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Increasing vitamin E content of canola (*Brassica napus* L.) by transferring *γ-tmt* gene

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Abstract

Vitamin E is one of the lipid soluble vitamins consisting of several isoforms, including tocopherols and tocotrienols amongst which the alpha tocopherol is the most active one. The conversion of γ tocophrol to α tocopherol takes place by the activity of *γ-tmt* enzyme. Many plants including canola lack the *γ-tmt* gene to enable them to convert γ tocophrol into α tocopherol. The aim of this study was to transfer *γ-tmt* gene into canola plants to enable them to produce α tocopherol and increase their vitamin E content. *γ-tmt* gene was isolated from tomato (*Lycopersicum esculentum* L.), Memory1 cultivar. Then, it was amplified using PCR, digested by *Xba*I enzyme, cloned into the pBluescriptII cloning vector and subcloned into *E. coli*. The gene was then transferred into pBI121 vector and subsequently the vector containing the *γ-tmt* gene was transferred into *Agrobacterium tumefaciens*. Two canola cultivars Zafam and Hayola 401 were used. The cotyledons of canola seeds were inoculated by *Agrobacterium tumefaciens*. Seven putative transformants from each cultivar (Zarfam 2 to 8 and Hayola 2 to 8) were chosen for further investigations. After the emergence of shoots and roots, the seedlings were assayed for the presence of *γ-tmt* gene, by PCR. Vitamin E content of the transformed plants was assayed by FTIR spectrophotometer. Results showed several fold increases in vitamin E contents of the transgenic plants compared to the control. The increases in α-tocopherol content were 2.61 and 2.71 times in Zarfam 6 and Hayola 8, respectively. This approach could be considered as a useful method for fortifying oil seed crops with vitamin E.

Key words: *Agrobacterium tumefaciens*, *Brassica napus*, Transformation, Vitamin E, *γ-tmt*.

INTRODUCTION

Nutrient deficiencies in crops are a serious threat to human health, especially for populations in poor countries. To combat this problem, the development of crops with nutrient-enhanced traits is inevitable. Biofortification of crops to improve nutritional quality is an alternative to this challenge (Zhu *et al.,* 2020).

For more than 80 years, tocopherols have been known to be essential nutrients as vitamin E, for humans and animals. In animals, tocopherols act as antioxidants that are able to scavenge and deactivate various reactive oxygen species (ROS). The other important function of tocopherols is the protection of polyunsaturated fatty acid chains (PUFAs) from lipid peroxidation by scavenging lipid peroxyl radicals (Sattler *et al.,* 2004). Vitamin E is soluble in lipids and its daily required dose is gained through consuming food. It contains eight isoforms which have common structural specifications and are divided into two groups. First group possesses saturated lateral isoprenoid chains and is classified as tocopherols group. The second group possesses lateral insoluble chains and is known as to coterinols (Groff *et al.,* 1995). Each group contains four forms of alpha, beta, gamma and delta. Alpha tocopherol is one of the eight isoforms of vitamin E and is a strong antioxidant, existing in plants naturally (Me` ne-Saffrane and Pellaud, 2017). Alpha tocopherol as a free radical

scavenger protects lipids and lipoproteins with low density from oxidation and also prevents athrosclerosis (Tucker and Townsend, 2005). Alpha tocopherol is the dominant form in mammals and birds. It plays an effective antioxidant role. However, the synthetic forms of vitamin E do not have this characteristic because of the lack of phytyl group (Lam *et al.,* 2010). Thus, increasing natural production of vitamin E in crops and vegetables is very important (Herbers, 2003). One alternative to increase alpha tocopherol is the conversion of other isoforms of vitamin E to alpha isoform. Gamma tocopherol methyl transferase (*γ-tmt*) catalyses the conversion of gamma tocopherol to alpha tocopherol (D'Harlingue and Camara, 1985; Shintani and Della Penna, 1998; Koch *et al.,* 2003). The expression of its gene was carried out for the first time in Arabidopsis (Shintani and Della Penna, 1998). They cloned the *γ-tmt* gene and sequenced its cDNA and reported that it could convert *A. thaliana* seed contents from 97% gamma tocopherol to 95% alpha tocopherol (Shintani and Della Penna, 1998). Canola (*Brassica napus* L.) is one the most important oil crop plants in China, Canada, Australia and north of Europe and has the third rank in edible oils after soybean and oil palm in the world (Block *et al.,* 1989; Cardoza and Stewart, 2003). Canola oil is used in foods and salads excessively and because of low contents of saturated fatty acids is preferred to the other edible oils (Sovero, 1993; Cardoza and Stewart, 2003). The ratio of linoleic acid to linolenic acid in canola oil is near1:2 that is considered a suitable ratio for human consumption. This oil is stored in the form of triglyceride in the cytoplasm of cotyledon (Purkrtova *et al.,* 2008). Canola plant is close to Arabidopsis and shares 85% similarity in DNA structure with it (Cavell *et al.,* 1998) and transferring gene to this plant is easily compared to the other plants. *Agrobacterium*-mediated gene transfer is an efficient approach for genetically improving plants (Mishra *et al.,* 2014; Li *et al.,* 2015). The aim of this research was to fortify canola oil by increasing its vitamin E content through transformation of canola plants by *γ-tmt* gene and conversion of γ-tocopherol into α- tocopherol.

