3 Biotech (2018) 8:485
https://doi.org/10.1007/s13205-018-1496-4

ORIGINAL ARTICLE

Rapid enhancement of α-tocopherol content in *Nicotiana benthamiana* by transient expression of *Arabidopsis thaliana* Tocopherol cyclase and Homogentisate phytyl transferase genes

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Received: 13 June 2018 / Accepted: 1 November 2018 / Published online: 15 November 2018

Abstract

Agrobacterium-mediated transient gene expression have become a method of choice over stable plant genetic transformation. Tocopherols are a family of vitamin E compounds, which are categorized along with tocotrienols occurring naturally in vegetable oils, nuts and leafy green vegetables. This is the first report involving *AtTC* and *AtHPT* transient expression in *Nicotiana benthamiana* and this system can be used efficiently for large scale production of vitamin E. Agroinfiltration studies were carried out in *N.benthamiana* for the expression of *Arabidopsis thaliana (At)* genes encoding homogentisate phytyltransferase (*HPT*) and tocopherol cyclase (*TC*) individually and in combination (*HPT* + *TC*). The transgene presence was analyzed by reverse transcription PCR, which showed the presence of both the vitamin E biosynthetic pathway genes. The gene expression analysis was carried out by (reverse transcription quantitative real-time polymerase chain reaction) RT-qPCR and α-tocopherol content was quantified using high performance liquid chromatography (HPLC). The relative gene expression analysis by RT-qPCR confirmed an increased expression pattern where *TC* + *HPT* combination recorded the highest of 231 fold, followed by *TC* gene with 186 fold, whereas the *HPT* gene recorded 178 fold. The α-tocopherol content in leaves expressing *HPT, TC*, and *HPT* + *TC* was increased by 4.2, 5.9 and 11.3 fold, respectively, as compared to the control. These results indicate that the transient expression of *HPT* and *TC* genes has enhanced the vitamin E levels and stable expression of both *A. thaliana* genes could be an efficient strategy to enhance vitamin E biosynthesis in agricultural crop breeding.

Keywords Transient expression · *Nicotiana benthamiana* · Vitamin E · Tocopherol biosynthesis · Gene expression

Introduction

Four distinct types—alpha (α), beta (β), gamma (γ), and delta (δ) tocopherols appear in nature due to the presence of number of methyl substituents at different positions in the chromanol ring. Out of the four, two (β and δ) are scarce in plants, whereas α-tocopherol present in the leaf tissues and γ-tocopherol accumulate in seeds (Grusak and DellaPenna 1999; Desel et al. 2007). Research in the past decades decoded the genes involved in the tocopherol biosynthesis (Shintani and DellaPenna 1998; Collakova and DellaPenna 2001). The prenylation of homogentisic acid (HGA) with phytyl diphosphate is a crucial step in the pathway, wherein homogentisate phytyl transferase (*HPT*) is the catalyst (Collakova and DellaPenna 2001). Both 2-methyl-6-phytyl benzoquinol (MPBQ) and 2,3-dimethyl-6-phytyl-1,4,-benzoquinol (DMPBQ) are the primary intermediates that act as substrates for tocopherol cyclase (*TC*) results in δ-tocopherol and γ-tocopherol (Shintani et al. 2002; Porfirova et al. 2002). Tocopherol cyclase (*TC*) performs a cyclization reaction with DMPBQ or MPBQ as substrate regardless of the methyl group on the aromatic ring (Stockert et al. 1996). This enzyme plays a key role in the formation of the chromanol ring structure of the tocopherols by the addition of oxygen heterocycle generated next to the aromatic ring produced from homogentisate. This chromanol ring formation determines their free radical scavenging function. Porfirova et al. (2002), suggested that *AtTC* catalyses the cyclization of 2,

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s13205-018-1496-4) contains supplementary material, which is available to authorized users.

