Chapter 5: Discussion
Vitamin E, an important fat soluble vitamin essential for human beings, is synthesized only by plants and some cyanobacteria. Of the 8 different forms of vitamin E, α-tocopherol has the highest biological activity and its intake at therapeutic doses is associated with decreased risk of several diseases and promotion of good health. Plant seed oils which are the major dietary source of tocopherols are usually poor in this form but abound in its immediate precursor, the γ-tocopherol. This observation led DellaPenna et al., (1998) to hypothesize that γ-tocopherol methyl transferase (γ-TMT) enzyme catalyzing the methylation of γ-tocopherol to α-tocopherol is limiting, and showed, subsequently, that its overexpression in transgenic Arabidopsis thaliana plants shifted the tocopherol pool in favour of the α-form. In the present study, the feasibility of such an approach was studied in engineering the α-tocopherol content of Brassica juncea, an important oilseed crop of the Indian subcontinent. The tocopherol content and composition of various Brassica species and different cultivars of Brassica juncea was checked to look for any naturally occurring high α-tocopherol containing varieties of Brassica. The Brassica juncea homolog of γ-TMT gene was also cloned and studied. As tocopherols have very potent antioxidant properties, the performance of high α-tocopherol containing transgenic plants was assessed under various oxidative stress causing abiotic conditions. The results obtained in the present study have been discussed in the following sections.

5.1 Cloning of γ-tocopherol methyl transferase homolog, BjTMT, from Brassica juncea

An RT-PCR based approach was used to clone γ-TMT cDNA from Brassica juncea cv. Varuna. A set of primers designed from the conserved regions of the aligned cDNA sequence of 3 closely related species B. napus, B. oleracea, and A. thaliana was used to amplify the total RNA from B. juncea leaves. Three fragments of sizes ~1.5 kb, ~1.1 kb, and ~0.9 kb were amplified. The ~1.1 kb fragment which is similar in size to the known γ-TMT cDNA sequence from other plants was cloned and sequenced. The 1186 bp sequence showed homology to other γ-TMT sequences
present in the genbank and, therefore, was confirmed to be the \( \gamma \)-TMT homolog in *Brassica juncea*. The clone was named as *BjTMT*. The amplification of 2 extra fragments in the RT-PCR reaction could be due to non-specific annealing of the primers or non-optimal conditions for the PCR. The *BjTMT* cDNA encoded a \( \gamma \)-TMT protein of 347 amino acids with a molecular weight of 38.2 kDa and a pI of 6.47. The analysis of the deduced amino acid sequence using the Chloro P1.1 program revealed the presence of an NH\(_2\)-terminal chloroplastid transit peptide of 51 amino acid length, consistent with the known intercellular locale of the tocopherol biosynthesis (Soll et al., 1985). The protein was also predicted to have 20 phosphorylation sites, which suggests the possible involvement of phosphorylation/dephosphorylation events in the regulation of its activity, though no such studies have been carried out, as yet. The deduced amino acid sequence also showed the presence of highly conserved SAM binding domains characteristic of methyl transferases (Kagan & Clarke, 1993).

The sequence alignment of *BjTMT* cDNA with the other known \( \gamma \)-TMT cDNA sequences present in the database showed a significant homology at both the nucleotide and amino acid level. It showed maximum identity with *B. napus* and *B. oleracea* (98% and 98%, respectively) at the nucleotide level and an identity of 99% and 97% at the protein level, respectively. The closest identity with *B. napus* was expected as both *B. juncea* and *B. napus* share a common parent, *B. rapa*.

A dendrogram formed from the sequences of \( \gamma \)-TMT present in the database showed that the sequences of \( \gamma \)-TMT from the dicotyledonous and monocotyledonous plants formed two separate groups. However, the *S. tuberosum* \( \gamma \)-TMT formed a separate branch and was more close to the monocot group.

To check for the number of copies of \( \gamma \)-TMT in the genome of *B. juncea* Southern blot analysis was performed. Multiple bands were seen in the blot probed with *BjTMT* cDNA suggesting the presence of multiple copies of \( \gamma \)-TMT in *B. juncea* genome. As *B. juncea* is an amphidiploid species derived from *B. rapa* and *B. nigra*, the presence of multiple copies of the gene could be due to the inheritance of the gene from the two different parents. The other possible reason for the multiple bands
seen in the Southern analysis could be the presence of the sites for the restriction enzymes used in the analysis within the intronic region(s) of the \( \gamma \)-TMT gene.