MATERIALS AND METHODS

Extraction of *γ-tmt* from PBluescript vector and its amplification

This experiment was carried out in Imam Khomeini International University (Lat 35, 40863 Long 51, 1548, Alt 1290 m), Plant Molecular Biology Lab. The *γ-tmt* gene extracted from tomato (accession number of JN62365) was used for this study. This gene had been cloned in pBluescriptII and subcloned into *E. coli* (Figure 1). A loopfull of sample was cultured in a 10 ml LB medium and incubated at 37 °C, 110 rpm for 12 to 16 h. PBluescript plasmids were extracted by the method described by Sambrook and Russel (2001). TMTF and TMTR specific primers were used for the amplification of the *γ-tmt* gene by PCR (Table 1). The PCR condition was set as the following initial denaturation at 94 °C for 3 min, 35 cycles including denaturation at 94 °C for 45 sec, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min, and final extension at 72 °C for 3 min. The products were run on a 1% (w/v) agarose gel. PCR products were purified by the K3035, Bioneer kit, (South Korea) cut by *Xba*I enzyme (Fermentas, Nedayefan, Iran) and were purified from the gel using accuprep kit (Bioneer, USA).

Extraction of pBI121 plasmid

E. coli cells containing pBI121 plasmid were cultured in the LB mediumfor12-16 h. Then pBI121 plasmids were extracted by the method described by Sambrook and Russell (2001). The extracted plasmids were digested by *Xba*I enzyme and verified on a 1% (w/v) agarose gel. To eliminate the 5´ end phosphate group, alkaline phosphatase (Fermentas, Nedayefan, Iran) was used and the plasmids were purified from the gel (Sambrook and Russell, 2001).

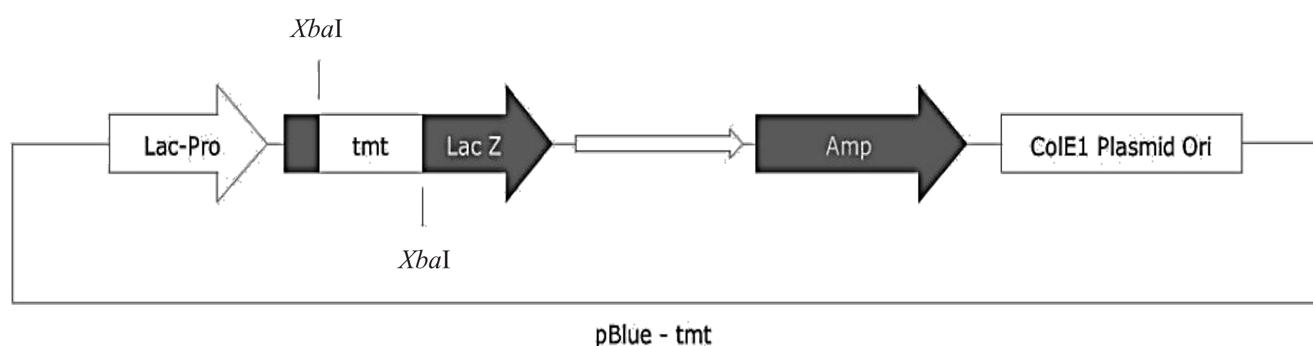

Figure 1. Recombinant phagemid containing gamma tocopherolmethyl transferase gene derived from tomato.

Table 1. Specific primers for gamma tocopherol methyl transferase with a restriction site for *Xba*I enzyme.

Primer	Sequence 5´-3´	Restriction enzyme	Annealing temperature (˚C)	GC (%)
TMTF	5´TATCTAGAATGGGCAGCCAATGCTATT	*Xba*I	61	40.7
TMTR	5´GCTCTAGATTATTCAGGTTTTCGACATGTG	*Xba*I	64	40

Transfer of *γ-tmt* gene into pBI121 plasmid
In order to transfer *γ-tmt* gene into the pBI121 plasmid, the required quantity of *γ-tmt* gene containing pBI121 was calculated using the following formula (Equation 1):

$$\frac{\text{the Concentration of plasmid (ng)}\times\text{size of target fragment kb)}\times 3}{\text{size of plasmid (kb)}} = \text{Concentration of target fragment (ng)} \quad (1)$$

