Characterization Of Plant Tocopherol Cyclases

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Introduction

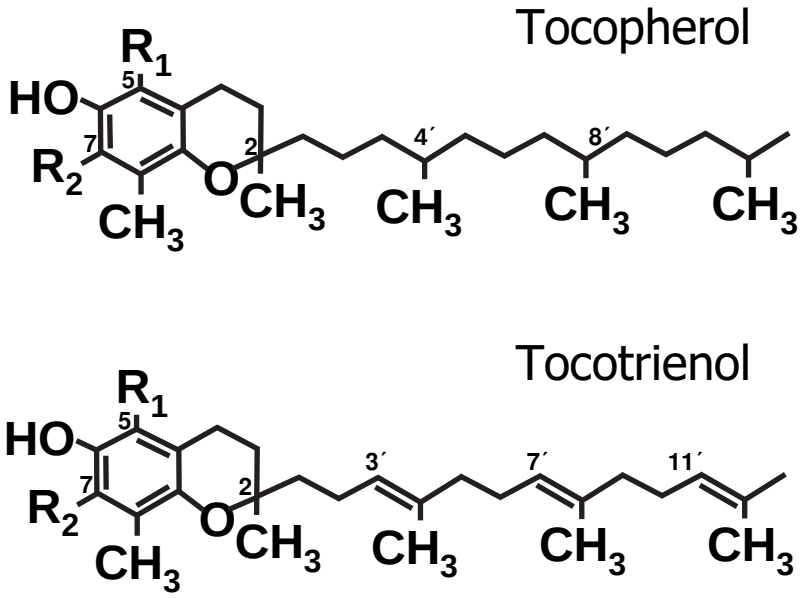
1.1 Vitamin E: What is it?

Plants produce a vast and diverse assortment of organic compounds that not only perform vital functions in plant cells but also are essential or beneficial in human nutrition. One such class of compounds consists of tocopherols, which are collectively known as vitamin E. Vitamin E is a fat-soluble vitamin that encompasses a family of eight structurally related tocopherols and tocotrienols each with a different biological activity, which is the measure of potency or functional use in the body (Traber and Packer, 1995). The IUPAC-IUB commission on Biochemical Nomenclature recommended that the term vitamin E should be used as a generic description for all tocopherol and tocotrienol derivatives qualitatively exhibiting the biological activity of α-tocopherol. Eavan and Bishop (1922) coined the term "vitamin E" for an essential micronutrient in reproduction of rats. Thereafter, physiological functions of vitamin E were associated in cellular antioxidant systems as factor 2 together with sulfur amino acids as factor 1 and selenium as factor 3 (Schwarz, 1965). Various antioxidant and non antioxidant functions of vitamin E, such as to protect the cells against the effects of free radicals, to prevent or delay the development of chronic diseases like cardiovascular diseases, atherosclerosis, and to prevent cancer, have been reported (Stampfer et al., 1993; Rimm et al., 1993; Ricciarelli et al., 2002; Schneider, 2005).

1.2 Chemical structure, occurrence and subcellular localization of vitamin E

Chemically, vitamin E comprises a group of lipophilic tocochromanols. The basic structural unit of these compounds is a chromanol ring system (2-methyl-6-hydroxychromanol) with a hydrophobic side chain of 16 C atoms, derived from a prenyl group. Hence, tocochromanols

are amphipathic in nature. It is assumed that the hydrophobic prenyl tail is located in the membrane associated with the acyl chains of the membrane lipids whereas the polar chromanol head group lies at the membrane-cytosol interface where it can interact with cytosolic biomolecules (Fryer, 1992). The two major homologous series of tocochromanols are the tocopherols and tocotrienols, both showing vitamin E activity in animals. The structure of tocopherol and tocotrienol homologues is shown in Fig. 1.1. Tocopherols contain the phytyl



Name	R1	R2
α-Tocopherol/Tocotrienol	-CH_3	CH_3
β-Tocopherol/Tocotrienol	-CH_3	-H
γ-Tocopherol/Tocotrienol	-H	-CH_3
δ-Tocopherol/Tocotrienol	-H	-H

Figure 1.1: The structure of tocopherols and tocotrienols.

side chain, obtained from reduction of prenyl tail. Unlike the side chain of the tocopherols, that of tocotrienols is derived from geranylgeranyl group. Consequently tocotrienols have an unsaturated side chain of 16 C with trans double bonds at its 3´, 7´and 11´positions. The number and substitution pattern of methyl groups at the chromanol head ring define the four different isomers (α, β, γ and δ) of tocopherols and tocotrienols. The stereo-chemistry of tocopherols and tocotrienols differs. Three chiral centers at carbons 2, 4´ and 8´ of the tocopherols enable eight stereoisomers (2^3). The naturally occurring isomers have the R-configuration at all three positions (RRR, 2D, 4´D, 8‘D, d-tocopherols or (+)- tocopherols), while chemically synthesized isomers are found in a racemic mixture of all stereoisomers.

Therefore, the antioxidant activity of chemically synthesized tocopherols and tocotrienols is almost one half of that of natural forms (Ingold et al., 1990). On the other hand, natural tocotrienols have a single isomer structure and the 2D, 3´trans; 7´trans configuration is found in all four isoforms (Kamal-Eldin and Appelqvist, 1996). The presence of the hydroxyl group at C-6 of the chromanol ring in the tocochromanols is important for their activity as an antioxidant. The chemical structure of vitamin E analogs favors a hydrogen donating power in the order $\alpha > \beta > \gamma > \delta$ (Pokorny, 1987).

Plants and other photosynthetically active organisms synthesize tocopherols but they are also found in fungi, algae, and animals, although they cannot synthesize them (Lichtenthaler, 1968; Singh et al., 1990; Grusak and DellaPenna, 1999). Lichtenthaler (1968) reported the occurrence of tocopherols in all photosynthetic organisms examined with the exception of the cyanobacterium *Anacystis nidulans*, and certain *Synechococcus* species, which are devoid of all forms of tocopherols (Powls and Redfearn, 1967; Dasilva and Jensen, 1971; Thomas et al., 1998).

The main source for the dietary uptake of tocopherols is plant food such as vegetables, fruits, seeds and plant seed oils. Tocopherols are also present in roots, tubers, cotyledons, hypocotyls, stems, leaves, and flowers of higher plants. α-tocopherol is most abundant in all parts of plants except seeds, where γ-tocopherol predominates with few exceptions (Franzen et al., 1991; Bartoli et al., 1997; Grusak and DellaPenna, 1999). The composition and the content of the different tocopherol components in plant tissues varies considerably, ranging from the low levels found in potato tuber ($<1\mu$g/g dry weight) to very high levels found in seeds (>1mg/g dry weight, Table 1.1) (Grusak and DellaPenna, 1999). Some plants also contain high levels of tocopherols in leaves. In contrast to tocopherols, tocotrienols are the major tocochromanols in seeds of most monocotyledonous plants and a limited number of dicots plants (Grusak and DellaPenna, 1999). They typically account for more than 50% of the total vitamin E antioxidants in the seed endosperm of monocots, including palm and agronomically important cereals such as rice, wheat and oats (Cahoon et al., 2003).

With in the plant cells, tocopherols and tocotrienols have been localized in plastids in either amyloplasts of seeds and tubers, chloroplasts of photosynthetic tissue, leucoplasts of petals or chromoplasts of fruits (Lichtenthaler et al., 1981; Fryer, 1992). Although a major fraction of α-tocopherol (48 to 57%) was obtained from chloroplasts, vacuoles of barley leaves and microsomal membranes of soybean roots were also found to contain small fractions of α-tocopherol (Rautenkranz et al., 1994; Caro and Puntarulo, 1996). In plastids, α-tocopherol is mainly detected in the inner envelope membranes, the site of synthesis (Soll et al., 1985;

Table 1.1: Tocopherol levels and composition in selected crops and plant oils (Grusak and Dell-Panna, 1999).

Plant and organ	Total tocopherol (μg/g fresh weight)	Percent α-tocopherol	Percent others and major types
Potato tuber	0.7	90	10% γ, β-tocopherols
Lettuce leaf	7.5	55	45% γ-tocopherol
Cabbage leaf	17	100	-
Spinach leaf	30	63	5% γ-tocopherol, 33% α-tocopherol
Synechocystis sp. PCC6803	10	95	5% γ-tocopherol
Arabidopsis leaf	40	90	10% γ-tocopherol
Arabidopsis seed	350	1	95% γ-tocopherol
Oil plam leaf	300-500	100	-
Palm seed oil	500	25	30% α-tocotrienol 40%γ-tocotrienol
Rapeseed oil	500-700	28	73% γ-tocopherol
Sunflower seed oil	700	96	4% γ, β-tocopherol
Corn seed oil	1000	20	70% γ-tocopherol

Arango and Heise, 1998), in plastoglobuli (Lichtenthaler et al., 1981; Grumbach, 1983) and in thylakoid membranes (Fryer, 1992; Havaux, 1998). It is assumed that most of the α-tocopherol is partitioned between the chloroplastidial envelope and the thylakoid and is stored in plastoglobuli in some cases only (Munne-Bosch and Alegre, 2002). In spinach, one third of the total α-tocopherol is located in the envelope membranes and the remaining two thirds in the thylakoids (Wise and Naylor, 1987). The molar ratios of α-, β-, and γ-tocopherol in the thylakoids of spinach is $1 : 0.06 : 0.02$, which is a reflection of their biological *in vivo* potencies in terms of their relative abundance (Asada and Takahashi, 1987).

1.3 Functional roles of vitamin E

1.3.1 Antioxidant functions

The antioxidant activity of tocopherols and tocotrienols is largely correlated with their ability to donate its phenolic hydrogen to lipid free radicals and is favored by (1) the degree of methylation of the chromanol ring ($\alpha > \beta \cong \gamma > \delta$), (2) the size of the heterocyclic ring, (3) the stereochemistry at position 2, and the length of the side chain. The degree of unsaturation at the side chain, for instance in tocotrienols, can also affect the solubility in membranes and ultimately contribute to improve their antioxidant capacity. The α-tocotrienol has been shown to be more effective than α-tocopherol to scavenge free radicals and reduce lipid peroxidation in a model membrane system (Serbinova and Packer, 1994; Packer et al., 2001).

The relative antioxidant capacity of tocopherols *in vivo* was found to be in the following order $\alpha > \beta > \gamma > \delta$ (Burton and Ingold, 1986; Burton and Traber, 1990), whereas the reverse order $\delta > \gamma \simeq \beta > \alpha$ was observed *in vitro* in fats, oils, and lipoproteins (Kamal-Eldin and Appelqvist, 1996).

The basic mode of action of tocopherols and tocotrienols to prevent autoxidation of fatty acids is to scavenge lipid peroxy radicals by donating a hydrogen atom. Fatty acids autoxidation or lipid peroxidation proceeds in three phases: initiation, propagation and termination. The action of one of the various forms of activated oxygen, lipoxygenase, heat, light, UV and γ-irradiation or transition metal ions initiate the formation of a carbon-centered radical by the abstraction of a hydrogen atom from the fatty acid substrate. The hydrogen atom located between two double bonds (bis-allylic methylene) of a polyunsaturated fatty acid (PUFA), such as linoleic acid (C18:2) or arachidonic acid (C20:4) is preferred because this C-H bond is the weakest in the molecule. This makes PUFAs prime targets for autoxidation. The carbon radical formed during initiation immediately reacts with the molecular oxygen to form a fatty acid peroxyl radical. This reaction is exceptionally fast and, therefore, reduces the probability of the action of antioxidants on fatty acid radicals. The fatty acid peroxyl radicals encounter another PUFA to abstract hydrogen, thereby forming lipid hydroperoxides and new alkyl radicals for chain propagation. Alternatively, the peroxyl radical can react in a 5-exo-cyclization with an additional double bond on the same fatty acid to yield a 5-membered cyclic peroxide (endoperoxide) and a carbon radical outside the endoperoxide ring. In the other incidence, β-fragmentation of a peroxyl radical leads to a rearrangement of the carbon chain configuration during prolonged autoxidation and produces molecular oxygen and fatty acid radicals due to the loss of oxygen (Schneider, 2005). To terminate the autoxidation, tocopherols and tocotrienols can impart a hydrogen atom to lipid peroxyl radicals before its propagation reaction to form fatty acid hydroperoxide. The abstraction of the 6-OH hydrogen results in the formation of a tocopheroxyl radical. This oxidation of tocopherols and tocotrienols may lead to the formation of various products *in vitro* depending on the type of substrate, polarity of the solvent, light, temperature, and other conditions of the medium. However, in the absence of antioxidants such as ascorbic acid and glutathione, which recycle tocopheroxyl radicals, the radicals may undergo radical-radical coupling with other peroxyl radicals to form adducts. Alternatively, it may disproportion to form quinines or may undergo self-coupling with other tocopheroxyl and tocotrienoxyl radicals to form dimers and/or trimers (Kamal-Eldin and Appelqvist, 1996; Munne-Bosch and Alegre, 2002).

The other essential antioxidant activity of tocopherols is molecular quenching of energy-activated singlet molecular oxygen ($^1O_2$), generated by the interaction of a triplet excited reaction center of chlorophyll (^{3}P680*) with molecular oxygen ($^3O_2$) exposed to high intensity

light (Foote et al., 1974; Melis, 1999; Hideg et al., 2000). In the quenching reaction physical deactivators, like α-tocopherol, donate an electron to the singlet oxygen to form a charge transfer complex that dissociates into α-tocopherol and molecular oxygen after intersystem crossing (Yamauchi and Matsushita, 1977; Thomas et al., 1998). The α-tocopherol could deactivate up to 120 $^1O_2$ molecules by resonance energy transfer before being degraded itself. Additionally, tocopherols may prevent, to a limited extent, the generation of $^1O_2$ by deactivation of the triplet excited endogenous membrane photosensitizers *in vivo* in a similar way to β-carotene (Fryer, 1992).

1.3.2 Membrane stability functions

The absence of tocopherols in cell membranes was found to have an influence on membrane permeability and, in turn, make them susceptible to degradation by endogenous phospholipases *in vivo* (Diplock and Lucy, 1973). Using deuterium-NMR and differential scanning calorimetry, tocopherol incorporation into multilamellar dispersions of deuterated phospholipids of model membranes was shown to induce the broadening of the gel-to-liquid crystalline phase transition curve (Wassall et al., 1986). In subsequent studies a different amount of α-tocopherol was used to determine the stability of model phospholipid bilayer membranes and was shown to inhibit the Ca^{2+} induced fusion of unilamellar vesicles of phosphatidylserine, which forms aggregations in the absence of tocopherol. α-tocopherol was believed to form a complex with membrane lipid components such as free fatty acids or lysophospholipids that have the tendency to destabilize the bilayer structure thereby countering their effects and rendering the membrane more stable (Kagan et al., 1989). This underlying principle of membrane stabilization by α-tocopherol was thought due to the fact that α-tocopherol and asymmetric phospholipids show complementary shapes in model membranes (Salgado et al., 1993).