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3-dimethyl-5-geranylgeranyl-1, 4-hydroquinone (DMGQ), to synthesize γ–tocotrienols, which indicates the existence of an enzyme common in the biosynthesis of vitamin E homologues. The *HPT* was shown to be a rate limiting enzyme in tocochromanol synthesis in *Arabidopsis* leaves and seeds, as overexpression of *HPT* increased the tocopherol levels (Collakova and DellaPenna 2001, 2003a). The identification of genes involved in the vitamin E biosynthetic pathway in recent decades has enabled researchers to facilitate their characterisation and to find the regulatory relationships (Kumar et al. 2005). Using metabolic and genetic engineering strategies, studies have been carried out to elevate the vitamin E levels in many plant species (Cahoon et al. 2003; Li et al. 2010, 2011). Among the vitamin E pathway enzymes, *TC* and *HPT* from various plant sources has been used in stable genetic transformation experiments, where over expression of *AtTC* resulted in sevenfold increase of vitamin E content in leaf tissues (Kanwischer 2005). Kumar et al. (2005) reported an increased vitamin E content of 18% and 28% with *Zea mays TC* and *AtTC*, respectively, in *Brassica napus*. Similarly, overexpression of *AtHPT* resulted in increased vitamin E content of 1.4 and 1.6 fold in *Arabidopsis* (Collakova and Dellapenna 2003a, b; Savidge et al. 2002).

A critical component of molecular biology research in plants involves gene expression in homologous or heterologous systems. A wide range of techniques has been developed to deliver the genes into plants or specific plant organs and to produce transgenic plants through stable or transient expression. Unlike stable transformation, transient system is rapid, as it does not require tissue culture system. Effective utilization of transient gene expression system have been executed successfully to analyse the gene function, characterization, gene silencing and recombinant antibody production (Scofield et al. 1996; Kapila et al. 1997; Vaquero et al. 1999; Negrouk et al. 2005). In this system, the experimental procedures are relatively easy, do not require expensive supplies and equipment and this method has been proved efficient in many different species (Mestre et al. 2000; Wroblewski et al. 2005; Santos-Rosa et al. 2008). Agroinfiltration is a technique to mechanically infuse plant cells with a suspension of *Agrobacterium tumefaciens* directed to deliver a target gene into the cytoplasm or vacuoles for the desired protein. In this system, any plant material can be infiltrated by applying vacuum and the desired protein is expressed transiently within 2–5 days, and also it eliminates the necessity to incorporate the foreign DNA in plant genome as in the case of stable genetic transformation, so its very safe in biosafety aspects. In this study, we demonstrate that the tocopherol biosynthetic pathway genes *AtTC* and *AtHPT* can enhance vitamin E/tocopherol using agroinfiltration mediated transient expression in the heterologous tobacco system.

Materials and methods

Plant material

Nicotiana benthamiana seeds were procured from Tamil Nadu Agricultural University, Coimbatore, India. The seeds were sown in pots containing a commercial mixture of soil-peat in the ratio 1:4 and the plants were germinated in the containment facility maintained at 25 °C with 16 h light/8 h dark photoperiod with a light intensity of 200–400 µmol m^{-2} s^{-1} and 75% relative humidity. The growth of plants was monitored continuously and plants having the optimal developmental stage (4–5 week old) with at least five fully developed true leaves and no visible flower buds were used for agroinfiltration studies.

Expression vectors and agrobacterial strains

The *AtHPT* cDNA (1.2 kb) (GenBank acc. number AT2G18950) procured from *RIKEN*, Japan was received in pDrive vector (Qiagen, Hilden, Germany) and subsequently cloned in a binary vector, pNutKan (Harish et al. 2013b) and the *AtTC* gene (GenBank acc. number NM_119430.5) was included in the pBinAR vector, gifted by Dr. Peter Dormann, Max-Planck-Institute for Molecular Plant Physiology, Potsdam-Golm, Germany (Harish et al. 2013b). The *TC* gene (1.4 kb) was further excised and cloned in pCambia1305.1 using restriction enzymes *Bam*H1, *Kpn*1 for this study. The two binary vectors carrying the gene cassettes (Fig. 1) were mobilized to *A. tumefaciens* LBA4404 (Hoekema et al. 1983) individually by triparental mating (Hooykaas et al. 1984).