The ratio of 1:3 vector to the gene was used. The ligation reaction was carried out using T4 DNA ligase according to the manufacturer's recommendation (Fermentas, Nedayefan, Iran). The sample was placed at 12-16 °C in a water bath for 10 h. Then, the cells became competent using the freeze and thaw method and calcium chloride 0.1M (Sambrook and Russell, 2001). Cells were cultured in the SOC medium without antibiotics. For the antibiotic resistance gene to be expressed the cultures were incubated in a water bath at 37 °C for 1 h. Then the cells were allowed to grow in a SOB medium containing Kanamycin (30 mgL^{-1}) up to 16 h. In order to identify the transformed bacteria, PCR was used. The colony samples with a positive result supposed to possess the desired fragment were further tested by digestion. By *Nco*I enzyme digestion, the orientation of the fragment i.e. its sense or antisense direction was determined. Plasmids with the sense direction of the fragment were extracted from *E. coli* and transferred into *Agrobacterium tumefaciens* strain GV3101.

Transferring *Agrobacterium* containing *γ-tmt* gene into canola plants
The seeds of two varieties of canola Hayola 401and Zarfam provided by the Center of Oil Seeds located at Lorestan province, Iran, were used for this study. In order to transfer the gene into canola plants, the method described by Moloney *et al.* (1989) was used. Canola seeds were surface sterilized by 2.5% sodium hypochlorite for 15 minutes and was washed by distilled sterile water 3 times. Then the seeds were dried on a filter paper cultured on 1/2 MS medium and placed in a plant growth room at 25 °C in the light and 18 °C during the dark period. Seeds started to grow after 5 to 6 days. The *Agrobacterium* containing pBI121 plasmids were cultured in the LB medium containing rifampicin and kanamycin antibiotics, incubated at 28 °C at 110 rpm. When the OD_{600} of the cultured bacteria reached 1, the growing bacteria were centrifuged at 13000×g and transferred into the inoculation medium (Agar 8 g/l, glucose 50 g/l, 1/2 MS) then the petioles were placed in the inoculation medium containing *Agrobacterium* for 30 sec and transferred into the co-culture medium (agar 4 g L^{-1}, BAP 5 mg L^{-1}, sucrose 30 g L^{-1}, MS) and placed at 25 °C, in dark for two days. Then they were transferred into the selective medium (agar 8 gL^{-1}, BAP 5 mg L^{-1}, sucrose 30 g L^{-1}, MS, kanamycin 10 mg L^{-1}, cefotaxime 200 mg L^{-1}) with 16/8 h light/dark photo period for a week. Then the samples were transferred into the shooting medium (agar 8 g L^{-1}, sucrose 20 g L^{-1}, MS, kanamycin 25 mg L^{-1}, cefotaxime 200 mg L^{-1}). After shooting, the samples remained in the medium for 4 to 5 weeks and were then transferred into the rooting medium (agar 8 g L^{-1} IBA 2 mg L^{-1}, sucrose 30 g L^{-1}, MS, kanamycin 10 mg L^{-1}, cefotaxime 200 mg L^{-1}). Then the rooted shoots were transferred into pots containing perlite and placed at 25 °C with a 16/8 photoperiod.

Extraction of vitamin E
Vitamin E was extracted (Bruni *et al.,* 2002) from the plants containing the recombinant *γ-tmt* gene. One g leaf tissue of the plant samples was macerated using a pestle and mortar in liquid nitrogen. Then, the samples were solved in 10 ml of 96% ethanol and placed at 25 °C for 24 h. Samples were centrifuged (20 min at 4000×g) and the upper phase was transformed to a new tube and were incubated at 25 °C in an oven until all ethanol in the samples had evaporated. Then the samples were dissolved in 10 ml of 86% hexane and placed in an ultrasonic apparatus (20 KHz), for 15 min (ultrasonic MISONIX model SONICATOR 4000). To avoid overheating, samples were placed on ice. The samples were centrifuged again (20 minutes at 4000×g) and filtered through a filter paper (type 2 MM) by vacuum and the volume of the samples was set to 20 µl using hexane. Samples were investigated by the quick method (Sambrook and Russell 2001) using an FTIR spectrophotometer set (TENSOR27 Company BRUKER).

