Vitamin E is an essential fat soluble vitamin, synthesized only by plants and some cyanobacteria. Thought of as just an antisterility vitamin at its discovery, vitamin E was soon found to have roles transcending its effect in the normal carriage and delivery of babies. It is valued, today, as a powerful antioxidant in the armoury of cells for overcoming the stress conditions that arise within a cell for a variety of reasons from time to time. In plants, tocopherols are believed to protect chloroplast membranes from photooxidation and help to provide an optimal environment for the photosynthetic machinery (Fryer, 1992; Munne-Bosch & Alegre, 2002a).

Vitamin E deficiency is rare in humans and the RDA is met quite well in the normal daily diets of an individual. But the real benefits of vitamin E lie in the higher intakes of this vitamin. When taken at therapeutic doses over a sustained period of time, vitamin E is implicated in decreased risk of cardiovascular diseases and some cancers, improved immune function and slowing of the progression of a number of degenerative conditions. The safe upper intake limits of the vitamin E are 100X RDA. Such high intakes of vitamin E are not possible through the normal dietary regimen even if deliberate attempts to ingest large amounts of vitamin E rich foods are made. This is so because majority of the sources are poor in the most active form of vitamin E, the α-tocopherol.

The observation that seed oils, the major dietary source of vitamin E, have much of their pools as γ-tocopherol whereas α-tocopherol makes only a minor fraction of this pool has led to the realization that the γ-tocopherol methyl transferase (γ-TMT) activity catalyzing the conversion of γ- to α-tocopherol could be limiting. This fact was testified by shifting the tocopherol profile in favor of α-tocopherol through the overexpression of γ-tocopherol methyl transferase in the model plant Arabidopsis thaliana (Shintani & DellaPenna, 1998) and later in Latusa sativa (Cho et al., 2005). As different oil seeds are prevalent in the food habits of people in different regions of the world, efforts are needed to tailor the α-tocopherol content in different crops so as to benefit the populace. Brassica juncea (Indian mustard) is an important oil seed crop of the Indian subcontinent. Mustard 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 *Brassica juncea* seeds, γ-tocopherol was found to be the major form present (86%) while α-tocopherol made only a minor fraction (10%) of the pool. Thus, there was a huge scope for converting the γ-tocopherol to α-tocopherol.

The objective of the present work was to increase the α-tocopherol content of *Brassica juncea* by overexpression of the *Arabidopsis thaliana* γ*TMT* cDNA through *Agrobacterium tumefaciens* mediated genetic transformation. Cloning of the *Brassica juncea* homologue of γ*TMT* gene was successfully achieved. The regulation of α-tocopherol levels as well as the performance of the transgenic plants under abiotic stresses was studied.

The highlights of the present investigation are as follows:

- A γ*TMT* cDNA (*BjTMT*, Accession no. DQ864978) of 1186bp was cloned from *Brassica juncea* cv. Varuna using an RT-PCR based approach. The sequence had an ORF of 1044 bp and a 3′ non coding region of 142 bp. The ORF encodes a protein of 347 amino acids with a molecular mass of 38.2 kDa and a pI of 6.47.
- The *BjTMT* cDNA sequence showed significant homology to the other known γ*TMT* sequences at both the nucleotide and amino acid level. At the nucleotide level it showed 98% and 97% homology, respectively, to the γ*TMT* sequences of other two *Brassica* species, *B. napus* and *B. oleracea* and 88% homology to γ*TMT* sequence of the closely related *Arabidopsis thaliana*.
- Sequence analysis using ChloR P1.1 server revealed a putative NH₂-terminal chloroplastid transit peptide of 51 amino acid length, consistent with the plastidic localization of tocopherol biosynthesis. The deduced amino acid sequence showed the presence of two highly conserved S-adenosylmethionine (SAM)-binding domains characteristic of sterol-C-methyl transferases.
- Dendrogram analysis of the *BjTMT* amino acid sequence with the other γ*TMT* sequences present in the database showed that *B. juncea* formed a separate group along with other dicotyledonous plants and was closely related to *B. napus*, *B. oleracea* and *A. thaliana*.
- PCR amplification of the genomic DNA of *B. juncea* with γ*TMT* specific primers resulted in a fragment of ~2.4 kb length suggesting the presence of
intron(s) in the gene. Southern blot analysis of the genomic DNA of *B. juncea* using *BjTMT* as a probe revealed that *γ-TMT* gene could have multiple copies in *B. juncea*.