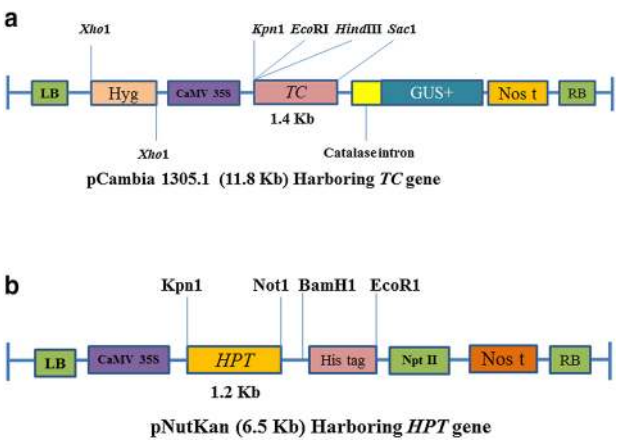

Fig. 1 Schematic representation of binary vectors harbouring the gene cassettes. **a** Binary vector pCambia 1305.1 harboring the gene *TC*; **b** pNUTkan vector harboring *HPT* gene

Agroinfiltration

A single colony of *A. tumefaciens* strain harboring the binary vectors were grown overnight in YEP medium (NaCl 10 g l^{-1}, yeast extract 5 g l^{-1}, peptone 5 g l^{-1}) supplemented with antibiotics (50 mg l^{-1} of kanamycin and 20 mg l^{-1} of rifampicin) in orbital shaker (220 rpm) at 28 °C. Saturated agrobacterial culture was centrifuged and the pellet was resuspended in the infiltration media containing 10 mM 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer, 10 mm $MgCl_2$ with 100 µM Acetosyringone (ACS) at 25 °C for 3–4 h and the final OD adjusted to 0.5–1 at 600 nm. Agroinfiltration was performed as per Garabagi et al. (2012) with slight modification. Vacuum infiltration was performed in *N. benthamiana* plants using vaccum chamber by submerging the whole plant in agrobacterial suspension (Fig S1c). Three plants were used for each experiment. Vacuum was applied at the rate of 4 bar applied for 5 min, which removes the air present in stomata and upon slow release of the vacuum, the agrobacterial solution penetrates through the stomata into the mesophyll due to the pressure difference (Fig S1e). The plant was removed from the chamber after the procedure and placed in the containment facility for 5 days under 25 °C, 16 h light/8 h dark photoperiod with 75% relative humidity. The plants were infiltrated with the two gene constructs individually as well as coinfiltrated followed by harvesting the leaf samples on 5 dpi (days post infiltration).

Gene expression analysis

Total RNA was isolated from the wild type (WT) and Agroinfiltrated leaves 5 dpi using Trizol reagent (Ambion, USA). All the consumables used for the RNA isolation were pretreated with diethyl pyrocarbonate (DEPC) and autoclaved. The samples were ground to a fine powder using liquid N_2 in sterile mortar and pestle. Chloroform (200 µl) was added to 1 ml of trizol and placed the tubes on ice for 5 min. The samples were then spun down at 12,000 rpm for 15 min at 4 °C. Transferred the upper aqueous layers to a clean fresh tube and to this half the volume of ice cold isopropanol was added. The tubes were placed on ice for 15 min followed by centrifugation at 12,000 rpm for 10 min at 4 °C. The supernatant was discarded and 500 µl of ice cold 75% ethanol (v/v) was added to the pellet and spun at 7500 rpm for 5 min. To the pellet, 50 µl of RNase free water was added and incubated at 55 °C in a water bath for 10 min. The concentrations of RNA (ng μl^{-1}) and purity ratios (260/280 nm and 260/230 nm) were analyzed using Nanodrop spectrophotometer 2000 (Thermo Scientific, USA). First strand cDNA was synthesized from 1 µg of total RNA using high capacity RNA to cDNA kit (PrimeScript™ 1st strand cDNA Synthesis Kit, Takara, Japan). A reverse transcription PCR was performed with *HPT, TC* and corresponding internal reference standard (F-box) (Liu et al. 2012) specific primers (Table 1) to check the integrity of the cDNA. The reaction was performed with 1 µl cDNA template with initial denaturation at 95 °C for 10 min followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, extension at 72 °C for 1 min and the final extension at 72 °C for 10 min. The PCR products were then electrophoresed on a 1.2% agaorse gel. Quantitive gene expression of both *HPT* and *TC* was analysed using Real-Time PCR system (Applied Biosystems, USA). RT-qPCR was carried out in a 20 µl reaction volume containing 1 µl of cDNA template, 5 µM of each primer and 10 µl of SYBR Green real-time PCR master mix (SYBR® Premix Ex Taq™, Takara, Japan). The experiments were performed in biological triplicates for each sample. F-box gene was used as a reference and the relative expression ratio was calculated using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008). The RT-qPCR reactions were carried out with initial denaturation at 95 °C for 30 s, denaturation at 95 °C for 5 s, followed by 40 cycles of annealing and extension at 60 °C for 34 s. The data acquisition was done at the end of each extension cycle. The gene-specific PCR primers are listed in Table 1.

