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BRIEF COMMUNICATION

Antioxidant value addition in human diets: genetic transformation of *Brassica juncea* with γ-TMT gene for increased α-tocopherol content

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Abstract α-Tocopherol, the most biologically active form of vitamin E, is implicated in decreasing the risk of several types of cancers, coronary heart disease and a number of degenerative human conditions, when taken in excess of the recommended daily allowance. Natural α-tocopherol has twice the bioavailability of the synthetic isomer. This study describes a successful attempt at fortifying human diets with natural α-tocopherol by taking recourse to genetic engineering of an important oilseed crop, *Brassica juncea*. γ-Tocopherol methyl transferase cDNA from *Arabidopsis thaliana*, coding for the enzyme catalysing the conversion of the large γ-tocopherol pool to α-tocopherol, was overexpressed in *B. juncea* plants. The successful integration of the transgene was confirmed by PCR and Southern blot analysis, while the enhanced transcript level was evident in the northern blot analysis. HPLC analysis of the seeds of the T_1 transgenic lines showed a shift in tocopherol profile with the highest over-expressors having α-tocopherol levels as high as sixfold over the non-transgenic controls. This study discusses the production of a transgenic oilseed crop with high α-tocopherol levels, which can provide a feasible, innocuous, and inexpensive way of taking the beneficial effects of high α-tocopherol intake to the masses.

Keywords *Agrobacterium tumefaciens* · α-Tocopherol · *Brassica juncea* · Transgenic

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Introduction

Vitamin E is an essential fat-soluble vitamin, known for its antioxidant effects in the body. Of the eight different naturally occurring forms of vitamin E, α-tocopherol has the highest activity and is more readily retained by the human body due to the preferential binding of the hepatic α-tocopherol transfer protein (Traber and Sies 1996). Several non-antioxidant functions of tocopherols have come to light in the last few years, which have been attributed to their role in the modulation of signal transduction pathways and transcription (Brigelius-Flohe and Traber 1999). In plants, α-tocopherol helps in keeping a check on the redox status in chloroplasts, in maintaining thylakoid structure and function during plant development, and in plant responses to stress (Munné-Bosch and Alegre 2002; Sattler et al. 2004). α-Tocopherol intakes in excess of the RDA (22.5 I.U. or 15 mg α-tocopherol) are associated with decreased risk of cardiovascular diseases, improved immune function, slowing of the progression of a number of degenerative human

conditions associated with aging such as, cataracts, arthritis, and disorders of the nervous system (Bramley et al. 2000). Plant seed oils, which are the major dietary source of vitamin E, have very low α-tocopherol content (Taylor and Barnes 1981) but relatively high levels of γ-tocopherol, the biosynthetic precursor of α-tocopherol, suggesting that the final step of the α-tocopherol biosynthetic pathway catalysed by γ-tocopherol methyl transferase (γ-TMT) is limiting. The overexpression of γ-TMT in *Arabidopsis thaliana*, a model plant, was reported to increase the vitamin E activity by ninefold over the wild-type controls (Shintani and DellaPenna 1998).

In this investigation, we report a sixfold increase in the α-tocopherol content in an important oil-yielding crop, *Brassica juncea*, by overexpressing the γ-TMT gene from *A. thaliana*.

Methods, results and discussion

The *A. thaliana* γ-TMT cDNA, obtained from the Arabidopsis Biological Resource Centre (ABRC) at Ohio State University, was cloned at the *Eco*RI–*Bam*HI site in *pRT101* vector in between *CaMV 35S* promoter and *polyA* terminator sequences. This promoter–γ-TMT cDNA–terminator cassette was released from *pRT101* vector by digestion with *Pst*I and cloned at the *Pst*I site in the multiple cloning site of *pCAMBIA 1301* (*pC1301*) vector to give rise to *pCAM-TMT* (Fig. 1a).

