

Alpha-tocopherol value added *Brassica juncea*: fortified human nutrition and abiotic stress tolerance to the plant

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Abstract

Of the eight different forms of vitamin E, α -tocopherol is the most biologically active, and is preferentially retained by the human body. α -tocopherol intake in excess of the recommended daily allowance is implicated in decreasing the risk of several types of cancers, coronary heart disease and a number of degenerative human conditions. Also, α -tocopherol, a lipid soluble compound is considered to play an important role as an antioxidant, protecting the integrity of the cellular membranes under normal and stress conditions. Edible oils that are the major source of vitamin E are poor in α -tocopherol content although its immediate precursor γ -tocopherol is present in large amounts. This study describes a successful attempt at enriching the human diets with natural α -tocopherol along with fortifying the antioxidant defense of the plants by taking recourse to genetic engineering of an important oilseed crop, *Brassica juncea*. γ -tocopherol methyl transferase (γ -TMT) c-DNA from *Arabidopsis thaliana*, coding for the enzyme catalyzing the conversion of the γ -tocopherol 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 transgenic lines showed a shift in tocopherol profile with the highest overexpressors having α -tocopherol levels as high as six fold over the non-transgenic controls. The transgenic *B. juncea* plants also showed enhanced tolerance to abiotic stress in comparison to the untransformed control. The study discusses the production of a transgenic oilseed crop with 6-fold higher α -tocopherol levels, that can withstand abiotic stress and can provide a feasible, innocuous and inexpensive way of taking the beneficial effects of high α -tocopherol intakes to the masses.

Key words: Antioxidant, *Brassica juncea*, α -tocopherol, γ -tocopherol methyl transferase, Transgenic

Introduction

Vitamin E is an essential fat soluble vitamin belonging to a class of compounds known as terpenoids and is known for its antioxidant effects in the body (Fryer, 1996; Bramley et al., 2000; Esterbauer, 1991). Intakes of vitamin E in excess of the recommended daily allowance (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 as cataracts, arthritis, and disorders of the nervous system (Traber & Sies, 1996). It is to be noted that the safe upper limit for vitamin E intake is 1000 mg per day.

Of the four naturally occurring tocopherols α -, β -, γ -, δ -, α -tocopherol has the highest vitamin E activity (Eitenmiller, 1997). Plant seed oils which are the major dietary source of vitamin E, have very low α -tocopherol content (Taylor & Barnes, 1981). The observation that many oil seeds contain relatively high levels of γ -tocopherol, the biosynthetic precursor of α -tocopherol, suggests that the final step of the α -tocopherol biosynthetic pathway catalysed by γ -tocopherol methyl transferase (γ -TMT) is limiting in these tissues. The overexpression of γ -TMT has been found to increase the levels of α -tocopherol in different plant species namely *Arabidopsis thaliana*, soybean, and lettuce (Shintani & DellaPenna, 1998; Tavva et al., 2006; Cho et al., 2005).

The objective of this work was to increase the α -tocopherol content of *Brassica juncea*, an important edible oilseed crop of the Indian subcontinent. Here we report that most of the commonly grown varieties of *Brassica juncea* have very low levels of α -tocopherol in comparison to γ -tocopherol. We show the modulation of α -tocopherol biosynthetic pathway and increased production of α -tocopherol in transgenic *Brassica juncea* cv. Varuna plants by overexpression of *Arabidopsis thaliana* γ -tocopherol methyl transferase cDNA through *Agrobacterium tumefaciens* mediated genetic transformation. The transgenic plants showed comparable growth parameters as the untransformed control ones under normal conditions but were found to be more tolerant to abiotic stress (NaCl induced) condition as observed in the retardation of leaf disc senescence assay.