1.3.3 Functions in photosynthetic electron transport

Studies on the role of α-tocopherol in photosynthetic electron transport have indicated that cyclic electron transport around photosystem II is inhibited by α-tocopherol and stimulated by α-tocopherol quinone, an oxidation product of α-tocopherol present in chloroplasts (Kruk et al., 1997). Later, Kruk and Strzalka (2001) showed that α-tocopherol quinone efficiently oxidizes the reduced cyt b559, and important in cyclic electron flow around photosystem II, when the photosynthetic electron transport chain in overreduced. Moreover, addition of exogenous α-tocopherol decreased the membrane permeability to small ions involved in the generation of a transmembrane electrochemical gradient for ATP synthesis in mitochondria

and chloroplasts (Fryer, 1992). Therefore, α-tocopherol also plays a role in the dissipation of excess energy in thylakoids for the protection of the photosynthetic apparatus.

1.3.4 Tocopherols in intracellular signaling

Over the last few years, tocopherols were found to have pronounced roles in intracellular signaling. They can affect the plant development and stress responses not only by controlling the redox state of chloroplasts but also by regulating the amounts of jasmonic acid, known to be involved in intracellular signaling (Munne-Bosch and Alegre, 2002). Jasmonic acid is synthesized as the secondary oxidation product of lipid hydroperoxides, reaction products of lipid peroxidation. Thus, tocopherols indirectly regulate the concentration of jasmonic acid in cells by controlling the accumulation of lipid hydroperoxides (Schaller, 2001). The intracellular signaling pathway of jasmonic acid is involved in the regulation of gene expression in the nucleus affecting photosynthesis, anthocyanin and antioxidant metabolism, in turn (Creelman and Mullet, 1997). Recently, jasmonic acid was shown to regulate some genes of the tocopherol biosynthetic pathway (Falk et al., 2002). Induction of such genes for jasmonic acid synthesis may explain the increased tocopherol content in plants under stress conditions (Chrost, 1999). Therefore, α-tocopherol could control its own synthesis by regulating lipid peroxidation in chloroplasts and jasmonic acid content within the cell.

On the other hand, intracellular signaling functions of α-tocopherol in animal cells are independent of its antioxidant activity. In animal cells, α-tocopherol inhibits protein kinase C, 5-lipoxygenase, phospholipase A2 and cyclooxygenase, while it activates protein phosphatase 2A and diacylglycerol kinase at a posttranscriptional level (Boscoboinik et al., 1991; Clement et al., 1997; Ricciarelli and Azzi, 1998; Devaraj and Jialal, 1999). The inactivation of protein kinase C was reported to be due to the dephosphorylation of the enzyme by the action of phosphatase 2A activated by α-tocopherol (Clement et al., 1997; Ricciarelli and Azzi, 1998). Alternatively, α-tocopherol could also act on the diacylglycerol pathway by activating diacylglycerol kinase and consequently decreasing diacylglycerol and protein kinase C activation (Koya et al., 1997). The change in physical properties of a membrane due to tocopherols was suggested to have inhibitory effects on phospholipase A2 activity (Grau and Ortiz, 1998). In this study the authors showed that β-, γ-, and δ-tocopherols are weaker inhibitors than α-tocopherol because they are located progressively deeper within the membrane.

1.3.5 Transcriptional regulation functions

Recently, the function of tocopherol in regulating gene transcription has gained considerable interest (Azzi et al., 1998). Dietary supplement of α-tocopherol resulted in an inhibition of

the liver collagen a1(I) gene expression (Chojkier et al., 1998). Human skin fibroblasts exhibit an age-dependent increase of collagenase expression that can be diminished by α-tocopherol (Ricciarelli et al., 1999). The α-tocopherol triggered down-regulation of transcription of the oxidized low-density lipoprotein (LDL) scavenger receptors SR-A and CD36 in smooth muscle cells, monocytes and macrophages, while β-tocopherol was ineffective (Ricciarelli et al., 2000; Devaraj et al., 2001). α-tocopherol can also weakly induce the expression of α-tropomyosin (Aratri et al., 1999) and connective tissue growth factor (Villacorta et al., 2003). Recently, a tocopherol-associated protein was identified as a ligand-dependent transcription factor which was translocated into the nucleus upon binding with α-tocopherol (Yamauchi et al., 2001).

1.4 Vitamin E in human disorders

1.4.1 Neurological disorders

Vitamin E deficiency in human beings is characterized by very low levels of tocopherol in plasma that cause severe debilitating spinocerebral lesions. An autosomal recessive neurodegenerative disease called ataxia with isolated vitamin E deficiency (AVED) is a rare form of vitamin E deficiency in which patients have an impaired ability to incorporate α-tocopherol into lipoproteins in liver (Gotoda et al., 1995). Reduced α-tocopherol transfer protein gene expression might result in a reduced plasma level of α-tocopherol (Wu et al., 1997). A number of mutations identified in α-tocopherol transfer protein gene of AVED patients have been defined as molecular basis of the AVED syndrome which causes reduced α-tocopherol concentrations in plasma and tissue (Gotoda et al., 1995; Ouahchi et al., 1995; Traber and Arai, 1999). These low levels of α-tocopherol in plasma are elevated by dietary vitamin E supplementation, which resulted in stabilization or improvement of the neurologic functions (Schuelke et al., 1999). It is unknown whether the degenerative neurological symptoms in patients with vitamin E deficiency syndrome are the result of an insufficient protection by antioxidant effects or of a lack of specific and non-antioxidant effects mediated by α-tocopherol.

1.4.2 Atherosclerosis

The development of atherosclerosis proceeds with the accumulation of oxidized LDL in the arterial wall, which is scavenged by specific scavenger receptors on macrophages. Subsequently, macrophages are converted into lipid-laden foam cells due to the uptake of oxidized LDL and are deposited as fatty streaks on the artery wall (Witztum and Steinberg, 1991). Compelling evidence shows that vitamin E inhibits the oxidation of LDL, which is probably

involved in lesion initiation and progression of atherogenesis (Ferns et al., 1993). The protective role as an antioxidant has been questioned by the discovery of the phenomenon of tocopherol-mediated peroxidation in which α-tocopherol can act as pro-oxidant. Studies on the action of α-tocopherol to decide whether it acts as anti- or pro-oxidants are inconclusive (Upston et al., 1999, 2003). Recently, nonantioxidative functions of α-tocopherol have gained considerable interest and suggested that it may also act in prevention of atherosclerosis by the following mechanisms (i) inhibition of monocyte-endothelial cell adhesion; (ii) inhibition of platelet adhesion and aggregation; (iii) inhibition of cyclooxygenase-2 and 5- lipoxygenase, and (iv) inhibition of SR-A and CD36 (Schneider, 2005).

Due to a large growing body of epidemiological evidence, several randomized and placebo-controlled trials with large populations have been conducted to assess the effect of vitamin E on the prevention of such diseases. The outcome of these clinical trials is apparently contradictory to the role of vitamin E. It is evident that the selection of the population in terms of age, sex, smoking habit and diet may have strongly affected the possible outcome on the efficacy of vitamin E (Ricciarelli et al., 2002).

1.4.3 Cataract

The abnormal growths of the lens in the eye is called cataract. It clouds vision and increases the risk of disability and blindness in aging adults. Observational studies on antioxidants to determine whether they can prevent or delay cataract growth have found that lens clarity was better in regular users of vitamin E supplements and in persons with higher blood levels of vitamin E (Leske et al., 1998). A study of middle-aged male smokers, however, did not demonstrate any effect from vitamin E supplements on the incidence of cataract formation (Kappus and Diplock, 1992). The effects of smoking, a major risk factor for developing cataract, may have overridden any potential benefit from vitamin E, but the conflicting results also indicate a need for further studies before researchers can confidently recommend extra vitamin E for the prevention of cataract.

1.4.4 Cancer

Vitamin E protects cell membranes against the detrimental effects of free radicals, which may lead to the development of chronic diseases such as cancer. Human observational studies provide further support by showing, on the one hand that oxidant stress increases with clinical progression of breast cancer and, on the other hand, that a diet rich in antioxidant-containing food reduces the risk of certain cancers (Steinmetz and Potter, 1996). As a cancer preventive agent, vitamin E acts in synergy with selenium, preventing cell transformation by

x-irradiation, suggesting its use in protecting normal cells against the potential late effects of secondary cancers following radiotherapy. Vitamin E has been found to act selectively as an anticancer drug, alone or in combination with chemotherapy and radiation.

An analogue of vitamin E, α-tocopherol succinate, has been shown to inhibit growth of a variety of cancer cells (Prasad et al., 2003). The molecular action of α-tocopherol succinate includes the induction of apoptosis by inhibition of protein kinase C via increasing protein phosphatase 2A activity (Neuzil et al., 2001). The apoptotic effect was more efficient in certain human prostate cancer cell lines. Additional treatment with the selenium agent, methylseleninic acid, resulted in synergistic effects (Zu and Ip, 2003). Zhang et al. (2004) showed that α-tocopherol succinate could inhibit cell invasiveness in three different prostate cancer cell lines possibly due to reduced levels of matrix metalloproteinases involved in the proteolysis of the basement membrane during invasion. Studies on the anti-cancer roles of γ-tocopherols demonstrate that γ-tocopherol has the ability to scavenge the mutagenic oxidant peroxynitrite by forming stable carbon-centered adducts (Christen et al., 1997) and is involved in the down-regulation of cyclins D1 and E to inhibit the cell cycle progression of prostate carcinoma cells (Galli et al., 2004). In summary, there is strong *in vitro* evidence for α-tocopherol succinate and γ-tocopherol to be useful in the prevention and treatment of cancer.

In the view of the suggested protective effects of vitamin E seen in observational studies, several clinical and intervention trials were carried out to evaluate the anti-cancer roles of tocopherols. The most persuasive evidence for a protective role of vitamin E is in carcinomas of the prostate and gastrointestinal tract (Hartman et al., 1998). There was less supportive indication for the beneficial role in breast, ovarian, lung, pancreatic, or urinary tract cancers. The main problem of most trials was in attributing the observed effects to vitamin E supplementation or to confounding factors such as generally healthier diets and lifestyles among participants taking vitamins (Sung et al., 2003). Hence, although *in vitro* studies gave evidence for the protective role of vitamin E, the efficacy of vitamin E has to be determined in clinical trials.

1.5 Biosynthesis of tocopherols

The biosynthetic pathway of tocopherols was unveiled in higher plants and algae three decades ago from precursor and product studies using radio tracer intermediates. These lipid-soluble antioxidants are only synthesized by photosynthetic eukaryotes and various

oxygenic cyanobacteria (Lichtenthaler, 1968; Soll et al., 1985). In plants, the site of tocopherol biosynthesis is located in the inner envelope membrane of the plastids along with the site for the synthesis of the multifunctional family of lipid soluble compounds called prenyllipids including phylloquinone and plastoquinone (Whistance and Threlfall, 1970; Soll et al., 1985). These prenyllipids share structural resemblance that comprises hydrophobic isoprenoid tails of various lengths attached to aromatic head groups. The schematic representation of the tocopherol biosynthetic pathway is shown in Fig. 1.2. Tocopherols are synthesized by the condensation of two building blocks, an aromatic head group and a phytyl tail, obtained from two convergent pathways. The aromatic part of the chromanol ring is derived from the precursor homogentisic acid. The formation of homogentisic acid in plants from *p*-hydroxyphenyl pyruvate and molecular oxygen is catalyzed by the cytosolic enzyme *p*-hydroxyphenyl pyruvate dioxygenase (HPPD) (Garcia et al., 1997, 1999; Norris et al., 1995).

The *p*-hydroxyphenyl pyruvate can be synthesized either from prephenate or tyrosine by the shikimate pathway, but the relative contribution of these two precursors to the total *p*-hydroxyphenyl pyruvate pool is unknown (Lopukhina et al., 2001). HPPD is a member of the large family of non-heme iron α-ketoglutarate dependent dioxygenases and located in the cytosol. This cytosolic localization of the HPPD suggests that homogentisate has to be transferred from cytosol into plastids. In plants, HPPD is involved in the biosynthesis of plastoquinone and vitamin E, whereas in mammals it is involved in tyrosine and phenylalanine catabolism. It catalyzes a complex reaction, the oxidative decarboxylation of the 2-keto acid side chain of *p*-hydroxyphenyl pyruvate, the hydroxylation of the aromatic ring, and a 1,2-shift of a carboxymethyl group. The overall reaction yielding homogentisic acid shortens the pyruvate side chain to acetate, moves the acetate side chain into a *meta*-position relative to the original hydroxyl group and adds the novel hydroxyl group at the *para*-position, the former position of the pyruvate side group (Fritze et al., 2004).

The second building block, phytyl tail, partly contributes for the formation of the oxygen containing ring of chromanol head group. The biosynthesis of phytyl side chain proceeds via the plastidial isoprenoid pathway called non-mevalonate pathway or 1-deoxy-D-xylulose-5-phosphate pathway, which synthesizes isopentenyl pyrophosphate (IPP) (Eisenreich et al., 1998; Lichtenthaler, 1999). The condensation of four molecules of IPP yields the C_{20} unit geranylgeranyl pyrophosphate. Geranylgeranyl pyrophosphate is then allocated to the synthesis of various end products such as phytyl pyrophosphate, chlorophylls, carotenoids, quinones or tocopherols and tocotrienols.