Table 1 The gene-specific primers for reverse transcription PCR and RT-qPCR

Reverse transcription PCR experiments	
TC-F	AGCTGGTACCTATGGAGATACGGAGCTTGATTGTTT
TC-R	GACTTCTAGAGTTACAGACCCGGTGGCTTGAAGAAA
HPT-F	CGC GTG GTA CCT CGA GTG AGT CTC TGC TCT CTA GTT CTT C
HPT-R	CGT CGT GAG CTC CAG TCA CTT CAA AAA AGG TAA C
RT-qPCR experiments	
qTC-F	GTCTCCCTGGGGTTATTGGT
qTC-R	GCCCAACTTCTGTGGTAGGA
qHPT-F	ATCTTGGAGGCTGTTGTTGC
qHPT-R	ATTCTCCTGATGCCAATGGA
F-box-F (reference gene)	GGCACTCACAAACGTCTATTTC
F-box-R (reference gene)	ACCTGGGAGGCATCCTGCTTAT

Quantification of α-tocopherol content

The infiltrated leaves (1 g) were ground with 1 ml of methanol and 20 µl of filter-sterilized extracts (1 mg ml^{-1}) and standard (1 mg ml^{-1} α-tocopherol, Sigma, USA) were injected into the column Phenomenex C18 5 µm (250 × 4.6 mm), which is maintained at 24 °C and isocratic elution was carried out with HPLC grade methanol: water (with 0.2% orthophosphoric acid) 65:35 ratio v/v at a flow rate of 1.0 ml min^{-1}. Photodiode Array Detector (PDA) was used for the detection of tocopherols at a wavelength of 295 nm. The analysis was carried out in triplicates and the quantification was calculated using the formula,

$$\text{Tocopherol}\left(\text{mg g}^{-1}\right) = \frac{\text{Peak area of the sample}}{\text{Peak area of the standard}} \times 1000.$$

Statistical analysis

All the samples were analysed in three biological triplicates. The values are expressed as means of triplicate analysis of the samples ($n = 3$) ± SD. The data were further analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test ($P < 0.05$) with the aid of SPSS (17 version) statistical package. $P < 0.05$ was considered as indicative of significance, as compared to either control or between the varieties.

Results and discussion

Pharmaceutically important proteins and metabolites can be produced and purified from leaves and seeds of a transgenic crop. The transgenic approach is a complicated process in terms of ecological safety and to produce larger quantities of desired compound. Without the integration of gene of interest/vectors into the genome (chromosome), the transient expression systems can be used to produce the desired protein and metabolites (Kapila et al. 1997; Fischer and Emans 2000). In the present study, *N. benthamiana* plants were infiltrated with vitamin E biosynthesis genes *TC, HPT* and combination of both the genes *TC* + *HPT*.