5.2 Tocopherol profile in \textit{Brassica} species

The total content and composition of tocopherols vary quite considerably in various plants and also within different organs of a plant. Seeds are the major repository of tocopherols but the content of \( \alpha \)-tocopherol in them is quite low. On the other hand, though \( \alpha \)-tocopherol is present in highest proportion in leaves, their total tocopherol content is very low. In this study, the tocopherol levels were estimated in the three diploid species, \textit{B. campestris} (\textit{B. rapa}), \textit{B. nigra} and \textit{B. oleracea}, and three amphidiploid species \textit{B. juncea}, \textit{B. napus} and \textit{B. carinata}. The total content varied from 432 ng/mg seed in \textit{B. nigra} to 781.1 ng/mg seed in \textit{B. campestris}. The proportion of \( \alpha \)-tocopherol was low as compared to that of \( \gamma \)-tocopherol, as is typical of other oil seeds (Ching & Mohamed, 2001). The tocopherol profile in the seeds of \textit{B. carinata} was an exception where the content of \( \alpha \)-tocopherol was ca. 1.3 times more than that of \( \gamma \)-tocopherol. Natural variation in tocopherol composition in 5 commonly grown cultivars of \textit{B. juncea} was also estimated in the present study. The content and composition in the varieties studied was not very much different and \( \alpha \)-tocopherol made only about 9-12% of the total tocopherol pool. Thus, this study shows that different \textit{Brassica} species that are the major source of edible oil and, are also used as vegetables, are poor in \( \alpha \)-tocopherol. Therefore, this system offered a great source of improvement in \( \alpha \)-tocopherol content.

5.3 Increase in \( \alpha \)-tocopherol content in \textit{Brassica juncea} transgenics by overexpression of \textit{Arabidopsis thaliana} \( \gamma \)-TMT gene

From the present study, the \( \alpha \)-tocopherol content in the seeds of different varieties of \textit{Brassica juncea} was found to be only 9-12% of the total tocopherol pool in comparison to its immediate precursor, the \( \gamma \)-tocopherol which contributes ~86%. This suggests that \( \gamma \)-TMT, the enzyme catalyzing the conversion of \( \gamma \)-tocopherol to \( \alpha \)-tocopherol, is probably limiting. Therefore, an attempt was made to increase the
content of α-tocopherol in B. juncea by overexpression of γ-TMT gene from A. thaliana using Agrobacterium mediated transformation.

The γ-TMT gene was expressed under the control of CaMV35S promoter using the method of Pental et al. (1993). The transgenic plants obtained were characterized for the stable integration of the transgene. The Southern blot analysis showed the presence of the γ-TMT cDNA in the genome of the transgenic plants. The copy number of the transgene was determined from both the right and left border of the T-DNA. This helps to identify the events which have more than one copy integrated as repeats at one locus at either end. While left border analysis showed single copy integration in all the 7 transgenic lines analyzed, two lines viz. 5 and 10.1 showed integration of two copies at the right border. The northern blot showed the overexpression of the γ-TMT mRNA in all the transgenic lines as compared to the untransformed control plant. The level of expression in the different lines varied. This could be due to the positional effect of the integration of the transgene. Also, the expression in the lines 5 and 10 was lower as compared to the other lines. This could be explained by the gene silencing effect of multiple copies of the transgene integrated in these lines (De Wilde et al., 2000; Wolters & Visser, 2000).

The levels of different tocopherol forms were determined in various transgenic lines and the untransformed control plants. It was observed that, though the total tocopherol content in the transgenic plants was similar to that in the untransformed control plants, the ratio of α-/γ- tocopherol was higher in the transgenic plants and was consistent with the overexpression of γ-TMT gene in the different lines. These results are in conformation with the earlier reports where a similar strategy was employed to increase the α-tocopherol content of plants. Overexpression of γ-TMT gene from A. thaliana has been shown to increase the α-tocopherol levels without significantly changing the total tocopherol content in Arabidopsis thaliana (Shintani & DellaPenna, 1998) and Lactuca sativa (Cho et al., 2005).

A correlation between γ-TMT transgene mRNA and the content of α-tocopherol indicates that the expression of the transgene is controlled at the transcriptional level. Similar transcriptional control of the γ-TMT transgene expression was also
demonstrated in *Arabidopsis* (Shintani and DellaPenna, 1998), and the modulations of many metabolic pathways have been directed by the transcriptional control of the transgenes (Stitt and Sonnewald, 1995).