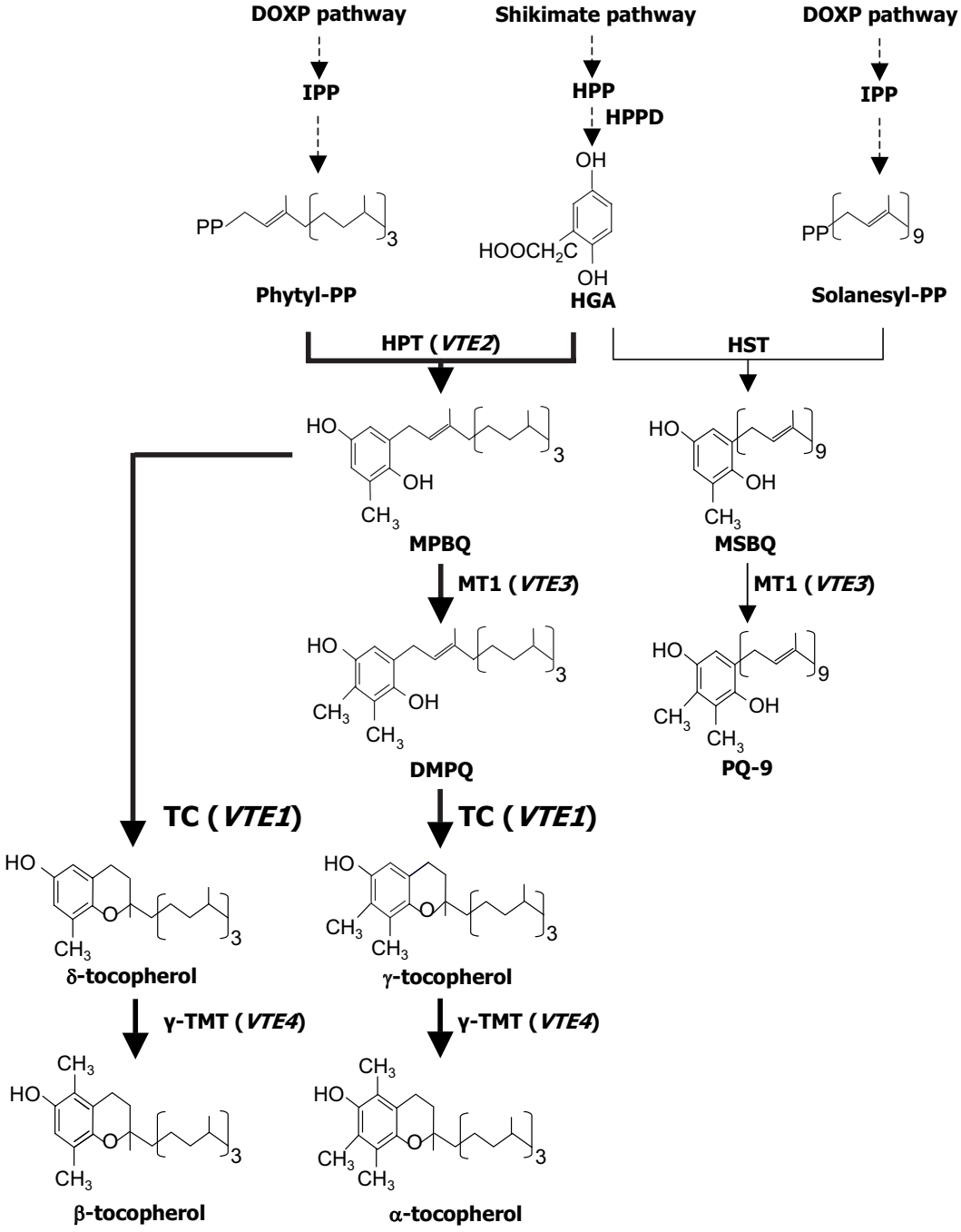


Figure 1.2: The biosynthesis of tocopherols and plastoquinone in plants. Thick arrows represent the steps leading to the synthesis of all four tocopherols homologues. The corresponding tocopherol biosynthetic genes that have been cloned from *Arabidopsis* are shown in brackets. Dashed arrows indicate multiple steps involved in the synthesis of Phytyl-PP and Solanesyl-PP from the DXOP pathway and the synthesis of HGA from the shikimate pathway. [IPP, isopentenyl pyrophosphate; HPP, *p*-hydroxylphenyl pyruvate; HPPD, p-hydroxylphenyl pyruvate dioxygenase; phytyl-pp, phytyl pyrophosphate; solanesyl-pp, solanesyl pyrophosphate; HGA, homogetisic acid; HPT, homogentisate prenyltransferase; HST, homogentisate solanesyltransferase; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; MSBQ, 2-methyl-6-solanesyl-1,4-benzoquinone; MT1, MPBQ/MSBQ methyltransferase; DMPBQ, dimethyl-5-phytyl-1,4-benzoquinol; PQ-9, plastoquinone 9; TC, tocopherol cyclase; γ-TMT, γ-tocopherol methyltransferase]

The synthesis of tocopherols requires the reduction of unsaturated double bonds of geranylgeranyl pyrophosphate to the saturated phytyl pyrophosphate by geranylgeranyl pyrophosphate reductase, whereas geranylgeranyl pyrophosphate is directly used for synthesis of the tocotrienols (Soll and Schultz, 1981; Keller et al., 1998).

The first committed step of tocopherol biosynthesis is the condensation of homogentisate and phytyl pyrophosphate, catalyzed by the homogentisate prenyltransferase (HPT). This reaction leads to the formation of the first tocopherol intermediate, 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ), a common precursor to all tocopherols. HPT catalyzes the fusion of the phytyl chain to the aromatic ring in the 6-position and the decarboxylation of the acetate group to yield a methyl group in the 2-position of the aromatic ring (Schledz et al., 2001; Collakova and DellaPenna, 2001; Savidge et al., 2002). The biosynthesis of tocotrienols was thought to occur using the common set of enzymes involved in tocopherol synthesis until the identification of homogentisate geranylgeranyl transferase (HGGT), which catalyzes the condensation of geranylgeranyl pyrophosphate with homogentisate to yield tocotrienols (Cahoon et al., 2003). In plants HPT and HGGT are specific for either phytyl pyrophostphate or geranylgeranyl pyrophosphate, respectively, while HPT from the cyanobacterium *Synechocystis* can utilize both substrates (Collakova and DellaPenna, 2001; Cahoon et al., 2003). As mentioned before, the biosynthesis of plastoquinone is also closely related to the tocopherol biosynthesis in plants. For the synthesis of plastoquinone, condensation of the solanesyl pyrophosphate to homogentisic acid yields 2-methyl-6-solanesyl-1,4-hydroquinone (MSBQ) by the homogentisate solanyltransferase, an activity distinct from HPT (Collakova and DellaPenna, 2001).

In the next step of the tocopherol biosynthetic pathway a *S*-adenosyl methionine dependent methylation of the first tocopherol intermediate, MPBQ, may occur to yield 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ). This methylation reaction is catalyzed by MPBQ methyltransferase (Shintani et al., 2002). The similarity of the MPBQ and MSBQ structures has led to the proposal that a single enzyme can perform the methylation of both compounds. The methylation of MSBQ results in the formation of plastoquinone (PQ-9). Recently, a MPBQ/MSBQ methyltransferase, an enzyme catalyzing the methylation of both MPBQ and MSBQ, has been identified in plants and *Synechocystis* (Shintani et al., 2002; Cheng et al., 2003). As shown in Fig. 1.2, at this point the tocopherol pathway splits for the synthesis of the four tocopherol homologues. On the one hand, cyclization and methylation transform DMPBQ into γ- and α-tocopherol, respectively, principally occurring isoforms in plants. On the other hand, MPBQ can be cyclized to δ-tocopherol and subsequently be methylated to β-tocopherol, representing minor tocopherol components in plants. A tocopherol cyclase (TC) performs a cyclization reaction with DMPBQ or MPBQ as substrate regardless of the

methyl group on the aromatic ring (Stocker et al., 1996). This enzyme plays a key role in the formation of the chromanol ring structure of the tocopherols, which is essential for the antioxidative radical scavenging function, by generating an additional oxygen heterocycle next to the aromatic ring originating from homogentisate. The chromanol ring cyclization of DMPBQ and MPBQ leads to the formation of γ-tocopherol and δ-tocopherol, respectively. The TC activity has been analyzed in chloroplasts and chromoplasts of higher plants and in *Anabaena varabillis* (Soll et al., 1985; Arango and Heise, 1998; Stocker et al., 1993, 1994, 1996). Recently, it has been shown that the TC from *Arabidopsis* can catalyze the cyclization of 2,3-dimethyl-5-geranylgeranyl-1,4-hydroquinone for the synthesis of γ-tocotrienols indicating the existence of an enzyme common in the biosynthesis of vitamin E homologues (Porfirova et al., 2002). The last step of the tocopherol pathway is the final transfer of a methyl group to the aromatic ring of γ- and δ-tocopherol to yield α- and β-tocopherol, respectively, which is catalyzed by the γ-tocopherol methyltransferase (γ-TMT) (d'Harlingue and Camara, 1985; Shintani and DellaPenna, 1998).

1.6 General considerations and approaches for improvement of tocopherol levels in transgenic plants

The ability of plants to synthesize vitamin E makes them valuable as vitamin E source for animals, which cannot produce vitamin E, and thus represents an essential component of the human diet. As described in previous sections, vitamin E activity has been implicated in a variety of health areas including possible benefits in preventing cardiovascular diseases, certain cancers and cataract formation. In order to gain these beneficial health effects, daily intakes of vitamin E are often quite high (100 to 1000 IU) compared to the recommended daily allowance (40 IU) (Shintani and DellaPenna, 1998). Furthermore, tocopherols protect unsaturated fatty acids from oxidation in fats and oils and are often included in the processed oil to help stabilize the fatty acids. Hence, it is of high interest to have plants with elevated tocopherol levels for human health, food, and animal feed utility. To manipulate the tocopherol content or composition or both, limiting steps with high flux coefficients must be identified in the tocopherol biosynthetic pathway. This requires the cloning of individual tocopherol biosynthetic enzymes and a detailed understanding of the molecular and biochemical regulation of each individual step of the pathway. The enzymes involved in tocopherol biosynthesis have low levels of activity and the membrane-bound nature, which has hampered the isolation of the corresponding genes via classical biochemical techniques. Over the past several years with the advent of genomic technologies in combination with molecular, genetic and biochemical approaches, the genes encoding enzymes directly or indirectly involved in the tocopherol biosynthetic pathway have been cloned and overexpressed in plants

to test whether they are limiting for tocopherol biosynthesis (Shintani and DellaPenna, 1998; Tsegaye et al., 2002; Collakova and DellaPenna, 2003).

To identify the limiting steps, enzymes involved in the tocopherol biosynthetic pathway can broadly be classified into (i) those that can increase the carbon flux through the pathway in quantitative manner (e.g., HPPD, HGGT and HPT) and (ii) those that mainly affect the overall tocopherol composition as qualitative aspect of the pathway (e.g., γ-TMT and TC). The overexpression of γ-TMT resulted in the conversion of the large pool of γ-tocopherol in seeds to α-tocopherol without changing the total tocopherol content of seeds (Shintani and DellaPenna, 1998). In contrast to this, the overexpression of HPPD in leaves and seeds of *Arabidopsis* resulted in the elevation of the total tocopherol levels to the 1.4-fold and 1.3-fold (Tsegaye et al., 2002). On the other hand, the overexpression of HPT gave 4.4 folds and 1.75-fold increase in total tocopherols in *Arabidopsis* leaves and seeds, respectively (Savidge et al., 2002; Collakova and DellaPenna, 2003). Cahoon et al. (2003) expressed barley HGGT transgene in *Arabidopsis* and corn seeds and achieved a 10- and 6-fold increase in tocotrienols, respectively, without affecting tocopherol levels in the seeds. Interestingly, expression of HPPD and a yeast prephenate dehydrogenase in tobacco leaves also resulted in a 10-fold increase of tocotrienols (Rippert et al., 2004). These studies demonstrate that flux into tocopherols predominantly regulated by HPPD or HPT. Nevertheless, the role of enzymes downstream in the tocopherol pathway e.g. TC is unclear with respect to the regulatory control. Biochemical studies provided evidence that the TC catalyzes a rate limiting step in tocopherol biosynthesis.

1.7 Tocopherol cyclase

TC catalyzes the key step in the biosynthesis of the chromanol substructure of the vitamin E family. The cyclization of DMPBQ to γ-tocopherol is an acid promoted cyclization and takes place in two steps (Fig. 1.3). In order to synthesize the oxygen containing heterocyclic ring, the ring closure proceeds by *Si* protonation of the double bond of DMPBQ followed by a re-attack of the phenolic oxygen atom to trap the intermediate carbocation (Stocker et al., 1994). It is assumed that pre-ionization of the phenol to a phenolate occurs in the enzyme binding pocket to favour double bond protonation, thereby resulting *de facto* in a single step process without an actual carbocation intermediate. In any case, the enantioselectivity of the enzyme catalyzed cyclization implies that the enzyme induces and immobilizes a single enantiomeric conformation of the substrate during the reaction (Manetsch et al., 2004).

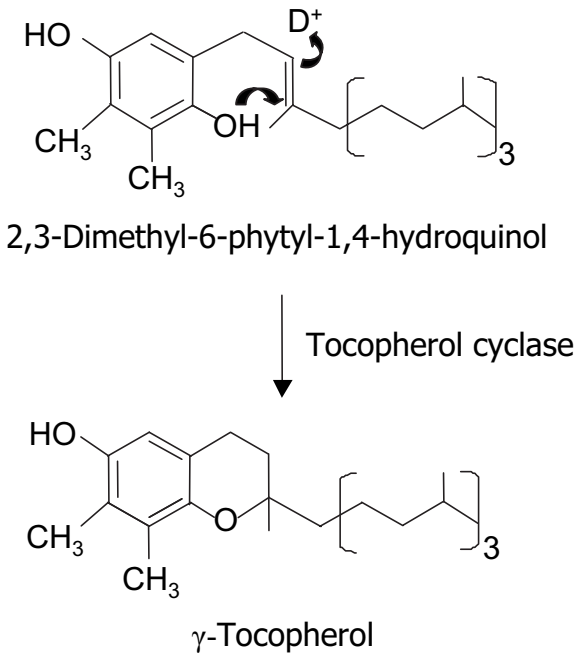


Figure 1.3: The chromanol head ring formation of γ-tocopherol from 2,3-dimethyl-6-phytyl-1,4-hydroquinol catalyzed by tocopherol cyclase.

Using a radio tracer intermediate, Soll et al. (1985) localized TC activity along with the other enzymes of tocopherol synthesis in chloroplasts of higher plants and also found that TC could be rate limiting for the synthesis of α-tocopherol. Later on, TC activity was also characterized in chromoplast membrane preparations, providing evidence for TC being a membrane associated protein (Arango and Heise, 1998). In another study, TC was identified in the cyanobacteria *Anabaena variabilis* using acetone precipitated protein powder as the enzyme source (Stocker et al., 1993). For optimal conversion of the hydrophobic TC substrates in the aqueous phase, Stocker et al. (1993) converted substrates into water-soluble inclusion complexes by formulation with cyclodextrin (Stocker et al., 1993). Using several chromatography steps, the TC protein was purified from *A. variabilis* and the mechanism of cyclization, catalyzed by TC, was investigated. Studies on substrate specificity revealed that TC recognizes three main features namely, the OH group at C1 of the hydroquinone, the E-configuration of the double bond, and the length of the lipophilic side chain (Stocker et al., 1993, 1996).