Gene expression analysis

A reverse transcription PCR was carried out, which proved the presence of both the vitamin E biosynthetic pathway genes. Expected size bands for *HPT* (1.2 kb), *TC* (1.4 kb), and *HPT* + *TC* (Fig. 2) confirmed the presence of respective genes in the infiltrated samples when subjected to electrophoresis. The relative gene expression levels of *HPT, TC* and *HPT* + *TC* in the agroinfiltrated leaves were calculated and

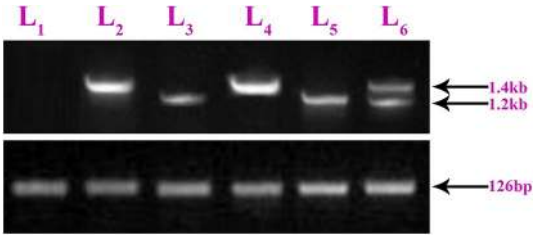

Fig. 2 Transient expression of *AtHPT, AtTC* and *AtTC* + *AtHPT* post agroinfiltration by reverse transcription PCR. Top panel: Target genes Lane 1 Wild type control Lane 2 *HPT* Lane 3 *TC* Lane 3 *HPT* + *TC* with *TC* primers Lane 3 *HPT* + *TC* with *HPT* primers Lane 6 *HPT* + *TC* with both *HPT&TC* primers. Bottom panel: Reference gene F-box

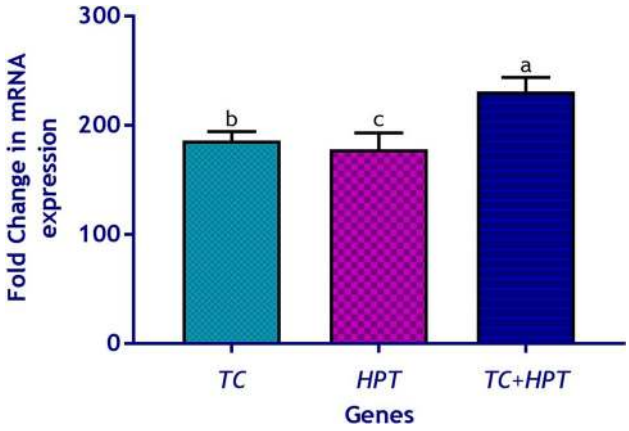

Fig. 3 Relative expression of tocopherol biosynthetic genes *TC* and *HPT* by RT-qPCR. All samples were analyzed in biological triplicates. The values are expressed as means of triplicate analysis of the samples ($n = 3$) ± SD. Means following the same letter are not significantly different, according to Duncan's multiple range test ($P < 0.05$)

compared with that of non-infiltrated leaves. Values were normalized with the reference gene F:box through RT-qPCR (reverse transcription quantitative real-time polymerase chain reaction) experiment. The gene expression analysis indicated that *TC* + *HPT* recorded the highest average gene expression of 231 fold followed by *TC* with 186 fold, whereas *HPT* recorded 178 fold. Transient gene expression of two tocopherol biosynthesis genes individually as well as in combination, which resulted in the upregulation of each transgene, which can invariably enhance α-tocopherol production (Fig. 3). These results suggests that the two tocopherol biosynthesis genes *TC* and *HPT* play a vital role in tocopherol production since their transient gene expression showed a marked increase in tocopherol levels as compared to that of controls. Therefore, our results confirmed that the two tocopherol biosynthetic genes can be used to enhance the tocopherol production in plants using a transient gene expression system.