The construct, *pCAM-TMT*, was mobilised into a disarmed *Agrobacterium tumefaciens* strain GV 3101, which was subsequently used to transform hypocotyl explants from 5-day-old seedlings of *B. juncea* cv. Varuna following the method of Pental et al. (1993). The transgenic plants were selected on MSB_1N_1 medium (MS medium supplemented with 1 mg/l each of 6-benzylaminopurine and α-naphthaleneacetic acid) containing hygromycin (20 mg/l). The plantlets were rooted on MSI_2 (MS medium supplemented with 2 mg/l of indol-3-butyric acid). The transformation frequency, as assessed by the number of transformed explants growing on hygromycin selection per total number of explants inoculated, varied between 28.5 and 35.1%. Putative transformants were also screened by the expression of *GUS* gene present in the construct. T_0 transgenic plants were checked for the integration of the transgene by PCR and Southern blot analysis (data not shown). Seeds of seven independent positive segregating T_0 lines, viz. 5, 10.1, 14.1, 16.1, 25.1, 44, and 53 were grown in the glass house to get T_1 transgenic plants which were used for further analysis.

All the seven T_1 transgenic lines selected were checked for the stable integration of the gene by PCR and Southern blot analysis. A ~1.2 kb band was amplified using γ-TMT gene specific primers in the transgenic plants while it was absent in the untransformed control plants (Fig. 1b). For Southern blot analysis, the genomic DNA (10 μg) was digested with *Pst*I restriction endonuclease in order to release the promoter–γ-TMT cDNA–terminator cassette (~2 kb). The blot was probed with ^{32}P-dCTP labelled γ-TMT cDNA. In all the seven transgenic lines analysed, the expected ~2 kb band lighted up whilst it was absent in the untransformed control plant (Fig. 1c). The overexpression of the transgene was evident in the northern blot analysis where ~1.2 kb band corresponding to γ-TMT mRNA was observed in those transgenic lines which were Southern positive (Fig. 1d).

The tocopherol content and composition in the lipid extract of T_1 seeds from all the seven transgenic lines and the untransformed control plants was checked using HPLC with an ultraviolet absorbance detector, following the method described by Cahoon et al. (2003). The seeds of untransformed control *B. juncea* plants were found to contain about 572 ng/mg seed weight of total tocopherol content. Of the total content, γ-tocopherol was the major form present (~86%) while α-tocopherol constituted only a minor fraction (~10%) of the total tocopherol pool. In the seven T_1 transgenic lines analysed, though the total tocopherol content was almost equal to that of untransformed control plants, there was a marked increase in the α- to γ-tocopherol ratio, with α-tocopherol levels as high as 62% of the total pool, that is, an increase of about sixfold over the control plants was achieved (Fig. 1e). Also, there was an increase in the β-tocopherol content with a corresponding decrease in the δ-tocopherol levels. The increase in the level of