In the contemporary studies to the present investigation, it was shown that the *vte1* mutant of *Arabidopsis* lacked all four tocopherols forms and was devoid of TC activity (Porfirova et al., 2002). The *vte1* mutants did not show any obvious phenotype. Under optimal conditions growth, chlorophyll content, and photosynthetic quantum yield were similar to the wild type. Genetic mapping of *vte1* identified *VTE1* as a gene encoding TC. *VTE1*

shows a high degree of similarity to the *Sucrose export defective1* (*SXD1*) gene from maize, suggesting that *VTE1* and *SXD1* represent single copy orthologous genes (Porfirova et al., 2002). Lately, functional equivalence of VTE1 and SXD1 was confirmed by *in vitro* assays and complementation studies (Sattler et al., 2003). In contrast to the *vte1* mutant, the corresponding maize mutant *sxd1* showed a phenotype of a severely altered plasmodesmata structure and function during leaf development. This was due to a photosynthate export deficiency phenotype characterized by an overall growth reduction and a source leaf specific accumulation of anthocyanins and starch (Russin et al., 1996; Provencher et al., 2001). Similarly, reduced growth and accumulation of carbohydrates was also observed upon down-regulation of *VTE1* expression in transgenic potato (Hofius et al., 2004). These investigations highlight the importance of TC in tocopherol biosynthesis and provide new insights in the functional role of tocopherols in plants.

1.8 Research objectives

The therapeutic benefits of vitamin E in human nutrition and health have been documented for more than 70 years. Vitamin E is an essential micronutrient whose supplementation in the human diet was shown to reduce the risk of cancer, cardiovascular diseases and cataract, as well as to limit the progression of several degenerative human diseases. Additionally, the antioxidant functions of tocopherol have also been reported to protect PUFAs from lipid peroxidation and to stabilize fats and oils. These beneficial functions of tocopherols often require a very high intake of vitamin E dosage, which cannot be met in the average diets derived from plant products.

Genetic engineering offers the possibility to modify plant storage lipids and valuable secondary compounds in order to meet specific nutritional and even therapeutic requirements. The manipulation of the metabolic pathway by genetic engineering is often successful if the regulation of the respective pathway is well characterized. The identification of the genes involved in a biosynthetic pathway, such as the tocopherol biosynthesis, facilitates the characterization of the enzyme involved in the pathway and permits new insights into the regulatory relationships among the respective enzyme activities. One of the potential key enzyme in tocopherol biosynthesis is the TC. Although the activity of TC was identified in plants, the respective enzymes have not been characterized so far.

Keeping the background information in mind, the present investigation was aimed at cloning and characterizing the TC genes from plants (*Arabidopsis* and maize) at biochemical and molecular levels. To gain access to the TC genes and proteins, the putative TC gene from

Synechocystis was available by the work of our group (Sadre et al., 2003). This was achieved by generating *Synechocystis* disruption mutants of several candidate genes by homologous recombination and subsequent analysis of the tocopherol composition by HPLC. These experiments indicated that the *SLR1737* gene might encode a putative TC. The deduced amino acid sequence should be used as query in database search to identify TCs from plants. Subsequently, the cloned plant genes should be overexpressed in suitable microorganisms to gain access to the proteins and to investigate their catalytic properties. In addition, the TC genes from plants were planned to be overexpressed in developing seeds of *Brassica napus* to improve the vitamin E content of the seed oil and gain new insights into the regulatory mechanisms involved in tocopherol biosynthesis.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals and consumables

If otherwise not stated, the chemicals used throughout the investigation were purchased from the following companies: Amersham Pharmacia Biotech (Freiburg), Applichem (Darmstadt), Biotrend (Köln), BioRad Laboratories GmbH (München), Calbiochem (Bad Soden), Carl Roth GmbH (Karlsruhe), Dynal (Hamburg), Duchefa Biochemie B.V. (Haarlem, The Netherlands), Eppendorf (Hamburg), Fluka (Taufkirchen), Hartmann Analytic (Braunschweig), Invitek (Berlin), Invitrogen (Karlsruhe), Macherey-Nagel (Düren), MBI Fermentas (St. Leon-Rot), Metabion (Planegg-Martinsried), Merck Biosciences GmbH (Darmstadt), New England BioLabs (Frankfurt), Novagen (Darmstadt), Pharmacia (Freiburg), Promega (Madison, USA), QIAGEN (Hilden), Roche Applied Science (Mannheim), Sigma (Taufkirchen), Serva (Heidelberg), VWR international (Darmstadt, Germany).

The consumables were obtained from: Applied Biosystems (Darmstadt), Biometra (Göttingen), BioRad Laboratories GmbH (München), Eppendorf (Hamburg), Fuji (Düsseldorf), Gibco BRL (Eggenstein), Greiner (Solingen), Hanna Instruments (Kehl), Heraeus (Osterode), Herolab (Wiesloch), Kodak (Stuttgart), Kontron Instruments (München), Labomedic (Bonn), Leica (Heidelberg), Millipore (Eschborn), MWG Biotech (München), Pharmacia (Freiburg), Raytest (Berlin), Serva (Heidelberg), Schott Glaswerke (Mainz), Sorvall (Bad Homburg), Wissenschaftliche Technische Werkstätten (Weilheim), Whatman (Maidstone, UK).

2.1.2 Enzymes and kits

Restriction endonucleases and DNA modifying enzymes were provided either from New England BioLabs (Frankfurt) or MBI Fermentas (St. Leon-Rot). M-MLV Reverse Transcriptase, *Pfu* Polymerase and *Taq* DNA Polymerase from Promega (Madison, USA) were used for PCR amplification. T_4-DNA ligase, T_4 polynucleotidekinase and Calf intestine alkaline phosphatase were obtained from MBI Fermentas (St. Leon-Rot). Lysozyme was bought form Merck for the disruption of bacterial cells.

The following kits have been used during the course of this study.

- Plasmid DNA Purification NucleoBond® Kit (Macherey-Nagel)
- Invisorb spin PCRapid Kit (Invitek)
- QIAEX® II Gelextraction kit (Qiagen)
- Dynabeads mRNA purification kit (Dynal)

2.1.3 Primary antibodies, secondary antibodies and substrate for Western blots

Western blotting was performed by using penta His Antibodies (primary antibodies) and Goat Anti-Mouse HRP conjugate (secondary antibodies) from Qiagen, Hilden. Lumi Light Western blotting substrate (Roche Applied Science, Mannheim) was used for chemiluminescence detection of His tagged proteins.

2.1.4 Instruments

Table 2.1: List of instruments.

Centrifuges:		
Centrifuge	5810 R	Eppendorf
Centrifuge	5417 R	Eppendorf
Centrifuge	RC-5B	Sorvall
Ultracentrifuge	L-50	Beckmann

HPLC:		
Pump	Agilent 1100 Series	Agilent
Auto Injector	Agilent 1100 Series	Agilent
Fluorescence Detector	Agilent 1100 Series	Agilent
UV Detector	Agilent 1100 Series	Agilent
Degasser	Agilent 1100 Series	Agilent
General:		
Agarose Gel Electrophoresis Units	19 cm x 13 cm	Work Shop, Bio 1
Scintilation Counter	LS 5000 TD	Beckmann
TLC Plates	Silica Gel 60, 0.25 mm	VWR
Power Pack	300	BioRad
Vivaspin Concentrator	10,000 MW cut off	Vivascience
Waring Blendor	Commercial Blender	Waring
pH-Meter	Hi 9321	Hanna Instruments
Bioimager	FLA - 3000	Fuji
CCD Camara	LAS - 1000	Fuji
Rotors	SS34, HB6, GS3, SLA3000	Sorvall
Spectrophotometer SmartSpec	3000	BioRad
Sterile Filters	0.2 μM	Sartorius
Thermocycler	Primus 96	MWG Biotech
Western Blotting Apparatus	V20 SDB	Carl Roth
Thermo-Mixer	Thermomixer Compact	Eppendorf
Ultrasonicator	Sonoplus GM70	Bandelin
SDS-PAGE Apparatus	Mini-Protean®3 Cell	BioRad
UV-Trans Illuminator	UVT-28M	Herolab
FPLC:		
Biologic Duoflow Chromatography System		BioRad

2.1.5 Solutions, buffers and media

Standard protocols (Sambrook et al., 1989) were used to prepare solutions, buffers and media unless they were supplied with kits. The pH of solutions was adjusted with either 1M NaOH, 1M KOH, 85% phosphoric acid, 95% sulphuric acid or 37% (v/v) hydrochloric acid. Solutions, buffers and media were sterilized either by autoclaving (20 min, 120°C, and 1 bar) or by filtration through 0.2 μM filters for thermo labile components.

Table 2.2: Buffers and media

Name	**Component(s)**	**Concentration**
10 x Agarose gel loading buffer	EDTA, pH 8.0	250 mM
	Ficoll	10% (w/v)
	Bromophenol blue	0.25% (w/v)
	Xylenxyanol	0.25% (w/v)
Acrylamide/Bisacrylamide	Acrylamide	30% (w/v)
	N´,N´-methylenbisacrylamide	0.8% (w/v)
Ammonium persulphate	(APS)	10% (w/v)
Anode buffer-I	Tris-HCl, pH 10.4	300 mM
Anode buffer-II	Tris-HCl, pH 10.4	25 mM
Antibody buffer	Milk powder in TTBS	0.5% (w/v)
BF mix	Sucrose	8.0% (w/v)
	Triton X-100	0.5% (w/v)
	EDTA	50 mM
	Tris-HCl, pH 8.0	10 mM
Bradford reagent	Coomassie Brilliant Blue G 250	100 mg/l
	Ethanol 96% (v/v)	50 ml/l
	Phosphoric acid 85% (v/v)	100 ml/l
Carbenicillin	Stock solution	50 mg/ml
Cathode buffer	Tris-HCl, pH 9.4	25 mM
	Aminocaproic acid	40 mM
Coomassie staining solution	Coomassie Brilliant Blue G 250	0.2% (w/v)
	Methanol	30% (v/v)
	Acetic acid	10% (v/v)
Destaining solution	Methanol	30% (v/v)
	Glacial acetic acid	10%(v/v)
DNA molecular weight standards (MBI fermantas)	1 kb ladder	
	100 kb ladder	
dNTP-mix	dATP	2.0 mM
	dCTP	2.0 mM
	dGTP	2.0 mM
	dTTP	2.0 mM
Isopropanol mix	Isopropanol	In 5:1 ratio
	5 M Ammonium acetate	
Isopropyl-β-D-thiogalactoside	IPTG	1 M
Kanamycin	Stock solution	50 mg/ml

LB-Carbenicillin	LB-Medium Carbenicillin	50 μg/ml
LB-Carb-plate	Bacto-tryptone Yeast extract NaCl Agar Carbenicillin	10 g/L 5 g/L 10 g/L 15 g/L 50 μg/ml
LB-Kan-plate	Bacto-tryptone Yeast extract NaCl Agar Kanamycin	10 g/L 5 g/L 10 g/L 15 g/L 50 μg/ml
LB-medium (Luria Bertani)	Bacto-tryptone Yeast extract NaCl	10 g/L 5 g/L 10 g/L
Lysozyme	Stock solution	20 mg/ml
Ponceau S-red-solution	Ponceau S-red Acetic acid	0.25% (w/v) 1% (v/v)
Protein elution buffer	NaH_2PO_4, pH 8.0 PMSF DTT Imidazole NaCl	50 mM 2 mM 1 mM 250 mM 300 mM
Protein lysis buffer	NaH_2PO_4, pH 8.0 PMSF DTT Imidazole NaCl	50 mM 2 mM 1 mM 10 mM 300 mM
Protein wash buffer	NaH_2PO_4, pH 8.0 PMSF DTT Imidazole NaCl	50 mM 2 mM 1 mM 20 mM 300 mM
Resolving gel buffer	Tris-HCl, pH 8.8	1.5 M

RNA lysis buffer	NaCl	600 mM
	Tris-HCl, pH 8.0	100 mM
	EDTA	20 mM
	SDS	4% (w/v)
RNase A	Stock solution	10 mg/ml
SDS	Sodium dodecyl sulphate	20% (w/v)
SDS-PAGE electrophoresis buffer	Tris-HCl, pH 8.3	25 mM
	Glycine	192 mM
	SDS	0.1% (w/v)
SDS-PAGE sample loading buffer	Tris-HCl, pH 6.8	12.5 mM
	SDS	1% (w/v)
	EDTA	0.08 mM
	Saccharose	12% (w/v)
	Bromophenol blue	0.01% (w/v)
	DTT	26 mM
Stacking gel buffer	Tris-HCl, pH 6.8	0.5 M
Storage buffer	KH_2PO_4	10 mM
	$MgCl_2$	200 mM
	β-Mercaptoethanol	7 mM
	Glycerol	50% (v/v)
	EDTA	1 mM
TAE buffer	Tris-acetate pH 8.0	40 mM
	EDTA	1 mM
TB buffer	PIPES pH 6.7	10 mM
	$MnCl_2$	55 mM
	$CaCl_2$	15 mM
	KCl	250 mM
TB medium	Bacto-tryptone	12 g/900 ml
	Yeast extract	24 g/900 ml
	Glycerol	4 ml/900 ml
	add 100 ml of K-PO_4 solution	23.1 g/l
	KH_2PO_4	125.4 g/l
	K_2HPO_4	
TTBS	Tris-HCl, pH 7.5	20 mM
	NaCl	500 mM
	Tween-20	0.05%(v/v)

YEP medium	Bacto-tryptone Bacto-yeast extract NaCl	10 g/L 10 g/L 5 g/L
YEP plates	Bacto-tryptone Bacto-yeast extract NaCl Bacto-agar	10 g/L 10 g/L 5 g/L 15 g/L

2.1.6 Chromatography matrices and membranes

Nickel ion charged Ni-NTA superflow resin from Qiagen was used to purify his-tagged fusion proteins. A prepacked Bio-Silect® SEC 125-5 gel filtration column (300 mm x 7.8 mm) from BioRad was used for the determination of the molecular weight of *Arabidopsis* TC overexpressed in *E. coli*. PVDF Western blotting membranes were provided by Roche Diagnostics GmbH, (Mannheim) for Western blot analysis.

2.1.7 Biological materials

2.1.7.1 *Escherichia coli (E. coli)* strains

Strain	**Genotype**
XL1-Blue MRF´(Bullock et al., 1987)	Δ(*mcr*A) 183, Δ(*mcrCB-hsdSMR-mrr*) 173, *endA*1, *supE*44, *thi*-1, *recA1*, *gyrA*96, *relA*1, *lac*, [F´*proAB*, *lacI*^{q}*Z*ΔM15, Tn10 (tet^r)].
OneShot Top10 (Invitrogen)	F$^-$, *mcr*A Δ(mrr-hsdRMS-mcrBC) Φ80*lac*ZM15 Δ*lac*X74 *deoR rec*A1 *ara*D 139 Δ*(ara leu)*7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G
DH5α (Hanahan, 1983)	F$^-$, Lambda$^-$, *rec*A1, *end*A1, *hsd*R17 (rK$^-$, mK$^+$), (*lac*ZYA-*arg*F), *supE*44, U169, Φ80d*lac*ZM15, *thi*-1, *gyr*A96, *rel*A1 Note - (*rec*A1), reduced recombination probability, (*end*A1) lacks endonuclease.

