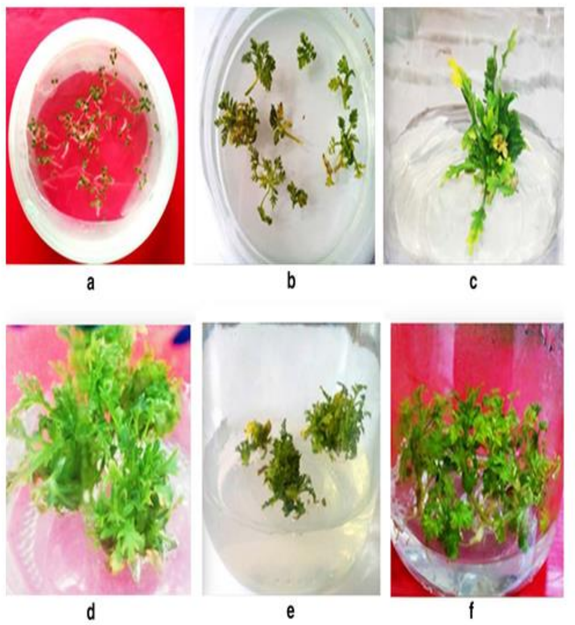
Vectors used for transformation



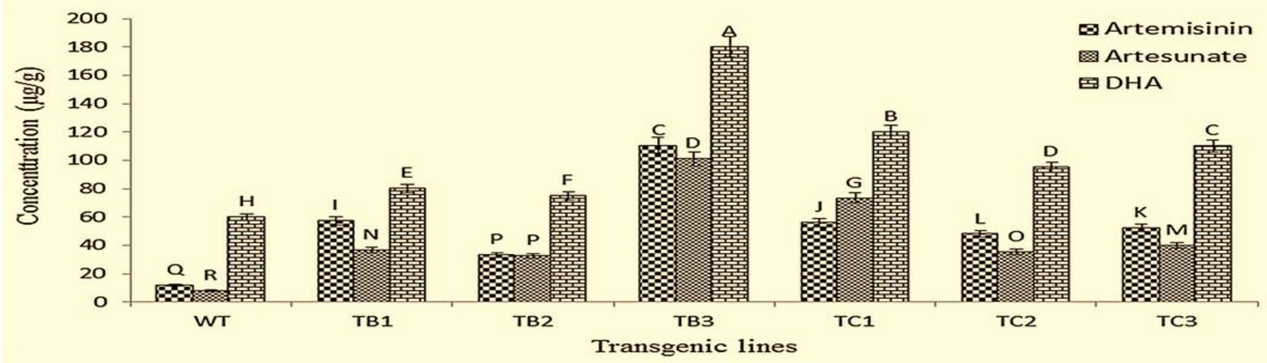
Conventional and semi quantitative reverse transcriptase PCR : 779bp=*rol* B, 540bp=*rol* C. Level of expression of *rol* B (c), and *rol* C gene (d), "e" and "f" show house keeping genes *GADPH* and β-*actin.*



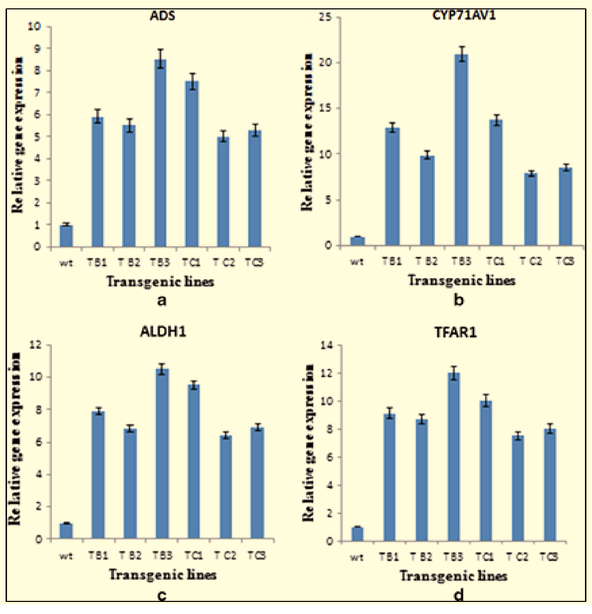
Seeds germination and vegetative propagation of wild type *A. annua* and *rol* gene transgenics.



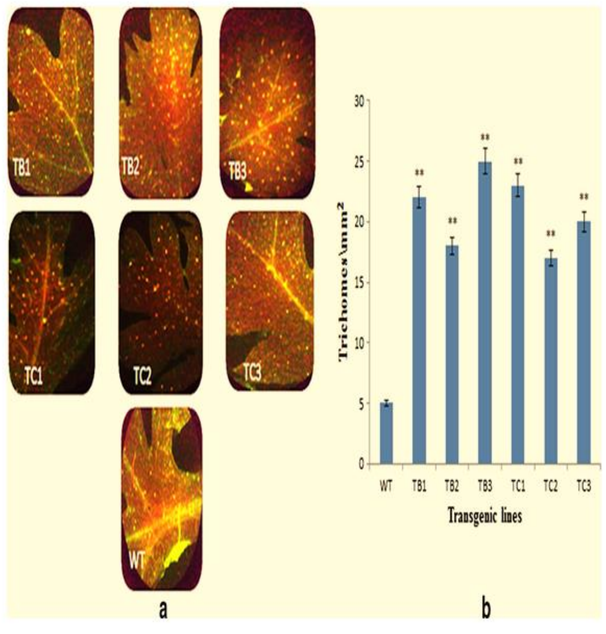
Analysis of artemisinin and derivatives by LC-MS: TB1-TB4 represent the *rol* B transgenic lines whereas TC1-TC3 represent *rol* C transgenic lines. "WT" indicates wild type plant.



Expression analysis of artemisinin biosynthetic pathway genes by real time qPCR.



Calculation of trichrome density of transgenics of *rol* genes and wild type plant of *A. annua*.



IV. CONCLUSION

Transformation of *A. annua* with *rol* B and *rol* C gene results in the enhancement of its secondary metabolites particularly the artemisinin and derivatives, *rol* B gene being more active to play part in the enhancement of artemisinin than *rol* C gene. Further the level of transcripts of the *rol* B and *rol* C gene found in transgenics also correlate with their artemisinin accumulation pattern. Altered expression of genes involved in biosynthesis of artemisinin and trichomes was observed.

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