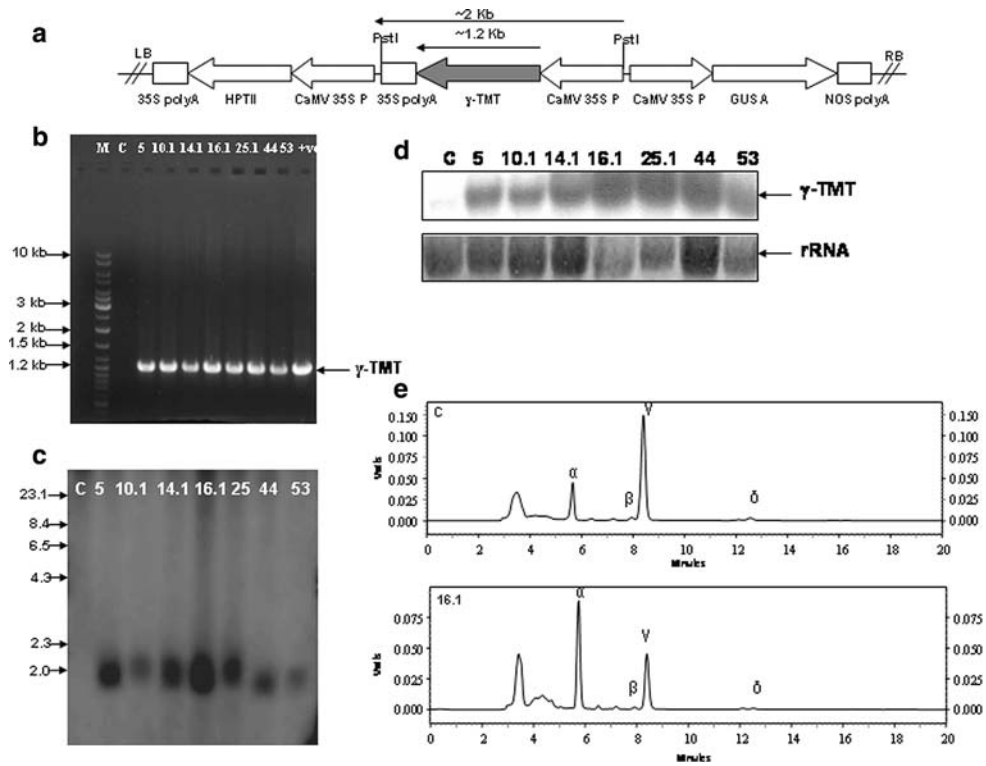

Fig. 1 Analyses of transgenic *B. juncea* plants for γ-TMT gene integration, overexpression, and tocopherol content. (a) *pCAM-TMT* construct used for *B. juncea* transformation; (b) PCR analysis of T_1 transgenic *B. juncea* plants using γ-TMT gene specific primers. A ~1.2 kb band was amplified in the transgenic plants while it was absent in the untransformed control plants. (c) Southern blot analysis of the T_1 transgenic *B. juncea* plants. Ten micrograms of genomic DNA from different T_1 transgenic lines and untransformed control plants was completely digested with *Pst*I, resolved on 0.8% agarose gel, blotted onto a positively charged nylon membrane and probed with ^{32}P-dCTP labelled γ-TMT probe. (d) Northern blot analysis of the T_1 transgenic and untransformed control plants of *B. juncea*. Twenty micrograms of the total RNA from the transgenic and untransformed control plants was electrophoresed on a denaturing agarose gel, transferred onto a nylon membrane, and hybridised with ^{32}P-dCTP labelled γ-TMT probe. *C*, untransformed control; *5*, *10.1*, *14.1*, *16.1*, *25.1*, *44*, *53*, T_1 transgenic lines; *M*, DNA size marker. (e) Representative HPLC profiles of various tocopherol forms in *B. juncea*. *C*, untransformed control; *16.1*, T_1 transgenic line

α-tocopherol in the transgenic plants corroborated well with overexpression of the transgene. Table 1 shows the amounts of the four forms of tocopherol in the untransformed control plant and in different transgenic lines, as analysed by HPLC.

The transgenic *B. juncea* plants had normal morphology and showed flowering and seed set like the healthy untransformed control plants. The performance of the transgenic *B. juncea* plants vis-à-vis the untransformed control plants was assessed by comparison of various growth parameters such as the height of the plant, number of seeds per pod, seed weight per pod, and chlorophyll content of the leaves. The values for these parameters were comparable in the transgenic as well as untransformed control plants (Table 2).

This study demonstrates a successful attempt at fortifying human diets with α-tocopherol using an important oilseed crop, *B. juncea.* The elevation of vitamin E levels in plants has been attempted by earlier workers through two strategies (i) quantitative elevation of the total tocopherol levels by increasing the flux through the biosynthetic pathway, and (ii) qualitative increase by shifting the tocopherol pool towards more α-tocopherol (Ajjawi and Shintani 2004). Few of

Table 1 Content of different tocopherols in seeds from untransformed control and T_1 transgenic *B. juncea* plants as determined by HPLC analysis