Sure cells (Stratagene)	e14$^-$ (McrA$^-$) Δ*(mcrCB-hsdSMR-mrr)171 end*A1 *supE*44 *thi*-1 *gyr*A96 *rel*A1 *lac recB recJ sbcC umuC*::Tn*5(Kan4^r) uvrC* [F´ *proAB lacI^{q}ZDM15* Tn*10* (Tet^r)]. Genes listed signify mutant alleles. Genes on the F´ episome, however, are wild-type unless indicated otherwise.
BL21(DE3) Star (Invitrogen)	F$^-$, *ompT hsdSB (rB$^-$ mB$^-$) gal dcm rne131* (DE3)

2.1.7.2 *Agrobacterium tumefaciens (A. tumefaciens)* strain

A. tumefaciens C58C1 ATHV Rif strain with the vir-plasmid pTiBo542 (=pEHA101; Hood et al. (1986)).

2.1.7.3 Plant material

- *Arabidopsis thaliana* Columbia C24
- *Zea mays* cv. Magister

2.1.8 Primers

The following oligonucleotides were synthesized by MWG GmbH and used for the PCR amplification of genes.

Name	**Sequence (5´ to 3´)**	**Tm (°C)**
For plant expression		
ATF	GATGGAGATACGGAGCTTGATTG	60.6
ATR	CTTACAGACCCGGTGGCTTG	60.0
ZMF	GATGAACCTCGCCGTCGCAGC	65.7
ZMR	GCTATAGGCCTGGGGGCTTAA	64.0
At_f_Sali	CGTCGACATGGAGATACGGAGCTTGATTG	68.1
At_r_xhoi	GCTCGAGTTACAGACCCGGTGGCTTG	68.5
Zm_f_Sali	CGTCGACATGAACCTCGCCGTCGCAGC	72.6
Zm_r_noti	CGCGGCCGCCTATAGGCCTGGGGGCTTAAG	75.0

For *E. coli* expression		
At_f_Ncoi	GCCATGGAGATACGGAGCTTGATTGTTTC	66.7
At_Ter-r_Xhoi	GCTCGAGCAGACCCGGTGGCTTGAAG	71.1
At_76a.f_Ncoi	GCCATGGGCACTCCTCACAGTGGATAC	69.5
At_98a.f_Ncoi	GCCATGGTTTCCATCCCAGAGAAGAG	66.4
Zm-F-Pagi	CTCATGAACCTCGCCGTCGCAGC	67.8
Zm_65a.Pagi.F	CATCATGACGCCGCATAGCGGGTAC	67.9
Zm-Ter-r_Noti	CGCGGCCGCTAGGCCTGGGGGCTTAAG	>75
1737_pagI.F	GATCATGAAATTTCCGCCCCACAG	62.7
1737_xhoi-ter.R	GACTCGAGGAATGGCACTGTTTTTTTGC	65.1
1737_xhoi.R	GACTCGAGTCAGAATGGCACTG	62.1

2.1.9 Vectors

The list of vectors, used during this study, is given below.

Plasmid	Selection Marker	References
pUC18	Amp^R	Stratagene
pUC19	Amp^R	Stratagene
pET28a	Kan^R	Novagen
pPZP111	$Chloramphenicol^R$	Hajdukiewicz et al. (1994)

2.2 Microbial methods

2.2.1 Culture of bacteria

2.2.1.1 *E. coli*

E. coli cells were cultured using standards protocols. The recombinant bacteria were grown either in LB medium or on LB-agar plates at 37°C containing the appropriate antibiotics (Sambrook et al., 1989). For the expression of recombinant proteins in *E. coli* cells TB medium was used as culture medium.

2.2.1.2 *A. tumefaciens*

A. tumefaciens C58C1 ATHV was cultured in YEP medium supplemented with 50 μg/ml rifampicillin at 28°C (Walkerpeach and Velten, 1994).

2.2.2 Transformation

2.2.2.1 Preparation of competent *E. coli* cells for $CaCl_2$-mediated transformation

E. coli competent cells for $CaCl_2$-mediated transformation were prepared as described by Inoue et al. (1990). 5 ml LB medium was inoculated with a single bacterial colony picked from a freshly cultivated *E. coli* on a LB-agar plate and was cultured overnight at 37°C with continuous shaking (200 rpm). On the following day, 100 ml of LB medium were inoculated with 1.0 ml of the preculture and cultivated until a OD $_{600nm}$ of 0.5 to 0.6 was reached. After cooling the cells on ice for 10 min, the cells were sedimented by centrifugation at 5000 x g at 4°C for 10 min. Then the pellet was resuspended in 40 ml TB buffer (Table 2.2) and incubated for 10 min on ice. The cells were again sedimented by centrifugation and finally resuspended in 8 ml TB buffer with 7% dimethyl sulfoxide (0.56 ml). After incubation for 10 min on ice, 200 μl aliquots of the suspension were dispensed into prechilled sterile eppendorf tubes, frozen immediately in liquid N_2 and stored at -80°C until further use.

2.2.2.2 Transformation of chemically competent *E. coli* cells

The competent *E. coli* cells were thawed on ice and incubated on ice for 30 min with the appropriate amount of DNA (100 to 200 ng) or ligation product (maximal 20 μl). A heat shock treatment of the cells at 42°C for 90 sec was followed by an incubation on ice for 2 min. 0.8 ml LB medium was immediately added to the tube and the cells were incubated at 37°C for 1 h with constant shaking. 200 μl of the transformed cell suspension was plated onto LB-agar plates supplemented with the appropriate antibiotics and incubated at 37°C overnight.

2.2.2.3 Preparation of electrocompetent *A. tumefaciens* cells

A modified method from Lin (1995) was used for the preparation of electrocompetent *A. tumefaciens* cells. 500 ml LB medium were inoculated with 2 ml from a log phase *A. tumefaciens* culture supplemented with 50 μg/ml rifampicillin and cultivated overnight at 30°C with shaking at 300 rpm to a OD_{600nm} 0.8 -1.0. The cells were harvested by centrifugation at 4000 x g and 4°C for 10 min in sterile centrifuge bottles and resupended in 500 ml sterile, ice cold 10% glycerol by gentle mixing. This wash step was repeated three times and the cells were finally pelleted by centrifugation at 4000 x g and 4°C for 10 min. The pellet was dissolved in 50 ml sterile, ice cold 10% glycerol and centrifuged with a Sorvall HB6 rotor at 3000 x g and 4°C for 10 min. Then the cells were resuspended in 2 ml sterile, ice cold 10% glycerol and 45 μl aliquots of the suspension were dispensed into prechilled sterile eppendorf tubes and frozen immediately in liquid N_2. The competent cells were stable for at least six months at -80°C.

2.2.2.4 Transformation of electrocompetent *Agrobacterium* cells

Transformation of electrocompetent *A. tumefaciens* cells was performed using a MicroPulser^{TM} electroporation apparatus from BioRad according to the manufacturer's recommendations. 0.2 to 1.0 μg DNA in 5 μl sterile dH_2O was added to thawed electrocompetent *A. tumefaciens* cells and incubated on ice for 10 min, then transferred into a chilled electroporation cuvette (0.2 cm, BioRad). After application of the pulse (25 μf, 2.5 kV, 200 Ω), 1 ml LB medium was added immediately and the cells were incubated at 30°C, 250 rpm for 3h. Finally, 50-200 μl of the cell culture were plated on LB-agar plates containing 50 μg/ml rifampicillin and 30 μg/ml chloramphenicol followed by incubation at 28°C for 2-3 days.

2.3 Molecular Methods

2.3.1 Isolation of plasmid DNA from *E. coli*

2.3.1.1 Mini preparation of plasmid DNA

For small scale preparation of plasmid DNA, a modified rapid boiling method was used as described by Riggs and McLachlan (1986). A single colony was inoculated in 1.5 ml of LB medium with the appropriate antibiotics and cultured overnight at 37°C. The cells were sedimented at 14000 x g for 20 sec and the cells were resuspended in 0.2 ml BF buffer (Table 2.2)containing lysozyme (final concentration, 1 mg/ml) and incubated at 100 °C for 1 min followed by quick cooling on ice for 3 min. The cell debris was pelleted at 20000 x g for 30 min at RT. The plasmid DNA was precipitated from the supernatant by adding 500 μl of isoprpanol mix (Table 2.2) and sedimented by centrifugation at 20000 x g for 30 min at RT. After washing the DNA pellet twice with 70% ethanol, the pellet was air dried and dissolved in 50-100 μl distilled H_2O containing 3 μl RNase A (stock solution 10 mg/ml) followed by incubation at 37°C for 30 min to remove RNA contamination. For restriction analysis, 5 μl of the DNA solution was digested with the appropriate restriction endonucleases.

2.3.1.2 Midi / Maxi preparation of plasmid DNA

The Nucleobond® plasmid DNA purification kit (Macherey Nagel) is based on the interaction of the negatively charged phosphate backbone of DNA with the methyl-ethylamine group coupled to silica beads. This kit was used to obtain high quality of plasmid DNA in high quantities. The procedure was performed according to the instructions of the manufacturer.

2.3.2 Isolation of plasmid DNA from *Agrobacterium tumefaciens*

Plasmid DNA was isolated from *A. tumefaciens* according to the instructions provided with the Nucleobond® plasmid DNA purification kit (Macherey Nagel) with an additional step of lysozyme treatment in S1 buffer containing 2 mg/ml lysozyme at 37°C for 30 min.

2.3.3 Determination of DNA yield and quality (Sambrook et al., 1989)

DNA yield was measured by UV spectrophotometry, using the following relationship:
1 OD 260 nm (1 cm path length) $\approx$ 50 μg DNA/ ml
DNA quality was checked by UV spectrophotometry (quotient 260 nm/280 nm) and/or analysis by agarose gel electrophoresis. A value of the ratio of 260 nm/280 nm in the range of 1.8 to 2.0 is an indication for pure DNA.

2.3.4 DNA restriction analysis and agarose gel electrophoresis

Restriction analysis of DNA with restriction endonucleases and DNA agarose gel electrophoresis were performed as described by Sambrook et al. (1989). The length of DNA fragments was determined by agarose gel electrophoresis. 0.1 vol of 10 x loading buffer (Table 2.2) was added to the DNA samples. Samples were loaded into the slots of 1% agarose TAE gel containg 30 ng/ml ethidium bromide and were separated by electrophoresis with 4 V/cm in horizontal gel chambers. DNA was visualized by excitation of fluorescence of the intercalated ethidium bromide under UV light. For documentation a gel documentation system with a CCD-camera was used.

2.3.5 DNA extraction from agarose gels

DNA fragments were extracted from agarose gels using the QIAEX® II Gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.3.6 DNA sequencing

DNA sequencing according to the dideoxynucleotide chain termination method (Sanger et al., 1977) was carried out at the sequencing service unit of the Institute for Molecular Biotechnology, RWTH Aachen.

2.3.7 Isolation of mRNA

2.3.7.1 Preparation of total RNA

Total RNA was isolated from leaves of *A. thaliana* and *Z. mays* by lithium chloride (LiCl) method (Menhaj et al., 1999). Frozen plant material was ground in liquid N_2 to a fine powder with a pestle and mortar, then transferred to a 50 ml falcon tube containing 2 to 3 ml of lysis buffer per g plant material and mixed well by vortexing. The mixture was extracted with an equal volume of a TE buffer saturated phenol:chloroform (P/C) mix (1:1 v/v) with shaking for 20 min at RT followed by centrifugation at 3000 x g and RT for 20 min to achieve phase separation. The aqueous phase was again extracted with 1 vol P/C mix. The RNA was precipitated from the aqueous phase by adding 0.75 volumes 8 M LiCl at 4°C and overnight incubation. The RNA was sedimented at 20,000 x g and 4°C for 20 min. The RNA pellet was resuspended in DEPC treated water and again precipitated by adding 0.1 volume 3 M sodium acetate, pH 5.2 and 3 volumes ethanol at -20°C for 2 hours followed by centrifugation at 20,000 xg at 4°C for 20 min. The pellet was washed with 70 % ethanol and finally dissolved in an appropriate amount of DEPC-H_2O after air drying.
The RNA concentration was measured photometrically.

1 OD 260nm (1 cm path length) $\approx$ 40 μg RNA/ml

2.3.7.2 Preparation of mRNA from total RNA

The isolation of mRNA from total RNA was performed according to the manufacturer's instructions using Oligo-dT-Dynabeads from DYNAL Biotech (Hamburg).

2.3.8 Two-step Reverse transcriptase-PCR (RT-PCR)

2.3.8.1. First strand cDNA synthesis from mRNA

For the synthesis of first strand cDNA, approximately 1 μg of mRNA and 2 pmol of 3'end gene specific primer in a sterile RNase free microcentrifuge tube in the total volume of 14 μl were incubated at 70°C for 5 min, then cooled quickly on ice for 5 min. The following components were added to the annealed primer/template:

dH_2O (to 25 μl final volume)	6.75 μl
M-MLV RT Reaction buffer	5.00 μl
10 mM dNTP mix	1.25 μl
M-MLV-RT (H-) (100 units)	1.00 μl

The reaction was performed at 50-55°C for 1 h followed by inactivation of the enzyme at 70°C for 15 minutes. The cDNA was further used as a template for PCR amplification.

2.3.8.2. Second strand cDNA synthesis and PCR amplification

5 to 10 μl of the first-strand cDNA reaction were used in subsequent PCR amplifications using gene specific primers (50 pmol), 1 x reaction buffer, 0.2 mM dNTPs, 3 U of *Pfu* polymerase and dH_2O in a final volume of 50 μl. The reaction was subjected to thermal cycling according to the following touch down-PCR programme.

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	5 min	
Denaturation	94°C	40 sec	
Annealing	70°C -1°C	30 sec	14-18 cycles
Extension	72°C	3.20 min	
Denaturation	94°C	40 sec	
Annealing	50°C to 54°C	30 sec	16-20 cycles
Extension	72°C	3.20 min	
Final Extension	72°C	5 min	
Soak	4°C	indefinite	

2.3.9 Polymerase chain reaction (PCR)

The polymerase chain reaction is a method for the enzymatic amplification and modification of a target DNA sequence flanked by two synthetic oligonucleotide (primers) complementary to (+) and (-) strands. The process uses multiple cycles of template denaturation, primer annealing and primer elongation to amplify DNA sequences. For high fidelity polymerization reactions, *Pfu* polymerase (Promega) was used to amplify the genes according to the manufacturer's instructions. Reaction conditions (template concentration, annealing temperature and extension duration) were optimized for individual experiments. PCR with Taq DNA polymerase was used to screen for transgenic *E. coli* colonies. Typical PCR reaction components and reaction conditions are given below:

Components	**Final concentration**
Template	100 to 200 ng
Primer1	0.1 - 0.5 μM
Primer2	0.1 - 0.5 μM
10 x reaction buffer	1 x
$MgCl_2$	1.0 - 3.0 mM
dNTP mix	200 μM each NTP
Taq DNA polymerase	1 - 4 units / 100 μl reaction

PCR reactions were performed using the following thermal cycler programme:

Step	**Temperature**	**Time**	**Number of cycles**
Initial denaturation	94°C	5 min	
Denaturation	94°C	40 sec	
Annealing	50°C to 54°C	30 sec	18-25 cycles
Extension	72°C	2-4 min	
Final Extension	72°C	5 min	
Soak	4°C	indefinite	

2.3.10 Phosphorylation and dephosphorylation of DNA

To phosphorylate the 5´-OH group of PCR amplified DNA by *Pfu* polymerase, T_4 polynucleotide kinase (MBI Fermentas, St. Leon-Rot) was used for phosphorylation as described in the manufacturer's protocol. Calf intestine alkaline phosphatase (CIAP), from MBI Fermentas (St. Leon-Rot), was used to dephosphorylate DNA fragments according the manufacturer's instructions.