RESULTS

The insertion of the *γ-tmt* gene into the MCS site related to lactose gene was investigated by PCR. The *γ-tmt* gene was amplified from PBluescript phagemid vector by PCR and cut by *Xba*I enzyme and a fragment with the size of 1089 bp was taken out on the gel. In order to transfer the gene into canola (*Brassica napus* L.) plants, the pBI121 expression vector was used. After purifying the PCR products, the *γ-tmt* gene and pBI121 were then ligated and transferred into *E. coli.* The grown bacteria in the medium containing kanamycin were selected. The best time to select the transformed colonies was 12-16 h after culturing bacteria. By doing so, the possibility of selection of non-transformed colonies was low. However, after 16 h, the number of non-transformed colonies increased. Grown colonies were assayed by PCR and the colonies having a positive result were separated. A problem arose at this step, as the colonies with a positive result in PCR, showed negative result with digestion. This may be for the reason that primers were matched with other areas of the pBI121 plasmid having sequence similarity with the gene of interest. In the next step, in order to determine the direction of *γ-tmt* gene in the pBI121 plasmid, the digestion pattern of the plasmid was explored with *Nco*I enzyme. Should the inserted gene be in the sense direction we would expect to observe fragments of the following sizes: 4513, 3602, 1196, 1897 and 3398 bp (Figure 2). This enzyme cuts pBI121 plasmid in four sites and cuts the gene of interest in one site. *Agrobacterium*-mediated transformation is one of most successful ways of transferring gene into dicotyledonous plants. In order to transfer the gene to canola plants, cotyledons were used as the starting material, since it has the advantages of shortening the transfer time, not involving the callus phase and consequently, omitting the possibility of mutations. Care was taken to remove the entire meristematic region at the end of the cotyledonary petioles as this region grows fast and is not controlled by the antibiotic. However, cutting the end of the petiole caused a reduction in the regeneration rate of the explants after transferring the gene (data not shown). Samples containing the gene remained green and viable and were sub-cultured weekly (Figure 3). The presence of the gene was investigated by PCR with specific primers of *γ-tmt* gene (Figure 4). After transferring plants to the pots, 1 g of leaf tissue was used to extract vitamin E. Then pure vitamin E sample was used in 1 mg/20 µl and 2 mg/20µl (Figure 5). For calibration, by increasing the amount of vitamin E from 1 mg to 2 mg, the rate of absorption increased by 6.34%. The obtained results of FTIR spectrophotometer for two canola cultivars (Hayola 401 and Zarfam) are given in the following. In Hayola 401 transformed plants, the highest increase in vitamin E content was observed in Hayola 4 and 8 (Table 2). Hayola 2 and 3 showed 1.20 and 1.14 times higher vitamin E contents compared to the control. Hayola 5 had no difference and Hayola 6 and 7 showed a small increase in relation to the control (Table 2). In Zarfam transformed plants (Figure 6), Zarfam 6 and 2 had the highest vitamin E contents, respectively followed by Zarfam 3. Zrafam 4 and 5 showed a slight increase in vitamin E content relative to the control (Table 2). In the transformed Zarfam 5, 6 and 7 the observed changes in vitamin E were very low. In Zarfam 2 and 3, the changes of vitamin E content were about 1.20, 1.14 and in sample 4 and 8, the increase in vitamin E was about 2.7 times that of the control (Table 2). In Zarfam 3, 4, 5 and 8 vitamin E content was not different to the control. However, in Zarfam 2 and 7, vitamin E content was 2 fold and in Zarfam 6 it showed approximately 2.6 times increase compared to the control (Table 2).

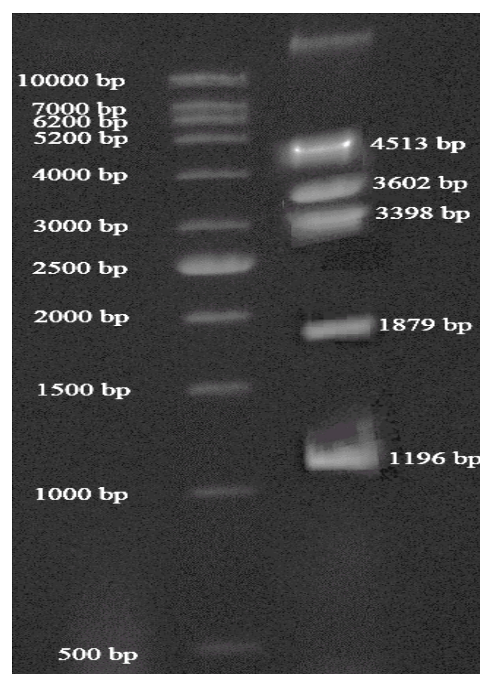

Figure 2. Digestion of PBI121- *γ-tmt* with *Nco*I. The resulted five bands indicate that the gene has been inserted in the sense direction.