Material and Methods

Transformation of *Brassica juncea* with γ -TMT cDNA binary vector construct: The *Arabidopsis* γ -TMT cDNA was obtained from *Arabidopsis* Biological Resource Centre at Ohio State University (ABRC). It was cloned in between CaMV 35S promoter and polyA terminator sequences in pCAMBIA 1301 (pC1301) vector. pC1301 has hygromycin as the plant selection marker and kanamycin as bacterial selection marker. This construct (Fig 1a) was confirmed and then mobilised into *Agrobacterium tumefaciens* strain GV 3101 using freeze-thaw procedure, which was used for the plant transformation experiments.

Brassica juncea cv. Varuna was used for the transformation experiments using *Agrobacterium* mediated transformation method. The transformation protocol was essentially as described by Pental et al (1993). The putative transgenic plantlets were

hardened and transferred to the green house. Untransformed control plants were also grown under similar conditions.

Molecular analyses of the transformants: The integration of the γ -TMT gene in the transgenic plants was confirmed by PCR and Southern blot analysis. For Southern blot analysis the genomic DNA was completely digested with *Pst* I and 32 P labeled γ -TMT cDNA was used as the probe. The overexpression of the transgene was checked by northern blot analysis. These molecular analyses were done using the procedure described in Sambrook et al (1989).

HPLC analysis: Total tocopherol in the lipid extracts of seeds was obtained using the method described by Cahoon et al. (2003). The tocopherol content in the lipid extracts of seeds was determined by HPLC. A Shimadzu HPLC system with ultraviolet absorbance detector was used for the analysis. The different tocopherol forms were resolved on a Phenomenex Luna 5u Silica 4.6 \times 250 mm HPLC column. The solvent system used was isopropanol:n-heptane (1:99 v/v) at a flow rate of 1ml/min. The absorbance was measured at a wavelength of 295 nm.

Leaf disc assay for abiotic stress tolerance: Leaf disc of equal diameter from fully grown mature leaves of control and transgenic plants were floated on NaCl (200 mM, 400 mM, 800 mM) solution for checking their performance under salinity stress. The difference in the chlorophyll content of the leaf discs was estimated after 7 days of treatment according to the procedure of Arnon (1949).

Results

The values of the different tocopherol forms in commonly grown varieties of *B. juncea* is shown in Table 1. The total tocopherol content in the seeds varied from 460 ng/mg to 532 ng/mg of the seed with γ -tocopherol present in highest proportion (~80-90%), while the α -tocopherol made only a small percentage (~9-12%). Seven T₁ transgenic lines selected were checked for the stable integration of the gene by PCR and Southern blot analysis and they were found to be positive (Fig. 1b & c). The overexpression of the transgene was evident in the northern blot analysis (Fig. 1d). The comparison of the HPLC profiles of different tocopherol forms in the untransformed control plants and different transgenic lines showed that there was a marked increase in the α - to γ -tocopherol ratio in case of transgenic lines (Fig. 1e). The α -tocopherol levels were as high as 62% of the total pool, that is, an increase of about six-fold over the control plants was achieved (data not shown). The transgenic *B. juncea* plants had normal morphology and showed flowering and seed set like the healthy untransformed control plants (data not shown). To compare the performance of the transgenic and untransformed control plants under abiotic stress (NaCl induced) leaf disc assay was performed. While the leaf discs from the transgenic plants (line 16.1) showed delayed senescence at all the concentrations of NaCl solution the leaf discs from the untransformed control plants showed early bleaching. Measurement of the chlorophyll content on the seventh day confirmed the phenotypic differences between the transgenics and the untransformed control plants. The chlorophyll content (Fig.) in the transgenic leaf discs floated on NaCl was 107, 67, and 23 μ g/g F. wt. while in the case of untransformed controls it was 75, 23, and 7 μ g/g F. wt. (at 200 mM, 400 mM, and 800 mM NaCl, respectively). At 200 mM NaCl the chlorophyll content of the transgenic plants was 1.4 times higher as compared to that in the control leaf discs. At 400 mM and 800mM NaCl the chlorophyll content was 2.9 and 3.3 folds, respectively, in the transgenic *B. juncea* plants as compared to the untransformed control plants. Thus, the result obtained from the retardation of leaf disc senescence assay suggests a role of γ -TMT gene in stress alleviation in the transgenic plants overexpressing it.