Quantification of α-tocopherol by HPLC

Overexpression of *HPT* and *TC* resulted in increasing the α-tocopherol levels in transgenic plants. For instance, the overexpression of the *AtHPT* increased the total α-tocopherol level in several plant species, including *Arabidopsis* leaves (4.4-fold) and seeds (fourfold) (Collakova and DellaPenna 2003a), tobacco leaves (5.5-fold) (Harish et al. 2013a, b), lettuce (two fold) (Lee et al. 2007) and potato tuber (106%) (Crowell et al. 2008). The overexpression of the *HPT* isolated from other plant species, including lettuce (*LsHPT*) and apple (*MdHPT*) when expressed in lettuce and tomato leaves have shown to increase the α-tocopherol levels to 18-fold and 3.6-fold, respectively (Ren et al. 2011; Seo et al. 2011). Fourfold and sevenfold increase in vitamin E content was reported by Harish et al. (2013a, b) in double transgenic tobacco expressing *TC* and *HPT*. Ghimire et al. (2011) reported that in transgenic leaves of *Perilla frutescens*, the α-tocopherol content increased to 1.81 fold. Similar results were reported in *At*, where overexpression of γ-*TMT* caused an 80 fold increase in seed α-tocopherol levels (Shintani and Della 1998). In other studies, over-expression of γ-*TMT* imparted a sixfold increase in α-tocopherol level in *Brassica juncea* (Yusuf and Sarin 2007), a twofold increase in lettuce (Cho et al. 2005) and a 10.4 fold and 3.3 fold increase in soybean (Tavva et al. 2007; Li et al. 2011). The HPLC results revealed that levels of α-tocopherol in the leaves of infiltrated plants were higher than that of control plants (Fig. 4). The infiltrated plants with *HPT* showed a 5.88 fold increase in the α-tocopherol content and 5.93 fold increase with *TC* (Fig. 4c, d). In the coinfiltration of *TC* + *HPT*, the α-tocopherol content was increased to 11.31 fold (Fig. 4 e). Transgenic plants in the recent decades have been successfully used to produce antibodies, vaccines, and enzymes (Peterson and Arntzen 2004). With the use of transgenic plants towards understanding and validating the genes involved in the pathway has been reported (Ghimire et al. 2011; Zhang et al. 2013). Arun et al. (2014) reported an increased vitamin E activity of 2.7–3.8 fold in four different cultivars of soybean seeds by overexpressing γ-*TMT* from *Perilla frutescens*. Enhanced levels of vitamin E has been reported by incorporating individual enzymes involved in the pathway including tocopherol cyclase (*TC*) (Yabuta et al. 2013), and γ-tocopherol methyltransferase (γ-*TMT*) (Ghimire et al. 2011; Zhang et al. 2013; Yabuta et al. 2013). Vitamin E activity can be dramatically increased by coexpression of pathway genes (Van Eenennaam. et al. 2003; Sattler et al. 2006). In canola seeds coexpression of *HPT* and γ-*TMT* resulted in 12-fold increase of vitamin E (Collakova and DellaPenna 2003b) whereas, in soybean a fivefold increase was reported by Van Eenennaam et al. (2003) using both *MT* and γ-*TMT*. Compared to bacterial, yeast, or mammalian expression systems, plants combine the key