2.3.11 Ligation

T_4 DNA ligase (MBI Fermentas) was used to catalyze the formation of a phosphodiester bond between juxtaposed 5´-phosphate and 3´-hydroxyl termini in duplex DNA as described by Sambrook et al. (1989).

2.3.12 Construction of TC expression chimeric plasmids

The open reading frames (ORFs) corresponding to the *VTE1* gene from *A. thaliana* and the *SXD1* gene from maize were amplified by PCR with *Pfu* polymerase (Stratagene, LaJolla,

CA, USA) using the specific primer pairs ATF: 5´-GATGGAGATACGGAGCTTGATTG-3´ and ATR: 5´-CTTACAGACCCGGTGGCTTG-3´ (*A. thaliana*) as well as ZMF: 5´-GATGAACCTCGCCGTCGCAGC-3´ and ZMR: 5´-GCTATAGGCCTGGGGGCTTAA-3´ (maize). The resulting PCR products were ligated into the *Sma*I site of pUC19. For functional expression studies in *E. coli*, cDNA fragments from *VTE1* and *SXD1* without the sequence encoding the putative plastidial transit peptide were amplified by PCR using the following specific primers At_76a.f_Ncoi: 5´-GCCATGGGCACTCCTCACAGTGGATAC-3´, At_98a.f_Ncoi: 5´-GCCATGGTTTCCATCCCAGAGAAGAG-3´, At_Ter-r_Xhoi: 5´-GCTCGAGCAGACCCGGTGGCTTGAAG-3´ (for *VTE1*) and Zm_65a.Pagi.F: 5´-CATCA TGACGCCGCATAGCGGGTAC-3´, Zm-Ter-r_Noti: 5´-CGCGGCCGCTAGGCCTGGGG GCTTAAG-3´ (for *SXD1*) containing *Nco*I/*Xho*I and *Pag*I/*Not*I sites, respectively. To introduce *Nco*I/*Xho*I and *Pag*I/*Not*I sites on the 5´ and 3´ends of *VTE1* and *SXD1* genes, cDNA were amplified by PCR with the following primer pairs At_f_Ncoi: 5´-GCCATGGAGA TACGGAGCTTGATTGTTTC-3´, At_Ter-r_Xhoi: 5´-GCTCGAGCAGACCCGGTGGCT TGAAG-3´ and Zm-F-Pagi: 5´-CTCATGAACCTCGCCGTCGCAGC-3´, Zm-Ter-r_Noti: 5´-CGCGGCCGCTAGGCCTGGGGGCTTAAG-3´, respectively. The *SLR1737* ORF was amplified from the genomic DNA of *Synechocystis* PCC 6803 with 1737_pagI.F: 5´-GATCAT GAAATTTCCGCCCCACAG-3´and 1737_xhoi-ter.R: 5´-GACTCGAGGAATGGCACTGT TTTTTTGC-3´, introducing *Pag*I/*Xho*I sites at the 5´and 3´end, respectively. The amplified DNA fragments were ligated into the corresponding *Nco*I/*Xho*I and *Nco*I/*Not*I sites of the pET28a vector (Novagen, Madison, WI, USA), so that the 6x His-tag sequence of the vector was added in frame to the 3´end of the truncated and non-truncated open reading frames. The resultant truncated constructs from *Arabidopsis* and maize were named pAtΔ98TC, pAtΔ76TC and pZmΔ65TC, respectively . The full-length chimeric TC constructs from *Arabidopsis*, maize and *Synechocystis* were designated pAtTC, pZmTC and pSyTC, respectively (Fig. A.3).

For the construction of the plant expression vectors, the coding regions of *VTE1* and *SXD1* were amplified by PCR using the following primer pairs At_f_Sali: 5´-GATCGTCGACAACA ATGGAGATACGGAGCTTG-3´, At_r_xhoi: 5´-GCTCGAGTTACAGACCCGGTGGCTTG-3´ and Zm_f_Sali: 5´-GATCGTCGACAACAATGAACCTCGCCGTC-3´, Zm_r_noti: 5´-CGCGGCCGCCTATAGGCCTGGG GGCTTAAG-3´ introducing *Sal*I/*Xho*I and *Sal*I/*Not*I sites at the 5' and 3' end, respectively. The PCR products were ligated to a corresponding *Sal*I site at the 3' end of the seed-specific napin promoter and to the *Xho*I or *Not*I sites at the 5' end of the transcriptional termination sequence of the nopaline synthase gene in vector pNapCassette. The *Sma*I excised plant expression cassettes of the resulting vectors were inserted into the *Smi*I site of the binary vector pPZP111. The chimeric constructs carrying either TC gene from *Arabidopsis* or maize to overexpress in developing seeds of *Brassica*

napus were named pPZP-AtTC and pPZP-ZmTC, respectively (Fig. A.4).

2.4 Biochemical methods

2.4.1 Expression of recombinant protein in *E. coli*

Chimeric pET constructs containing TC sequences from *Arabidopsis* and maize were transformed in BL21(DE3) star strain (Invitrogen) and a single colony harboring the recombinant plasmid was used to cultivate a 50 ml LB culture with 50 μg/ml kanamycin at 37°C for overnight. 2 l TB selection medium (Table 2.2) was inoculated with 5 ml of the preculture and cultivated at 37°C with constant shaking until a cell density of OD_{600nm} 2 was reached. Then the culture was incubated on ice for 10 to 15 min to cool down and induced for 2 hours at 22-25°C with IPTG (final concentration 1 mM). The cells were harvested by centrifugation at 5000 x g and 4°C for 10 min, followed by washing with 20 mM sodium phosphate buffer, pH 8.0. The resultant cell pellets were immediately frozen in liquid N_2 and stored at -20°C until further use. The presence of recombinant protein was confirmed by Western blotting using anti-His antibodies.

2.4.2 Purification of His-tagged TC proteins by Ni-NTA affinity chromatography

The induecd cells from 1 l culture were resuspended in 50 ml ice cold protein lysis buffer (Table 2.2) with lysozyme (final concentration 1 mg/ml) and incubated on ice for 30 min followed by ultrasonication for cell disruption. Ultrasonic cell disintegration was performed on ice by 50 % duty cycle, 2 x 30 sec with an interval of 1 min. DNase I and RNase A (final concentration each 10 μg/ml) were added to the cell lysate and incubated on ice for 15 min. The cell debris was sedimented by centrifugation at 12,000 x g, 4° C for 20 min and the supernatant was subjected to ultracentrifugation at 160,000 x g and 4°C for 60 min (rotor Beckmann 50.2 Ti). The cleared supernatant was transferred into a new tube containing 300 μl Ni-NTA agarose matrix (Qiagen) and incubated at 4°C for 1 hour with continuous shaking. Then the suspension was poured into an empty column (BioRad, 0.5 cm x 20 cm) and the unbound proteins were collected in the flow through. To remove non-specifically bound proteins, the column was washed with 15 column volumes (CV) protein washing buffer (Table 2.2) and the His-tagged recombinant protein was eluted with 5 CV protein elution buffer (Table 2.2). To exchange the elution buffer to storage buffer and to concentrate the eluted proteins, Viva spin ultrafiltration (10,000 MWCO) tubes were used according to the recommendations of VWR International. Protein concentration was determined by the Bradford

method and small aliquots of the purified protein in storage buffer (Table 2.2) were freezed in liquid N_2 and stored at -80°C until used for TC assays.

A preparation of enriched TC protein from *Arabidopsis* was also subjected to precipitation with ammonium sulphate at 45% saturation on ice for 1h. The precipitated proteins were sedimented by centrifugation at 10,000 x g and 4°C for 15 min and dissolved in 1-2 ml storage buffer. The residual ammonium sulphate from the protein suspension was removed using viva spin ultrafiltration tube against the same buffer and small aliquots were stored in -80°C.

2.4.3 Ammonium sulphate precipitation of recombinant TC

The frozen cell pellet was resuspended in ice cold protein lysis buffer (50 μl/ml culture) with lysozyme to a final concentration of 1 mg/ml and incubated on ice for 30 min followed by ultrasonic cell disruption on ice by 50% duty cycle, 2 x 30 sec with an interval of 1 min. Then the cell lysate was treated with DNase I and RNase A (final concentration 10 μg/ml) on ice for 15 min. The resultant cell lysate was centrifuged at 12000 x g, 4° C for 20 min to remove cellular debris. 1 ml supernatant was labelled as clear lysate and the rest was ultracentrifuged at 160,000 x g, 4°C for 60 min (rotor Beckmann 50.2 Ti). To precipitate the recombinant TC proteins, supernatant was brought to 45% saturation with fine powder of ammonium sulphate and stirred on ice for 1h followed by centrifugation at 10,000 x g and 4°C for 15 min. The pellet was resuspended in 1-4 ml 50 mM sodium phosphate, pH 8.0 containing 1 mM DTT and 5% glycerol. Subsequently, the suspension was dialyzed against the same buffer for 1h at 4°C using 10,000 MWCO dialysis membrane. The protein concentration were estimated and small aliquots were frozen in liquid N_2 and stored in -20°C until further use.

2.4.4 Molecular weight determination by gel filtration chromatography

To determine the native molecular mass of the purified TC, gel filtration chromatography was performed using the Duo Biologic FPLC system and a Bio-Silect SEC 125-5, 300 mm x 7.8 mm column from BioRad with 100 mM NaH_2PO_4, pH 7.0, 150 mM NaCl at a flow rate of 1 ml*min^{-1}. The column was calibrated with a protein standard kit containing thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), vitamin B-12 (1.35 kDa) from BioRad (Munich, Germany). 1 to 2 mg purified TC from *Arabidopsis* was applied on the previously equilibrated column with 100 mM NaH_2PO_4, pH 7.0, 150 mM NaCl and the fractions were collected after elution with the same buffer. Fractions containing TC activity were identified by TC assays. Subsequently the fractions containing significant

TC activity were checked by SDS-PAGE and Western blotting. The molecular weight of the native TC from *Arabidopsis* was determined from a K_{av}:log Mr calibration curve calculated for the standard proteins.

2.4.5 Estimation of protein concentration by the Bradford method

Protein concentrations were determined as described by (Bradford, 1976). 2 μl to 10 μl protein solutions were mixed with 1.0 ml Bradford reagent (Table 2.2) and incubated for 5 min at RT. Then the absorbance at 595 nm was measured against a blank containing 2 to 10 μl of the protein buffer in 1 ml Bradford reagent. 5 μg to 20 μg bovine serum albumin (Sigma) were used in standard assays for calibration.

2.4.6 SDS-Polyacrylamide gel electrophoresis

The discontinuous Laemmli system (Laemmli, 1970) was used for separation of protein samples using the Mini-Protean® 3 Cell apparatus (BioRad). If otherwise not stated, 4.5% stacking gels and 12.5% resolving gels were prepared according to following pipetting scheme.

Components	**Stacking gel (4.5%)**	**Separating gel (12.5%)**
Acryl/ bis acrylamide solution	0.375 ml	1.65 ml
dH_2O	1.45 ml	1.30 ml
Resolving gel buffer	-	1 ml
Stacking gel buffer	0.625 ml	-
20% (w/v) SDS	12.5 μl	20 μl
10% (w/v) APS	12.5 μl	32 μl
TEMED	5 μl	5 μl

Sample preparation

A final concentration of 10 % TCA was used to precipitate 30 to 40 μg proteins, depending on the experiment, on ice for 15 min, then centrifuged at 20,000 x g for 15 min. The pellet was resuspended in 1 X SDS-PAGE sample loading buffer (Table 2.2) and the pH was adjusted with 1 or 2 μl of resolving gel buffer. Denaturation of proteins was achieved by incubation at 95°C for 5 min. An amount of 10 to 15 μg protein per lane alongside of the prestained broad range protein marker (New England Biolabs) was separated using 1 x SDS-PAGE electrophoresis buffer (Table 2.2) under constant (200 V) volts until dye front reached at bottom of the gel.

Visualization

Visualization of proteins on gel was performed by coomassie brilliant blue G-250 staining (Table 2.2) as described by Meyer and Lamberts (1965).

2.4.7 Western blotting

Elcetrophoretically separated proteins were transferred from the SDS-polyacrylamide gel to a hydrophobic PVDF membrane (Roche, Mannheim) by a semi dry blotting method (Kyse-Anderson, 1984). A piece of PVDF membrane and six pieces of Whatman filter paper (Schleicher & Schüll, GB004) were cut according to the dimensions of the gel. The membrane was treated with methanol for a few seconds followed by rinsing with dH2O and soaked in anode buffer-II, while three pieces of filter and the protein gel were equilibrated in cathode buffer. From the remaining three filter pieces, two filter papers were equilibrated in anode buffer-I and the other one was equilibrated in anode buffer-II. Then all equilibrated filter pieces, membrane and protein gel were placed in a stack assembly as shown in Fig. 2.1 on western blotting semi dry apparatus (Carl Roth).