Line	Total tocopherol content (ng/mg of seed)	α-Toc %	β-Toc %	γ-Toc %	δ-Toc %
C	572.0 ± 20.2	9.8 ± 0.4	0.82 ± 0.1	86.82 ± 1.0	2.56 ± 0.2
5	593.2 ± 15.5	35.48 ± 0.9	1.06 ± 0.2	63.25 ± 0.3	0.21 ± 0.2
10.1	609.7 ± 16.2	24.36 ± 0.5	1.02 ± 0.3	74.05 ± 0.5	0.67 ± 0.1
14.1	520.8 ± 16.7	60.55 ± 0.4	1.37 ± 0.2	37.59 ± 0.6	0.51 ± 0.1
16.1	590.2 ± 18.3	62.29 ± 1.0	1.34 ± 0.4	35.84 ± 0.5	0.53 ± 0.2
25.1	578.7 ± 13.8	61.68 ± 0.5	0.97 ± 0.2	36.72 ± 0.3	0.63 ± 0.3
44	605.3 ± 11.9	48.36 ± 0.6	1.45 ± 0.3	49.52 ± 0.2	0.67 ± 0.4
53	585.6 ± 21.2	41.96 ± 0.3	0.94 ± 0.2	56.44 ± 0.4	0.68 ± 0.2

Values are represented as mean ± SE of three replicate seed samples from each of the lines

Table 2 Comparison of the growth parameters of untransformed control and T_1 transgenic plants of *B. juncea*

Parameter	Control	Transgenic line
Height (cm)[a]	219 ± 10.5	210 ± 15.3
No. of seeds per pod[b]	8.3 ± 1.0	8.0 ± 0.5
Seed weight per pod (mg)[b]	104.4 ± 3.7	99.3 ± 5.6
Chlorophyll content (μg/g)[c]	120.2 ± 2.3	125.6 ± 3.9

[a] Values are the mean ± SE of three plants from each line

[b] Values are mean ± SE of 50 pods from each line

[c] Values are mean ± SE of fifth leaf from the top for each line

the enzymes catalysing the reactions at the flux control points have been overexpressed with the aim of increasing the total tocopherol levels. The studies include overexpression of hydroxyphenyl pyruvate dioxygenase by Tsegaye et al. (2002) and Falk et al. (2003); deoxyxylulose phosphate synthase (Estevez 2001); homogentisate phytyl transferase by Collakova and DellaPenna (2003) and Savidge et al. (2002). The success rates in these studies have varied but not very drastically. Methylphytylbenzoquinone methyl transferase, tocopherol cyclase, and γ-TMT are the enzymes important in determining the tocopherol composition (Ajjawi and Shintani 2004). The overexpression of MBPQMT and γ-TMT in soybean seeds resulted in an increase of α-tocopherol by greater than eightfold, at the expense of δ-, β-, and γ-tocopherols (Van-Eenennaam et al. 2003), while the overexpression of γ-TMT in the model plant *A. thaliana* increased the seed α-tocopherol levels by 80-fold (Shintani and DellaPenna 1998). In lettuce it led to an over twofold elevation (Cho et al. 2005).

These findings need to be extended to the commonly used oilseed crops for human beings to benefit from them. As different oil seeds are prevalent in the food habits of people in various regions of the world, efforts are needed to tailor the α-tocopherol content in different crops so as to benefit the general populace. In this investigation, we achieved the goal to increase the α-tocopherol content of an important oilseed crop of the developing Indian subcontinent, *B. juncea* (Indian mustard). In India alone, *B. juncea* is grown on six million hectares of land. Mustard oil is the main cooking medium and the seeds and oil are used as a condiment in the preparation of pickles and for flavouring curries and vegetables. Of the total tocopherol content in *B. juncea* seeds, γ-tocopherol was found to be the major form present (86%) while α-tocopherol constituted only a minor fraction (10%) of the pool. Thus, there was a huge scope for converting the γ-tocopherol to α-tocopherol.

The overexpression of γ-TMT gene increased the α-tocopherol levels by over sixfold. The

change in tocopherol profile was not found to have any adverse effect on the growth and yield of the transgenic plants. The transgenic plants were healthy and compared well with the various growth parameters of normal untransformed control plants.

Further increase in the α-tocopherol content could be envisioned by making double transgenics overexpressing γ-TMT along with a flux-controlling enzyme, upstream in the pathway and marks the next milestone for this work. Studies on the performance of high α-tocopherol containing transgenic plants of *B. juncea* under abiotic stress conditions are in progress.

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