DISCUSSION

This study was carried out in order to increase the amount of alpha tocopherol as the most important supply of vitamin E. In this regard, *γ-tmt* gene was

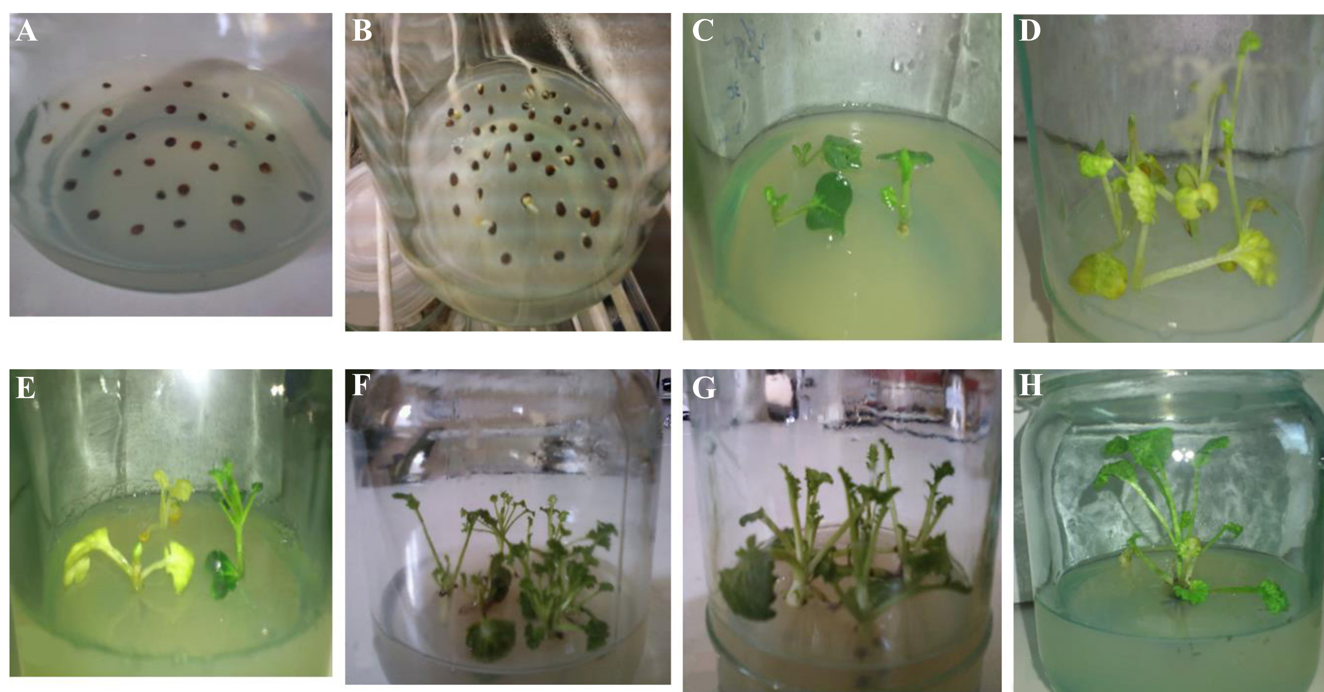

Figure 3. The steps of transformation and the growth of *Brassica napus* (canola), **A:** Planting of canola seeds in MS medium, **B:** Budding of canola seeds after two days, **C:** The growth of seedlings after transformation by *Agrobactearium tumefaciens* containing the *γ-tmt* gene, **D:** Non- transgenic seedlings in the MS medium containing antibiotics, **E:** Transgenic seedlings in the MS medium containing antibiotics, **F:** Transgenic seedlings in the shooting medium,**G:** Transgenic seedlings at the beginning of rooting stage, **H:** Rooted seedling.