The overexpression of a pathway enzyme and change in tocopherol profile was not found to have any adverse effect on the growth and yield of the transgenic plants, signifying that there was no undue drain of intermediates from any other metabolic pathway. The transgenic plants appeared healthy and compared well with the various growth parameters of normal untransformed control plants.

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 phytol 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 *Lauca 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 our country and is recommended for health purposes, is obtained from *B. juncea*. 

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5.4 Regulation of tocopherol levels under abiotic stress conditions

The tocopherol levels and composition in plants vary in different tissues and are regulated during the developmental stages and in response to abiotic stresses. While the seeds predominantly accumulate γ-tocopherol, in leaves α- form makes the bulk of the pool (Bramley et al., 2000; Franzen & Haas, 1991; Shintani & DellaPenna, 1998). Increases in the α-tocopherol levels have been reported to occur with ageing and senescing of plants (Rise et al., 1989; Molina-Torres & Martinez, 1991; Tramontano et al., 1992). The tocopherol levels have also been found to increase in response to a variety of abiotic stresses including high light, drought, salt, and cold. These increases in the tocopherol content have been suggested to possibly protect cellular components from increased oxidative stress (Munne-Bosch & Alegre, 2002) and may provide an additional line of defense against oxidative damage (Havaux et al., 2000; Munne-Bosch & Alegre, 2002).

In the present investigation, the regulation of tocopherol levels was studied under three different abiotic stresses, namely, salt, heavy metal and drought stress, in the cotyledonary leaves of untransformed control and high α-tocopherol containing transgenic plants. The tocopherol content was found to increase in the beginning with the level of stress under all the three conditions but decreased after a threshold level of stress. The levels of both α- and γ-tocopherol initially but later γ-tocopherol accumulated. This could signify that γ-TMT activity might be limiting. This could be resolved by the observation that the transgenic plants accumulated more α-tocopherol in comparison to the levels present in the untransformed control plants exposed to the same degree of stress. Similar results have been reported by Collakova & DellaPenna (2003) in Arabidopsis thaliana leaves where they observed an increase in the total content of tocopherols and accumulation of γ-tocopherol under stress. The accumulated γ-tocopherol was converted to α-tocopherol in the transgenic Arabidopsis plants overexpressing the γ-TMT gene.
5.5 Increased α-tocopherol and tolerance to abiotic stresses

The tocopherols are one of the most important antioxidants present in the cell and are involved in quenching and scavenging various reactive oxygen species and act as recyclable chain reaction terminator of poly-unsaturated fatty acid free radicals generated by lipid peroxidation (Fryer, 1992; Kamal-Eldin & Appelquist, 1996; Bramley et al., 2000; Fukuzawa & Gebicky, 1983; Neely et al., 1988; Tappel, 1962; Burton & Ingold, 1986; Esterbauer, 1991). To test the plausibility of the assumption that the increased α-tocopherol content should confer advantage to the plants in tolerating the abiotic stresses better, the T1 transgenic plants of *B. juncea* over expressing the γ-TMT gene and the untransformed control plants were subjected to stress tolerance tests. Their performance was compared using leaf disc senescence test and by the germination of the seeds and growth of the seedlings on medium supplemented with the stress inducing agents. The leaf discs from the transgenic *Brassica juncea* plants over-expressing γ-TMT gene showed significant tolerance against high concentrations of NaCl, CdCl₂ and mannitol as compared to the untransformed control plants. This was evident by delayed bleaching or senescence and presence of high chlorophyll content in the leaf discs obtained from the transgenic plants as compared to the untransformed control plants. Also, the shoots of transgenic *Brassica juncea* plants kept on medium supplemented with NaCl, CdCl₂ and mannitol appeared healthy and showed better growth than those of untransformed control plants.

The mechanism for the better performance of the transgenic *Brassica juncea* plants as compared to the untransformed control plants still remains to be elucidated. However, it can be speculated that the higher levels of α-tocopherol in the transgenic plants could be strengthening the defense mechanism of the plants against the reactive oxygen species that are known to be produced during the abiotic stresses (Fryer, 1992; Mittler, 2002). Tocopherols are thought to represent key antioxidants protecting the membrane lipids from peroxidation by quenching and scavenging.
these reactive oxygen species (Fryer, 1992). Further work needs to be done to unravel the mechanism involved and explore the interaction of increased α-tocopherol in the transgenic plants with other antioxidants present in the cell, like ascorbate and glutathione, and the different antioxidant enzymes.