- The tocopherol content and composition was determined in three diploid (*B. campestris, B. nigra*, and *B. oleracea*) and three amphidiploid (*B. juncea, B. napus* and *B. carinata*) species of *Brassica*. The total tocopherol content varied from 432 ng/mg seed in *Brassica nigra* to 781.1 ng/mg seed in *B. campestris*. Alpha tocopherol was present in lesser amounts as compared to γ-tocopherol that made the bulk of the pool. The only exception was *B. carinata* where the content of α-tocopherol was more than the γ-form. The α/γ tocopherol ratio was 0.41 in *B. oleracea*, 0.08 in *B. nigra*, 0.73 in *B. napus*, 1.34 in *B. carinata*, 0.44 in *B. campestris*, and 0.15 in *B. juncea*.

- The different forms of tocopherols present in the commonly grown Indian cultivars of *Brassica juncea* viz., Varuna, Pusa Bold, Jaikisan, Jagannath and Agarni, were analysed to check for any naturally occurring varieties having high content of α-tocopherol. The total tocopherol content varied from 460 ng/mg to 532 ng/mg of the seed. The tocopherol profile in various varieties of *B. juncea* was similar to that observed in other plants with γ-tocopherol present in highest proportion (~80-90%), while the α-tocopherol made only a small percentage (~9-12%).

- The *γ-TMT* cDNA from *A. thaliana* was cloned under the control of constitutive CaMV35S promoter in a binary vector, pCAMBIA 1301. The vector had *gus* as the reporter gene, *hygromycin phosphotransferase II* (*hptII*) as the plant selectable marker gene, and *neomycin phosphotransferase II* (*nptII*) as the bacterial selection gene. The construct was named as pCAM-ΤMT.

- *B. juncea* cv. Varuna was transformed using *A. tumefaciens* strain GV3101 harbouring pCAM-ΤMT.

- The T0 and T1 transgenic plants were characterized at the molecular level by PCR, Southern and Northern blot analysis. All the transgenic lines analyzed had a single copy insertion barring two lines which had two copies of the transgene. The *γ-TMT* transgene was found to be over expressed in all the transgenic lines.
The tocopherol content and composition was analyzed in the seeds of the T1 transgenic lines and the untransformed control plants using HPLC. The seeds of untransformed control *Brassica juncea* plants had a total tocopherol content of 572 ng/mg seed weight with ~86% γ-tocopherol and ~10% α-tocopherol. In the seven T1 transgenic lines analyzed, though the total tocopherol content was almost equal to that of untransformed controls, α-tocopherol levels were as high as 62% of the total pool, that is, an increase of about 6 fold over the control plants was obtained. The increase in the level of α-tocopherol in the transgenic plants corroborated well with expression levels of the transgene.

The transgenic plants overexpressing the γ-*TMT* gene had similar morphology and growth pattern as compared to the untransformed control plants and there was no significant yield penalty with respect to plant biomass and seed set.

The regulation of tocopherol levels was studied under salt (NaCl), drought (mannitol) and heavy metal (CdCl₂) stress conditions. The tocopherol content was found to increase initially with the level of stress under all the three conditions but decreased after a threshold level of stress. Both α- and γ-tocopherol levels were increased initially but later γ-tocopherol accumulated, signifying that γ-*TMT* activity could be limiting. This fact was testified by the observation that the transgenic plants accumulated more of α-tocopherol in comparison to the levels present in the untransformed control plants exposed to the same degree of stress.

Retardation of leaf disc senescence and the germination and growth of seeds on medium supplemented with the stress inducing agents were used as preliminary tests to confirm the plausibility of the assumption that increased α-tocopherol content should confer advantage to the plants in tolerating the abiotic stresses better. While the leaf discs from the transgenic plants showed delayed senescence at all the concentrations in each of the three cases (salt, heavy metal and drought stress), the leaf discs from the untransformed control plants showed early bleaching. Measurement of the chlorophyll content of the leaf
discs confirmed the phenotypic differences. The germination and growth of the T1 transgenic seeds on medium supplemented with different concentrations of stress inducing agents was found to be better than those of the untransformed control plants, thereby, further strengthening the notion of increased α-tocopherol levels being helpful to the plants in withstanding the abiotic stresses.

Conclusions and Future Prospectives

Alpha tocopherol is one of the most powerful antioxidants present in the living organisms. In animals and human beings it is known to help in keeping several diseases at bay, while in plants it is important in overcoming the environmental stress conditions. 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 intakes to the masses and helping the plant itself to withstand the abiotic stresses.

The studies on animal models by feeding the transgenic seeds enriched with vitamin E would confirm the therapeutic effects. This would thus, require the removal of marker genes to avoid the controversies of GMOs. There are several approaches which can be used for getting rid of the marker genes based on the cre-lox system and co-transformation.

Further increases 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 which will mark the next milestone for this work. Studies on the better performance of high α-tocopherol containing transgenic plants of Brassica juncea under abiotic stress conditions as compared to the untransformed control plants needs to be carried forward by looking at the lipid peroxidation status of the transgenic plants vis-à-vis the untransformed control plants and the interplay of the increased α-tocopherol content with the other antioxidants in the cell.