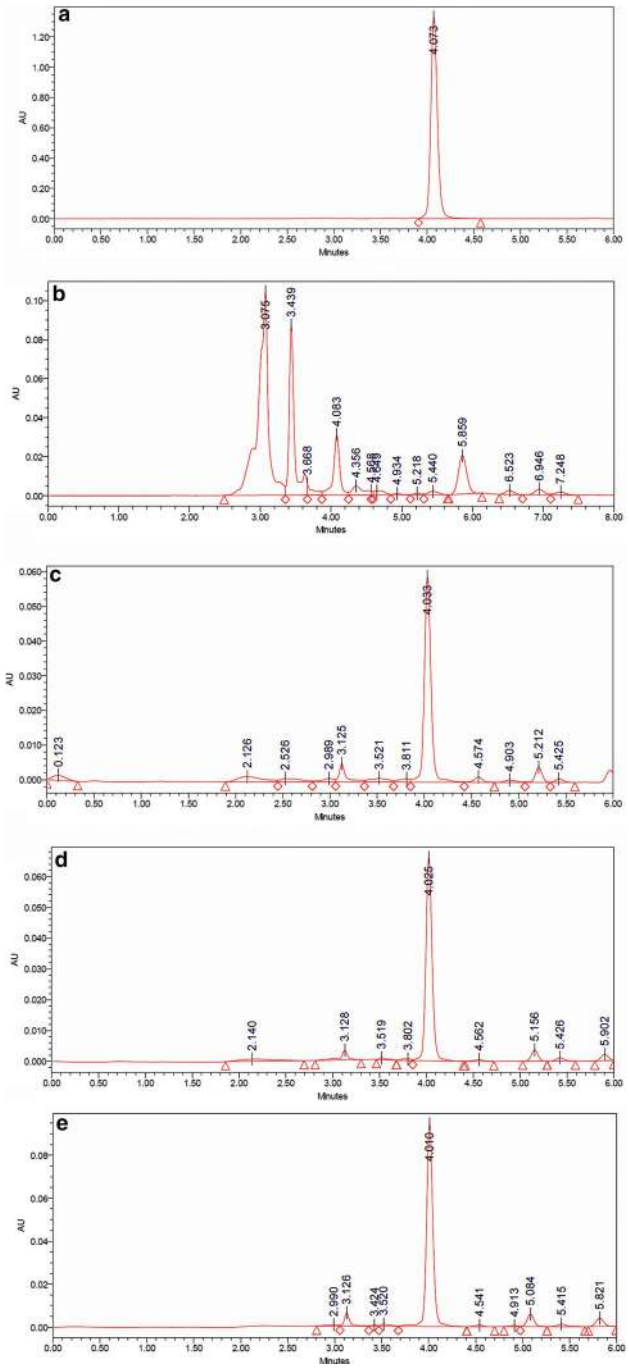

Fig. 4 Quantification of α-tocopherol content by HPLC analysis. **a** Tocopherol standard; **b** Control WT; **c** Chromatogram of vitamin E in leaf sample infiltrated with genes *HPT*; **d** *TC*; **e** *HPT* + *TC*

advantages of eukaryotic post-translational modification, relatively higher protein and metabolite production levels at low cost (Fischer and Emans 2000). Maize, tomato and lettuce are few crops where the potential of biofortification through metabolic engineering has been realized in alleviating vitamin deficiencies (Tang et al. 2012; Castorena-Torres et al. 2014; Kiekens et al. 2015). In contrast to

other transgenic systems, agroinfiltration does not expose transgenic plants or vectors to the environment. Transient expression system can be an effective, rapid and acquiescent system in transgenic plants for the production of high-value proteins and therapeutics. Reports suggest that the levels of transient expression using agroinfiltration was higher than stably transformed lettuce and petunia plants (Vaquero et al. 1999; Wroblewski et al. 2005). Transient expression using agroinfiltration is a promising technology for producing recombinant proteins and therapeutic drugs. Recently, Wunnakup et al. (2017), reported that the overexpression of *HPT* from *Clitoria ternatea* enhanced the metabolic flow of the α-tocopherol biosynthetic pathway using transient expression system. As mentioned above, most reports on metabolic engineering of vitamin E used only conventional stable genetic transformation system till now and this is the first study involving *AtHPT* and *AtTC* to elevate vitamin E levels using transient expression system. The use of plants for metabolite production system also eliminates potential contamination of the therapeutic drug with animal pathogens such as prions, viruses and mycoplasmas.

Conclusion

Transient expression of two tocopherol biosynthesis genes in *N.benthamnia* leaves increased the vitamin E content significantly. The results of our study suggests that metabolic engineering of tocopherols may help to enhance levels of vitamin E in staple food crops. We have used a very efficient, reproducible, and inexpensive transient expression system, which can be used for production, characterization and testing of dietary and pharmaceutically important compounds by employing appropriate and more efficient viral component based vectors. The advantages are low cost, less time consuming and not tedious. In stable genetic transformation system, large scale production of vitamin E and other therapeutic compounds are feasible. Though we report the laboratory scale production of vitamin E using transient expression system, with further optimization, outcome of this study can be used as a tool to enhance vitamin E production.

Acknowledgements The first author thanks Bharathiar University for University Research Fellowship. We would also like to thank, UGC-SAP and DST-FIST for the financial support to carry out this research. The authors would like to thank Dr. S. Girija, Assistant Professor, Department of Biotechnology, Bharathiar university for support in performing the HPLC analysis.

Author Contributions SS designed the work, executed experiments and prepared the manuscript. KSP did the experimental analysis. RV contributed substantially to the manuscript. RS mobilized the funds and critically evaluated the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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