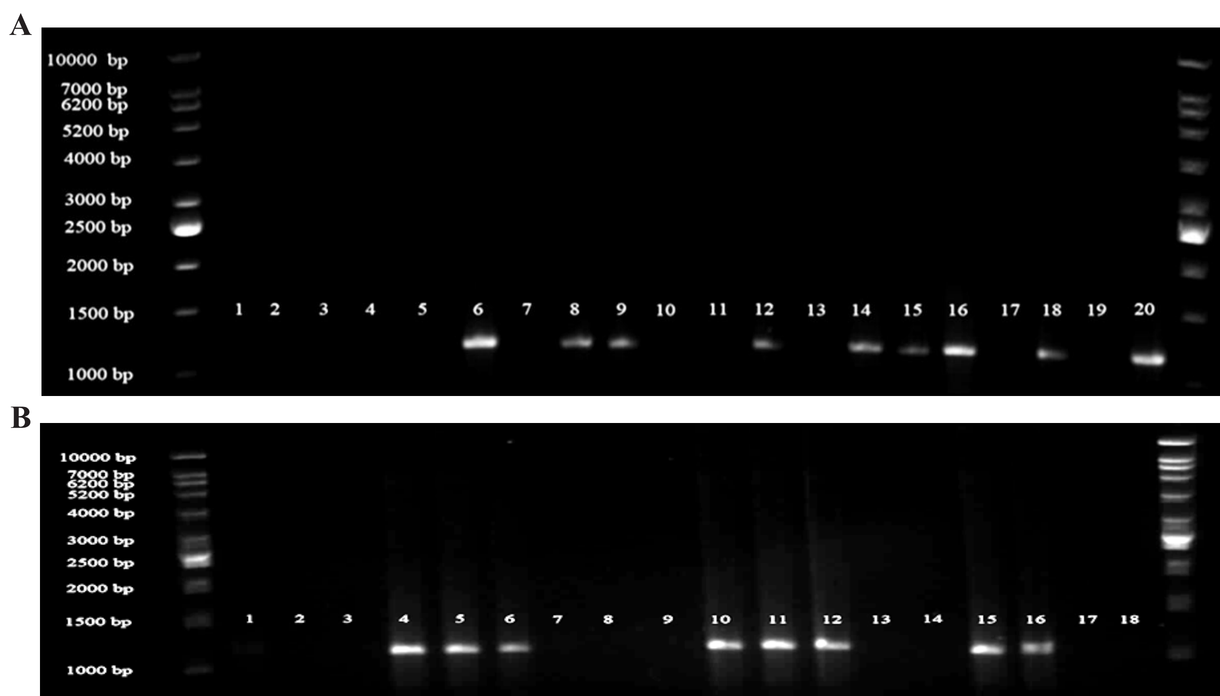

Figure 4. PCR amplification of *γ-tmt* gene in the regenerated plants using specific primers.
A: Hayola401 putative transformants. The left and right lanes are 1kb DNA ladder. Lane 1 is the control, lanes 6, 8, 9, 12, 14, 15, 16, 18 and 20 show positive results and lanes 2, 3, 4, 5, 7, 10, 11, 13, 17 and 19 show negative results on putative transgenic seedlings.
B: Zarfam putative transformants. The left and right lanes are 1kb DNA ladder. Lane 1 is the control, lanes 4, 5, 6, 10, 11, 12, 15 and 16 have positive results, lanes 2, 3, 7, 8, 9, 13, 14, 17 and 18 show negative results on putative transgenic seedlings.