Discussion

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 & Shintani, 2004). Few of the enzymes catalyzing 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 (HPPD) by Tsegaye et al. (2002) and Falk et al. (2003); deoxyxylulose phosphate synthase (Estevez, 2001); homogentisate phytyl transferase (HPT) by Collakova and DellaPenna (2003) and Savidge et al. (2002). The success rates in these studies have been varied. Methylphytylbenzoquinone methyl transferase (MPBQMT), tocopherol cyclase (TC), and γ -tocopherol methyl transferase (γ -TMT) are the enzymes important in determining the tocopherol composition (Ajjawi & Shintani, 2004). The overexpression of MBPQMT and γ -TMT in soybean seeds resulted in an increase of α -tocopherol by greater than eight fold, at the expense of δ -, β -, and γ -tocopherols (Van-Eenenmam et al., 2003), while the overexpression of γ -TMT in the model plant *Arabidopsis thaliana* and *Latuca sativa* increased the seed α -tocopherol levels by nine and two fold, respectively (Shintani & DellaPenna, 1998; Cho et al., 2005). These findings need to be extended to the commonly used oilseed crops for human beings to benefit from. This makes our study significant as the mustard oil, which is widely used in the Indian subcontinent and is recommended for health purposes, is obtained from *B. juncea*. We obtained transgenic lines with α -tocopherol content as high as six fold over the untransformed controls. Also, as α -tocopherol is a very potent antioxidant, it is expected to impart tolerance to plants against the abiotic stresses (in which the antioxidant status of the cell is compromised). The preliminary result obtained in the present study using the retardation of leaf disc senescence assay is a pointer to the better performance of the α -tocopherol enriched transgenic plants. The mechanism of the stress tolerance in these transgenic plants at the cellular level is being investigated further.