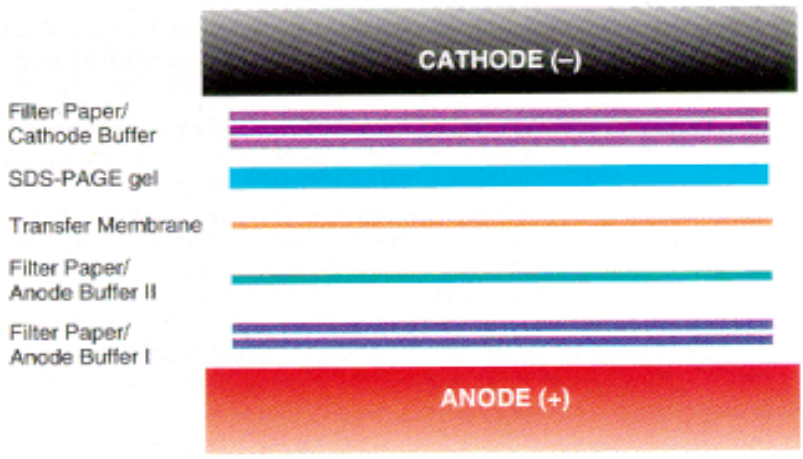


Figure 2.1: Semi-dry Western blot transfer stack assembly

Care was taken to remove air bubbles between membrane and protein gel with the help of a glass pipette. Protein transfer was performed at 95 mA for 30 min. The protein transfer was verified by staining the membrane with Ponceau S stain (0.1% Ponceau S in 5% acetic acid) and unoccupied protein binding sites on the membrane were blocked by placing the membrane in blocking buffer (10% milk powder in TTBS buffer) for 1 h with constant shaking at RT. The blocked membrane was washed twice with antibody buffer (Table 2.2) and incubated in the penta His antibody buffer solution (Qiagen) containing penta His

antibodies in 1:2000 dilution with antibody buffer either for 1 h at RT or for overnight at 4°C. Then the membrane was washed trice with TTBS (Table 2.2) for 10 min at RT followed by incubation in antibody buffer solution of Goat anti-mouse HRP Conjugate (Qiagen) in 1:1000 dilution for 1 h at RT. The non-specifically bound Goat anti-mouse HRP Conjugates were removed by washing thrice with TTBS buffer for 10 min. For chemiluminescent detection, a Lumi-Light Western blotting substrate (lumi-light stable peroxide solution: Lumi-light luminal/enhancer solution = 1:1 from Roche diagnostics GmbH, Mannheim) was applied on the membrane and incubated for 5 min. Then the membrane was exposed under the LAS 3000 CCD camera (Fuji) and the luminescence signals were recorded according to the manufacturer's recommendations.

2.4.8 Tocopherol cyclase assays

Assays of TC activity were performed *in vitro* using formulated substrates with purified recombinant TC proteins from *A. thaliana* and maize. The reaction products were detected with a fluorescence detector after separation on a normal phase HPLC column (Agilent).

2.4.8.1 Formulation of cyclase substrates (Stocker et al., 1993)

In the biosynthesis of tocopherols, TC utilize two substrates, which are 2,3-dimethyl-6-phytyl-1,4- benzoquinol (DMPBQ) and 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) (Fig. 1.2). Since TC substrates are not commercially available, MPBQ and DMPBQ were synthesized according to Soll (1987) by Dr. Schüßeler in Prof. Enders´group (Institute for Organic Chemistry, RWTH Aachen University, Aachen, Germany). The DMPBQ and MPBQ are highly hydrophobic in nature and their solubility in aqueous phase had to be enhanced by formulation with methyl-β-cyclodextrin, which masks the hydrophobic tail of DMPBQ and MPBQ. If otherwise not mentioned, the substrate-cyclodextrin inclusion complex was achieved by incubation of 2 mg (2.4 mMol) DMPBQ or MPBQ in 1 ml of 45.5 mM cyclodextrin solution (prepared in 50 mM potassium phosphate buffer pH 7.0) with continuous stirring for 15 min at 40°C. For the reduction of substrate-cyclodextrin complex, 1 ml 500 mM ascorbic acid, 50 mM potassium phosphate, pH 7.0 was added to the suspension and incubated at 30°C for 15 min with constant shaking. The formulated substrates were stored at -20°C. Based on the recovery of the substrate after formulation, the optimal ratio of substrate and cyclodextrin concentration was achieved.

2.4.8.2 TC assays

TC activity was determined with the recombinant TC from *Arabidopsis* and maize using DMPBQ as substrate in a 100 μl reaction volume at 30°C and 40°C for 30 minutes, respec-

tively. 250 ng to 50 μg of recombinant TC protein was used in the TC assays. The reaction mixture was composed of 200 mM potassium phosphate, pH 7.3, 4 mM dithiothreitol, 75 mM ascorbic acid, and 180 mM formulated DMPBQ. The cyclization reaction was stopped with 200 μl ethanol followed by extraction of lipophilic compounds in 1 ml hexane. The lipophilic components were separated by HPLC (Agilent 1100 series) on a EC 250/4 Nucleosil 100-5 column (Macherey-Nagel GmbH & Co. KG, Düren, Germany) with an isocratic solvent system of n-heptane and Isopropanol (99.5:0.5) and were detected by excitation at 295 nm and emission at 325 nm using a fluorescence detector. The cyclization of DMPBQ results in the formation of γ-tocopherol, the reaction product of the TC assays. 80 ng of α-tocopherol was used as an internal standard per assay reaction to quantify the γ-tocopherol.

2.5 Computer programmes

National Center of Biotechnology Information (NCBI)	http://www.ncbi.nlm.nih.gov/BLAST/
BLAST	Altschul et al. (1997)
Caynobase	http://www.kazusa.or.jp/cyano/cyano.html
CLUSTAL(X 1.81)	Higgins and Sharp (1988)
TreeView	http://taxonomy.Zoology.gla.ac.uk/rod/rod/html
CHROMAS 1.4.3	http://trishil.sci.gu.edu.au/ conor/chromas.html
Clone manager	Clone Manager for Windows, Version 4.1, 1995-1996, Scientific & Educational Software
ChloroP	www.cbs.dtu.dk/services/ChloroP
TMHMM	http://www.cbs.dtu.dk/services/TMHMM

Chapter 3

Results and Discussion

3.1 Identification of tocopherol cyclase sequences

To identify tocopherol cyclase (TC) genes from plants, BLAST (Altschul et al., 1997) searches in the NCBI public database (http://www.ncbi.nlm.nih.gov/BLAST/) were performed with the deduced amino acid sequence corresponding to the ORF of the *SLR1737* gene from *Synechocystis* PCC 6803. Two lines of evidence support the assumption that the *SLR1737* gene product is involved in tocopherol biosynthesis.

(i) The *SLR1737* gene is located in the same operon as the *SLR1736* gene in the *Synechocystis* genome. The *SLR1736* encodes the homogentisate prenyltransferase, shown in Fig. 1.2 (Schledz et al., 2001). The bacterial genes involved in the same metabolic pathway are often organized in operons (Shintani and DellaPenna, 1998). Therefore, the *SLR1737* gene was a likely candidate for an enzyme involved in tocopherol biosynthesis.

(ii) The disruption of the *SLR1737* gene resulted in the loss of all forms of tocopherols in the *Synechocystis* null mutant, but it still showed HPPD and HPT activities very similar to those of wild type cells (Sadre et al., 2003).

As shown in Fig. 1.2, only the lack of HPPD, HPT and TC activities would result in the complete loss of tocopherols. These data validated the essential role of *SLR1737* in tocopherol biosynthesis and substantiated the putative function of *SLR1737* as a tocopherol cyclase.

Based on this *SLR1737* sequence from *Synechocystis*, non redundant BLAST queries in the NCBI database resulted in the identification of further putative TC sequences for TCs from plants and cyanobacteria, which share significant identities in their deduced amino acid sequences (Table 3.1). As expected, no orthologs of the *Synechocystis SLR1737* gene from

Table 3.1: Sequence homologs of the *SLR1737* sequence from plants and cyanobacteria.

Organism	**ID/Accession**	**Protein Name**	**Encoded protein**		
			amino acids	**kDa**	**pI**
Arabidopsis thaliana	gi 24212569 At4g32770 Q94FY *VTE1*	tocopherol cyclase, chloroplast precursor (Vitamin E deficient 1)	488	54.72	5.95
Eucalyptus gunnii	gi 33188419 AAP97931.1 Q7XAF0	putative tocopherol cyclase	515	56.74	6.78
Solanum tuberosum	gi 47078321 gbAAT09809.1 Q6E6T1	tocopherol cyclase	501	56.21	5.8
Zea mays	gi 14334010 AF302187_1 Q94FY8 *SXD1*	probable tocopherol cyclase, chloroplast precursor sucrose export defective 1	474	52.67	5.33
Oryza sativa	Q6K7V6	putative tocopherol cyclase	470	52.19	6.81
Trichodesmium erythraeum IMS101	gi 48893042 ZP_00326335.1	hypothetical protein Tery02003363	358	41.53	6.32
Nostoc punctiforme PCC 73102	gi 23130125 ZP_00111944.1	hypothetical protein Npun02000631	365	41.83	5.53
Anabaena variabilis ATCC 29413	gi 46135297 ZP_00162682.2	hypothetical protein Avar03000564	358	40.83	6.07
Nostoc sp. PCC 7120	gi 17227741 NP_484289.1	hypothetical protein all0245	363	41.37	5.77
Crocosphaera watsonii WH 8501	gi 46120075 ZP_00179394.2	hypothetical protein Cwat03000312	366	42.15	5.3
Gloeobacter violaceus PCC 7421	gi 37522658 NP_926035.1	hypothetical protein GLR3089	363	40.33	5.63
Synechocystis sp. PCC 6803	gi 1652857 S74814 *SLR1737*	hypothetical protein SLR1737	363	41.48	8.33

fungi, animals and non-photosynthetic bacterial genomes could be found since tocopherols are only synthesized in photosynthetically active organisms. A corresponding orthologe is also missing in some photosynthetic bacteria such as certain strains of *Synechococcus*, lacking tocopherols (Dasilva and Jensen, 1971; Thomas et al., 1998).

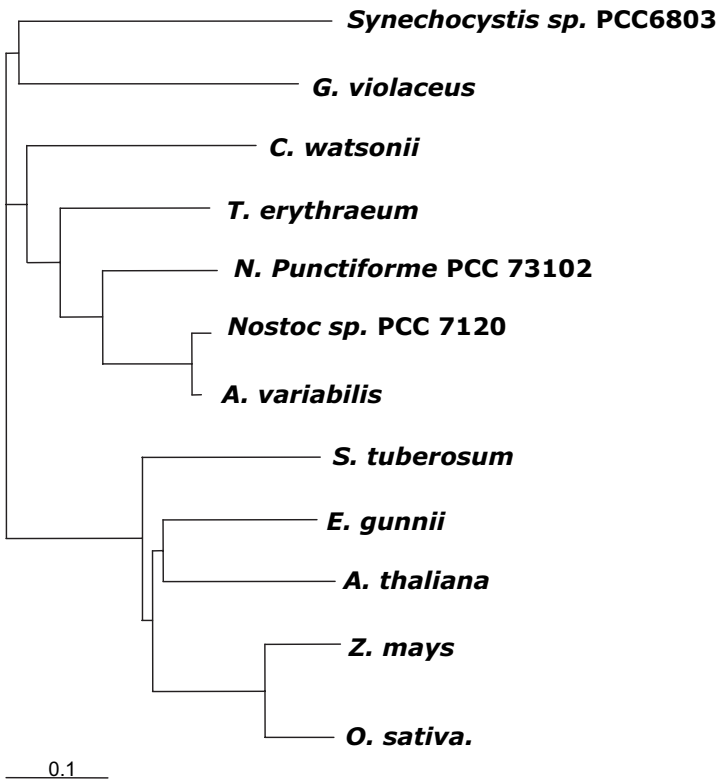


Figure 3.1: Phylogenic relationship amongst the putative TCs from cyanobacteria and plants. The dendrogram was drawn using TreeView (Version 1.5.2, http://taxonomy.zoology.gla.ac.uk/rod/rod/html) after Clustal X analysis. [ID/Accession No - *Arabidopsis thaliana*, Q94FY7; *Eucalyptus gunnii*, Q7XAF0; *Solanum tuberosum*, Q6E6T1; *Zea mays*, Q94FY8; *Oryza sativa* (japonica cultivar-group), Q6K7V6; *Trichodesmium erythraeum* IMS101, gi 48893042; ZP_00326335.1; *Nostoc punctiforme* PCC 73102, gi 23130125, ZP_00111944.1; *Anabaena variabilis* ATCC 29413, gi 46135297, ZP_00162682.2; *Nostoc* sp. PCC 7120, gi 17227741, NP_484289.1; *Crocosphaera watsonii* WH 8501, gi 46120075, ZP_00179394.2; *Gloeobacter violaceus* PCC 7421, gi 37522658, NP_926035.1, *Synechocystis* sp. PCC 6803, gi 1652857, S74814]

The putative TC amino acid sequences were aligned using the CLUSTAL X algorithm (Fig. A.1) and the phylogenic relationships of these sequences were drawn with the TreeView programme (TreeView, http://taxonomy.Zoology.gla.ac.uk/rod/rod/html; Fig. 3.1). According to the dendrogram, the putative TCs can be classified into three subgroups. In the first subgroup, SLR1737 from *Synechocystis* and the hypothetical protein GLR3089 from *Gloeobacter violaceus* PCC 7421 were placed. The second subgroup comprised the remaining cyanobac-

terial sequences and the third subgroup consists of the plant sequences. On the whole, the phylogenic relationship among the analyzed sequences is in accordance with the taxonomic classification of these organisms and clearly distinguishes between eukaryotic plant and prokaryotic bacterial sequences.

Table 3.2: Sequence identity (%) index of putative tocopherol cyclase orthologs from plants and cyanobacteria.

		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
1	*Arabidopsis thaliana*											
2	*Eucalyptus gunnii*	77										
3	*Solanum tuberosum*	66	76									
4	*Zea mays*	61	69	68								
5	*Oryza sativa* (cv. japonica)	63	71	66	79							
6	*Trichodesmium erythraeum* IMS101	47	47	48	47	48						
7	*Nostoc punctiforme* PCC 73102	44	45	48	45	46	70					
8	*Anabaena variabilis* ATCC 29413	43	46	46	45	46	72	80				
9	*Nostoc* sp. PCC 7120	44	46	46	44	45	72	79	97			
10	*Crocosphaera watsonii* WH 8501	39	41	41	41	42	58	60	62	62		
11	*Gloeobacter violaceus* PCC 7421	41	40	39	37	36	50	49	51	50	48	
12	*Synechocystis* sp. PCC 6803	36	38	34	37	35	48	47	48	48	46	41

The CLUSTAL X alignments were further utilized to calculate the sequence identity (%) among the putative TCs from plants and cyanobacteria, summarized in Table 3.2. The analysis showed that the cyanobacterial orthologs of *SLR1737* have a significant degree of identity of 41 to 97%, whereas the highest sequence identity of 97% was found between *Nostoc* sp. PCC 7120 and *A. variabilis* ATCC 29143 sequences of unknown function. Likewise, the plant orthologs of putative TCs have a high degree of identity ranging from 61 to 79 % at amino acid level. The orthologs from two monocot plants, namely rice (Q6K7V6) and maize (SXD1), share 79% identity and other plant sequences showed more than 60% identity. The cyanobacterial and plant orthologs share 35 to 48% amino acid identity, sufficient to identify the plant orthologs from the putative cyanobacterial TC by this *in silico* approach.

The alignments of the amino acid sequences of the putative TCs showed a high degree of similarity between cyanobacterial and plant sequences, especially three regions within the N-terminal and one towards the C-terminal part of the sequence are highly conserved (Fig. A.1). The plant orthologs have additional N- and C-terminal domains that are absent in all seven cyanobacterial sequences. Using the transit peptide prediction software ChloroP (http://www.cbs.dtu.dk/services/ChloroP), the poorly conserved N-terminal domains of the plant sequences were predicted to encode cleavable chloroplast transit peptides. The pre-

dicted subcellular localization of the plant proteins is consistent with previously reported TC activity and tocopherol biosynthesis found in plastids (Soll et al., 1980, 1985; Arango and Heise, 1998).