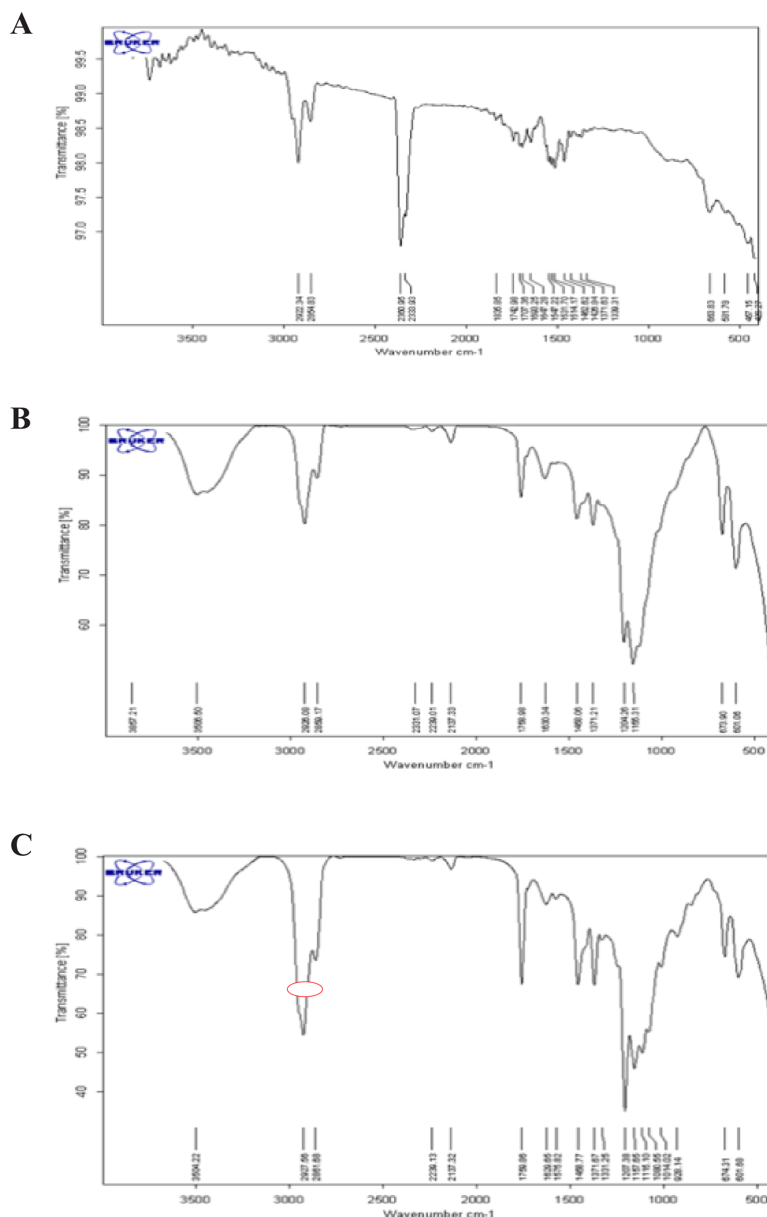

Figure 5. Absorption spectrum of spectrophotometer of FTIR with Hexane and pure vitamin E. The red circle shows the peak related to vitamin E. **A:** Absorption spectrum of pure Hexane, **B:** Absorption spectrum of pure vitamin E 1 mg/20µl, **C:** Absorption spectrum of pure vitamin E 2 mg/20µl.

transferred into canola. The biological activities of α, β, γ and δ tocopherol varies depending on the number and position of methyl groups on the chromanol ring. α, β, γ and δ tocopherol show 100, 50, 10 and 3% equivalent activity to that of α tocopherol activity. Tocopherols are synthesized only by photosynthetic organisms. Usually, α tocopherol is the main form found in leaves while γ tocopherol and tocoterinols accumulate to higher levels in seeds of many plant species. The aromatic ring of tocopherol interacts with reactive oxygen species (Fryer, 1992) and for this reason tocopherol is believed to be critical to protect fatty acids in membranes from oxidative degradation (McKersie *et al.,* 1990). With the methylation of C-5 site of γ-tocopherol, it is converted to α-tocopherol (Tavva *et al.,* 2007). To increase the α-tocopherol content, different plants have been the subject of various studies. For this purpose, γ-tocopherol methyl transferase gene has been transferred into plants and the increase in their α-tocopherol contents measured (Konda *et al.,* 2020). The efficiency of the gene transferred into a plant depends on various factors including the *Agrobacterium* strain, incubation time, the promoter type, vector and inoculation time. In

Table 2. Changes in vitamin E content in transformed plants compared to the control. Sample concentration of 1 mg/20 µl was adjusted for all extracts.

Sample	Increase in absorption (%)	Increase in Vitamin E (unit)
Vitamin E pure	52.26	1.00
Hayola 1 (control sample)	56.95	0.00
Hayola 2	49.28	1.20
Hayola 3	49.66	1.14
Hayola 4	39.82	2.70
Hayola 5	57.54	0.00
Hayola 6	55.60	0.21
Hayola 7	52.03	0.77
Hayola 8	39.71	2.71
Zarfam 1 (control sample)	45.87	0.00
Zarfam 2	32.88	2.04
Zarfam 3	46.18	0.00
Zarfam 4	43.57	0.36
Zarfam 5	44.68	0.18
Zarfam 6	29.26	2.61
Zarfam 7	33.80	1.90
Zarfam 8	45.95	0.00