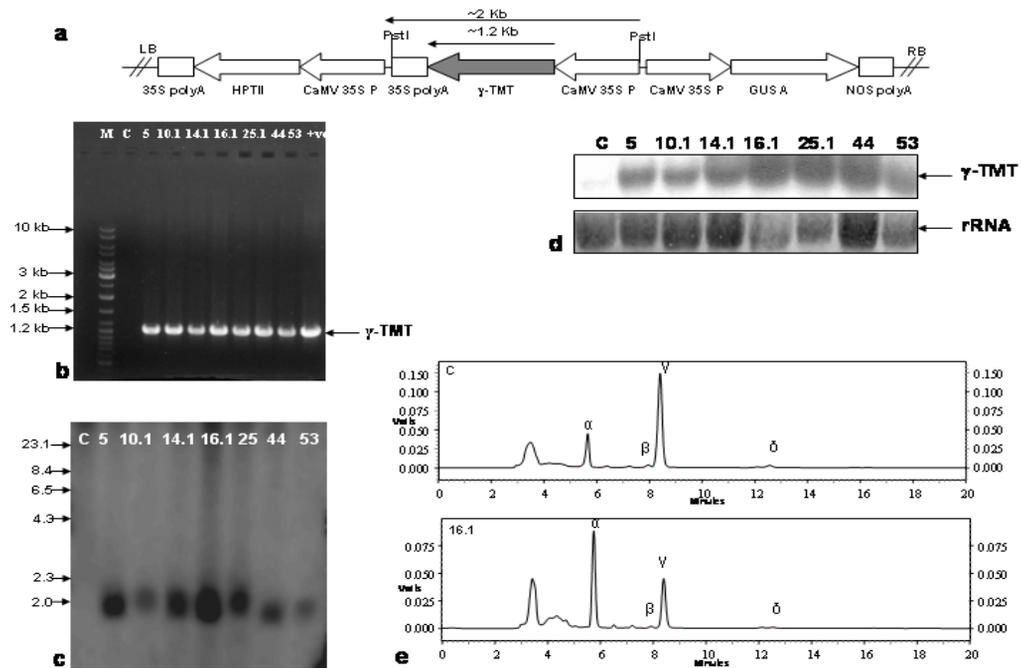


Fig. 1. Analyses of transgenic *Brassica juncea* plants (a) *pCAM-TMT* construct used for *Brassica juncea* transformation (b) PCR analysis of T₁ transgenic *B. juncea* plants using γ -*TMT* gene specific primers. A ~1.2 kb band was amplified in the transgenic plants while it was absent in the untransformed control plants. (c) Southern blot analysis of the T₁ transgenic *B. juncea* plants. 10 μ g genomic DNA from different T₁ 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 ³²P-dCTP labelled γ -*TMT* probe. (d) Northern blot analysis of the T₁ transgenic and untransformed control plants of *B. juncea*. 20 μ g 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 ³²P-dCTP labelled γ -*TMT* probe. C, untransformed control; 5, 10.1, 14.1, 16.1, 25.1, 44, 53, T₁ transgenic lines, M, DNA size marker. (e) Representative HPLC profiles of various tocopherol forms in *Brassica juncea*. C, untransformed control; 16.1, T₁ transgenic line.

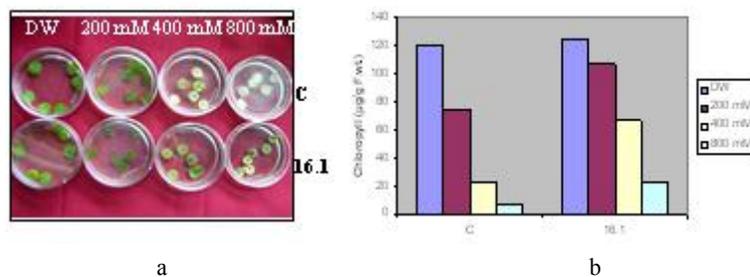


Fig. 2 Retardation of salinity induced senescence of leaf discs in transgenic *Brassica juncea*.

(a) Leaf discs of untransformed control and transgenic line (16.1) kept in 200 mM, 400 mM, and 800 mM NaCl solution for 7 d. Distilled water served as the experimental control. Chlorophyll content of the leaf discs from (a) estimated on 7th day.

Table 1. Tocopherol content of commonly grown Indian varieties of *B. juncea*. Tocopherol content was estimated in the lipid extract of the seeds and tocopherol profile generated using HPLC. Values are the mean \pm SE of three replicate samples.

| Variety | Tocopherol content (ng/mg seed) | α -toc. (%) | β -toc. (%) | γ -toc. (%) | δ -toc. (%) |
|-----------|---------------------------------|--------------------|-------------------|--------------------|--------------------|
| Varuna | 508.3 \pm 24.2 | 9.2 \pm 0.4 | 0.6 \pm 0.2 | 87.7 \pm 1.0 | 2.5 \pm 0.2 |
| Pusa Bold | 460.6 \pm 7.9 | 12.3 \pm 0.2 | 1.0 \pm 0.5 | 82.5 \pm 0.9 | 4.2 \pm 0.1 |
| Jaikisan | 532.3 \pm 20.5 | 11.7 \pm 0.4 | 0.7 \pm 0.1 | 86.3 \pm 0.9 | 1.3 \pm 0.3 |
| Jagannath | 490.8 \pm 26.3 | 8.9 \pm 0.9 | 0.7 \pm 0.2 | 89.5 \pm 0.4 | 0.9 \pm 0.3 |
| Agarni | 510.5 \pm 18.0 | 9.7 \pm 0.5 | 0.5 \pm 0.3 | 88.2 \pm 0.3 | 1.6 \pm 0.5 |

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