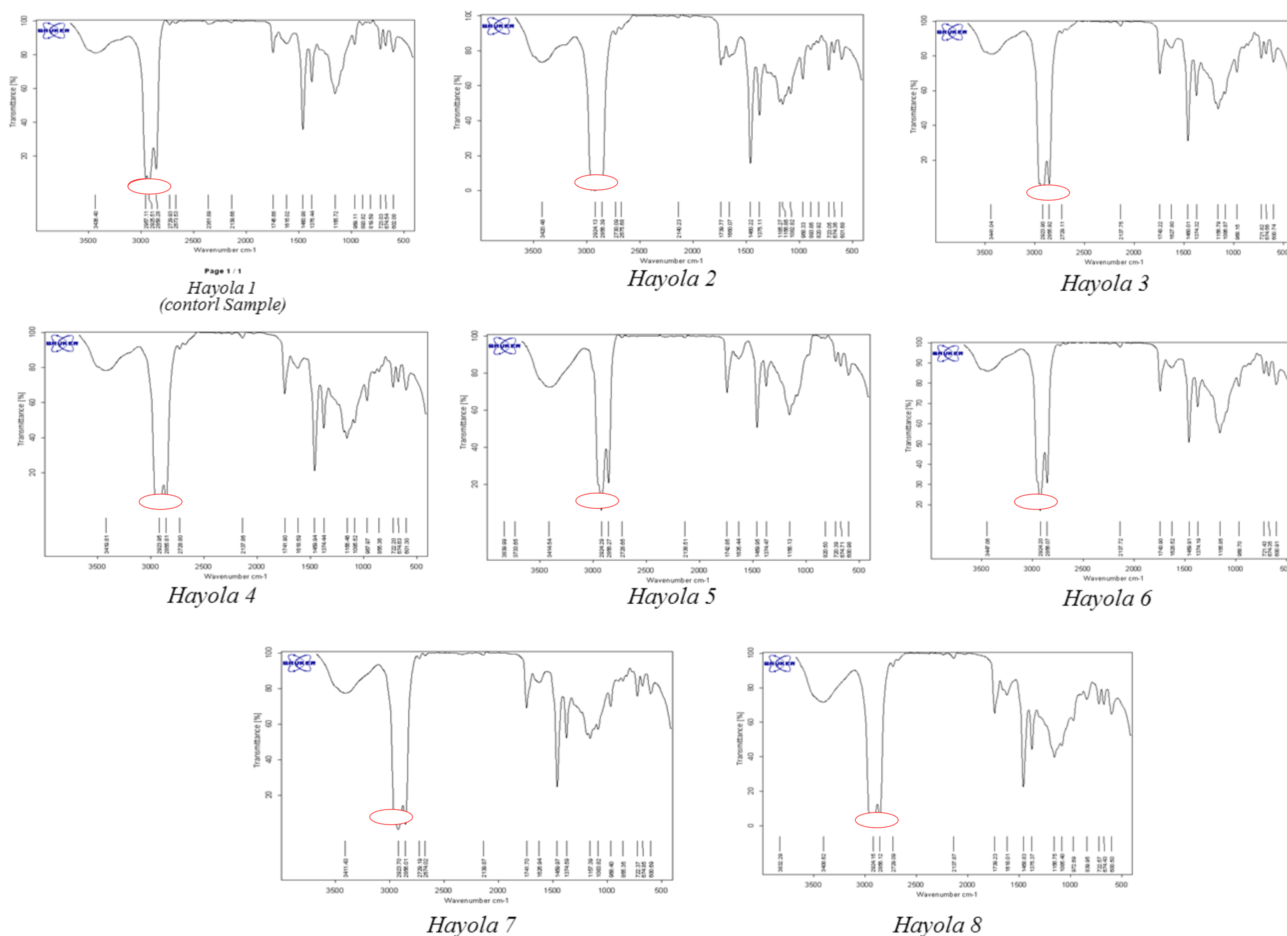

Figure 6. Absorption spectrum of vitamin E extracted from transformed canola plants, Hayola 401 cultivar. The red circle shows the peak related to vitamin E.

this regard two strains of *Agrobacterium tumefaciens*, GV3101 and LBA4404, vector PBI 121 and CaMV 35S promoter were used in this study. Strain LBA4404 was not used for gene transfer process since it has slower growth and lower transforming ability compared to strain GV3101. We also used two canola cultivars Zarfam and Hayola 401. The co-cultivation was 2 days at 25 ºC and inoculation was carried out for 10 Sec. The increases in α-tocopherol content were 2.61 and 2.71 times in Zarfam 6 and Hayola 8, respectively. Chimirre *et al.* (2011) used *Agrobacterium* strain 4404, vector pYB1130 and CaMV 35S for transferring *γ-tmt* into *Perilla frutescens*. The co-cultivation used was 3 days at 24 ºC and inoculation for 7 min. They found 1.8 fold increase in α-tocopherol content in the transformed plants. Arun *et al.* (2014) also used *Agrobacterium* strain EHA 105, pCAMBIa vector and vicillin promoter in soybean and reported 5.2 fold increase in α-tocopherol content. Tavva *et al.* (2007) used vector pCAMBIA1304 and vicillin promoter for transferring *γ-tmt* into *Perilla frutescens*and and could recover only one transgenic plant with 10.4 fold increase in α-tocopherol content. Lee *et al.* (2008) developed transgenic plants harboring *γ-tmt* under vicillin promoter, in pBK I vector with much higher α-tocopherol composition compared to the non-transformed plants. Chen *et al.* (2012) used *Bn-tmt* gene, under FAE1 promoter to transform soybean plants. They obtained transformed lines with 11.1 fold increase in α-tocopherol content. The expression of a heterologous gene in a plant cell is influenced by a number of factors, among them promoter plays the central role (Chen *et al.,* 2012). In this particular case, i.e. the increase in α-tocopherol content, also depends on the presence of γ-tocopherol in the plant. Zhang *et al.* (2013) reported that transferring *γ-tmt* into rice showed no effect in α-tocopherol content, since γ-tocopherol is present in a small amount in rice seeds. An approach to increase α-tocopherol content is the transformation of *γ-tmt* into plants possessing γ-tocopherol. This alternative has been employed by many researchers with success. We used canola plants with the purpose of increasing its α-tocopherol content. Two cultivars were used and reasonable results were obtained. The transgenic plants are being grown to obtain next generations and examined by more informative techniques such as Real Time PCR and Southern blotting and HPLC for the inheritance of the transferred gene and α-tocopherol content.

ACKNOWLEDGEMENTS

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