The TC preprotein from maize was predicted to have a transit peptide of 65 amino acids while that from *Arabidopsis* was predicted to have a longer stretch of 98 amino acids. The prediction with regard to the *Arabidopsis* protein appeared unlikely, because removal of such a long transit peptide leads to the deletion of two of the conserved N-terminal regions of TC sequences (Fig. 3.2). With regard to the maize protein, Provencher et al. (2001) experimentally demonstrated that the N-terminal region is in fact required for import into plastids. In contrast to the N-terminal region, the C-terminal domain of the putative plant TCs is highly conserved suggesting that it might be of functional importance. It is, however, unlikely that the C-terminal domain is essential for enzymatic activity because of the absence of this region in cyanobacterial TC sequences. As expected for the proteins localized in plastids, search for posttranslational modification motifs failed to detect any typical protein motifs except for some putative phosphorylation sites. The hydrophobic nature of the putative TC proteins with low pI values except that of the *Synechocystis* protein is also in accordance with characteristics of membrane-associated proteins (Stocker et al., 1993, 1994, 1996).

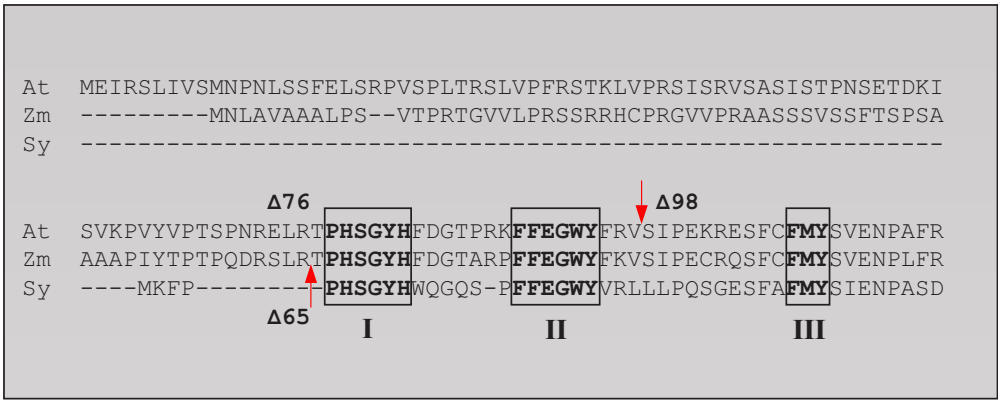


Figure 3.2: Alignment of the N-terminal regions of TC orthologs from plants and *Synechocystis*. Arrows indicate the predicted N-terminal sequence as transit peptide in *Arabidopsis* and maize TC sequences. Amino acids in boxes are conserved regions in the putative TC sequences. At, TC from *Arabidopsis*; Zm, TC from maize; Sy, TC from *Synechocystis*.

In summary, the analysis of the putative TC sequences from both prokaryotic and eukaryotic photosynthetically active organisms revealed high similarities suggesting that they are derived from a common ancestral gene. To provide direct evidence that the identified sequences encode TCs essential for tocopherol biosynthesis and to characterize the enzymes, the sequences from *Synechocystis* sp. PCC 6803, *Zea mays* and *Arabidopsis thaliana* were

cloned and overexpressed in *E. coli*.

3.2 Expression of tocopherol cyclases in *E. coli*

In order to demonstrate that the proteins encoded by the *VTE1* gene (At4g32770) from *Arabidopsis*, *SLR1737* from *Synechocystis* and the maize *SXD1* gene are functional TCs, the respective sequences were cloned and overexpressed in *E. coli* to perform *in vitro* TC assays. For this purpose cDNA was synthesized from the mRNA isolated from leaves of *Arabidopsis* and maize and used as template in subsequent PCR reactions for the amplification of the *VTE1* and *SXD1* ORFs with gene specific primers. The ORF of the *SLR1737* gene from *Synechocystis* was amplified from genomic DNA of *Synechocystis* PCC 6803 with gene specific primers by PCR. The amplified PCR products were cloned in pUC19 and the fidelity of all constructs were confirmed by sequencing.

For functional expression studies in *E. coli*, the cDNA of plant TCs, with and without the 5´-sequences predicted to encode the plastidial transit peptides, were cloned into a pET28a expression vector in which 5´-region encodes the N-terminal region of 98 amino acids appeared unlikely. Therefore, in addition to a Δ98 construct of the *Arabidopsis* TC (pAtΔ98TC), a further construct, pAtΔ76TC, was developed lacking the sequence of the N-terminal 76 amino acids only (Fig. 3.2). The maize construct pZmΔ65 was prepared according to the ChloroP prediction (Fig. 3.2). The chimeric construct carrying the *SLR1737* gene from *Synechocystis* was named pSyTC. The full-length constructs with the TCs from *Arabidopsis* and maize were designated pAtTC and pZmTC, respectively. To express plant and *Synechocystis* TCs as fusion proteins, the stop codon was eliminated in order to add 6xHis-tag in frame at the 3´end of the truncated and non-truncated open reading frames. All chimeric TC constructs with *Arabidopsis*, maize and *Synechocystis* sequences were transformed in BL21(DE3) star cells. The protein synthesis of the TC genes was induced with 1 mM IPTG and subcellular fractions of the transgenic *E. coli* cells were subsequently used for *in vitro* TC assays. These assays were established by modifying the protocol of Stocker et al. (1993). Since TC substrates are not commercially available, MPBQ and DMPBQ were synthesized according to Soll (1987) by Dr. Schüßeler in Prof. Enders´group (RWTH Aachen University, Institute for Organic Chemistry, Aachen, Germany).

To test the TC activity of the recombinant proteins in *E. coli*, cell lysate harboring one of the TC constructs from *Arabidopsis*, maize and *Synechocystis* or the empty vector as a control, *in vitro* TC assays were performed using DMPBQ as substrate. The lipophilic compounds were extracted from the assays with hexane and the reaction product, γ-tocopherol, was

separated from the substrate by normal phase HPLC (see materials and methods). The

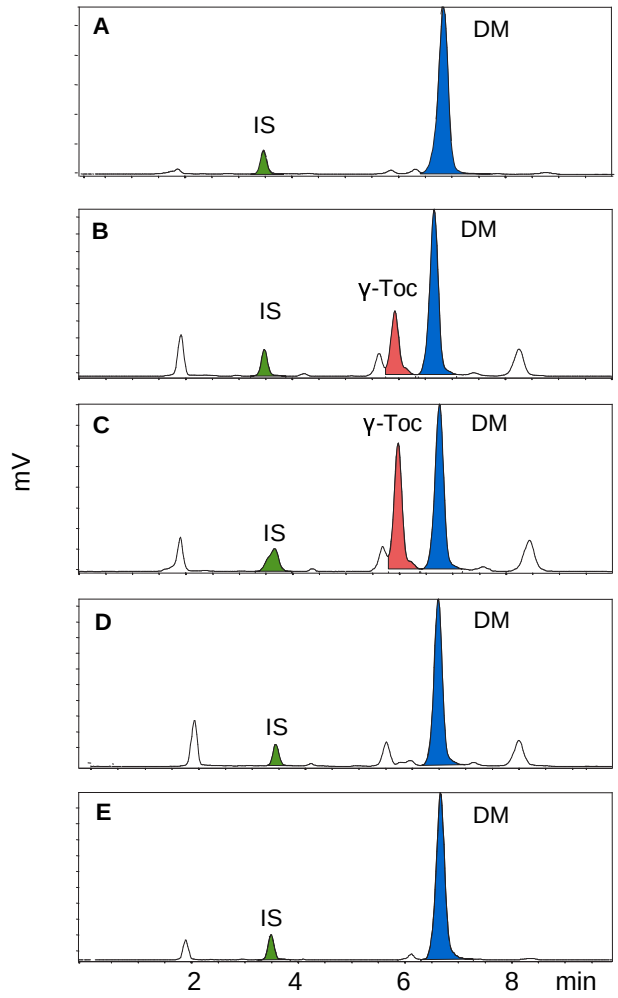


Figure 3.3: Functional expression of TC orthologs in *E. coli*. HPLC chromatograms of *in vitro* TC assays using lysate of *E. coli* cells harboring (A), pET28a as a control; (B), pZmΔ65TC; (C), pAtΔ76TC; (D) pAtΔ98TC; (E), pSyTC; IS, internal standard α-tocopherol; γ-Toc, γ-Tocopherol; DM, 2,3-dimethyl-6-phytyl-1,4-benzoquinol.

product was quantified using α-tocopherol as an internal standard. As shown in Fig. 3.3, TC assays with cell extracts harboring the Δ65TC from maize converted DMPBQ into γ-tocopherol demonstrating high TC activity for the synthesis of γ-tocopherol from DMPBQ. TC assays with pAtΔ76TC cell lysate also followed the same pattern and showed high TC activity. On the other hand, the formation of γ-tocopherol was undetectable with cell lysate expressing the Δ98TC construct of *Arabidopsis*, similar to TC assays performed with lysate of cells harboring the empty vector. To test the expression levels of the recombinant Δ98TC, which might be critical for activity of recombinant TC, cell lysate of *E. coli* cells overexpressing Δ98TC and Δ76TC proteins were analyzed by Western blotting (data not shown). The analysis showed that both the TC proteins from *Arabidopsis* were expressed in similar levels and may not be a limiting factor for *in vitro* TC activities. These findings provide

clear evidence that the *VTET1* (At4g32770) as well as the *SXD1* genes encode a tocopherol cyclase and they are consistent with recent reports from Porfirova et al. (2002) and Sattler et al. (2003). The deletion of the conserved N-terminal regions in the AtΔ98TC construct is a convincing reason for the loss of TC activity and suggests the necessity of this region for the protein to acquire a catalytically active conformation. The cell lysate expressing the pSyTC also failed to show TC activity (Fig. 3.3). The failure to detect TC activity in protein extracts of *E. coli* cells might be due to low expression levels of the recombinant TC protein or stability of the cyanobacterial protein in *E. coli*.

To investigate whether the TC from *Synechocystis* accumulated in distinctly lower levels in *E. coli* cells than the respective proteins ZmΔ65TC and AtΔ76TC from maize and *Arabidopsis*, the levels of the recombinant TCs expressed as His-tagged fusion proteins were examined in cell lysate of transgenic *E. coli* cells after separation of inclusion bodies by Western blotting (see materials and methods). Fig. 3.4 illustrates the expression levels of the recombinant TC proteins from *Arabidopsis*, maize and *Synechocystis* that accumulated in the bacterial cells. In each case a single band of the expected molecular mass (47 kDa for AtΔ76TC, 45.9 kDa for ZmΔ65TC and 42 kDa for SyTC) was detected on Western blots and the highest band intensity was recorded for the *Synechocystis* protein. Hence, in spite of the high expression levels of the *Synechocystis* protein compared to the respective proteins of maize and *Arabidopsis*, it failed to acquire a catalytically active conformation, unlike the plant TC proteins. Perhaps this is due to the pI value of the *Synechocystis* protein, which

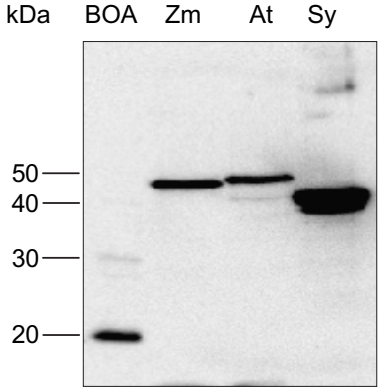


Figure 3.4: Western blot analysis of the expression levels of the recombinant TC proteins from *Arabidopsis*, maize and *Synechocystis*. Total soluble proteins of transgenic *E. coli* harboring one of the TC constructs induced under optimized conditions were precipitated by 45% ammonium sulphate saturation and 12 μg of each fraction was used for Western blotting. Similar levels of expression were achieved with the Δ98TC construct of *Arabidopsis*. (BOA, His-tagged protein ladder; Zm, pZmΔ65TC; At, pAtΔ76TC; Sy, pSyTC).

differs from those of all other TC proteins (Table 3.1) but this awaits clarification. Sattler and co-workers (2003) were also unable to detect TC activity with the protein encoded by

the cyanobacterial *SLR1737* gene under *in vitro* conditions. Despite the vast conservation of the tocopherol biosynthetic pathway and the high sequence similarity between TC proteins from cyanobacteria and plants, there seem to be different requirements for the ambient conditions on hand.

Some enzymes can be catalytically active in their preprotein forms upon heterologus expression in *E. coli* (Williams et al., 2000). To exploit whether the preproteins of *Arabidopsis* and maize display enzymatic TC activities, the cell lysate of transgenic *E. coli* cells expressing either pAtTC or pZmTC was tested in TC assays using DMPBQ as substrate. *Arabidopsis* preprotein showed cyclization of DMPBQ into γ-tocopherol, whereas in TC assays with the maize preprotein formation of γ-tocopherol was undetectable. To investigate expression levels of the TC preproteins from *Arabidopsis* and maize, cell lysates were subjected to Western blotting.

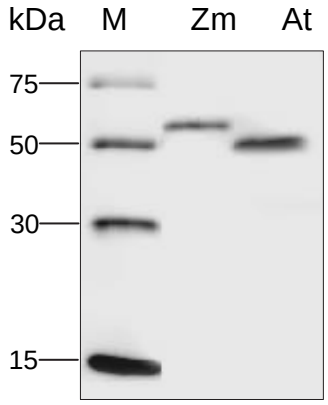


Figure 3.5: Western blot analysis of the expression levels of TC preproteins from *Arabidopsis* and maize. Soluble fraction of transgenic *E. coli* cells (10 μg) harboring either pAtTC or pZmTC were used for Western blotting. (M, qiagen His-tagged ladder; Zm, *Zea mays*; At, *Arabidopsis thaliana*).

As depicted in Fig. 3.5, both recombinant TC proteins accumulated approximately in similar levels in *E. coli* but the *Arabidopsis* preprotein was accumulated in cells as a 50 kDa TC protein instead of the expected 54.7 kDa preprotein. In contrast to the *Arabidopsis* preprotein, a band of 53 kDa was detected in *E. coli* extracts overexpressing the maize preprotein, which corresponds to the molecular mass of unprocessed preprotein. These data suggest that the preprotein of *Arabidopsis* unlike that of maize is rapidly degraded in *E. coli* to a catalytically active protein. Hence, although AtTC and ZmTC have functional similarity i.e. catalysis of cyclization reaction but the properties of these two proteins differ noticeably.

The enzymes involved in the tocopherol biosynthesis pathway have been localized in the plastids, more specifically in the inner envelope membrane of plastids (Soll et al., 1980, 1985;

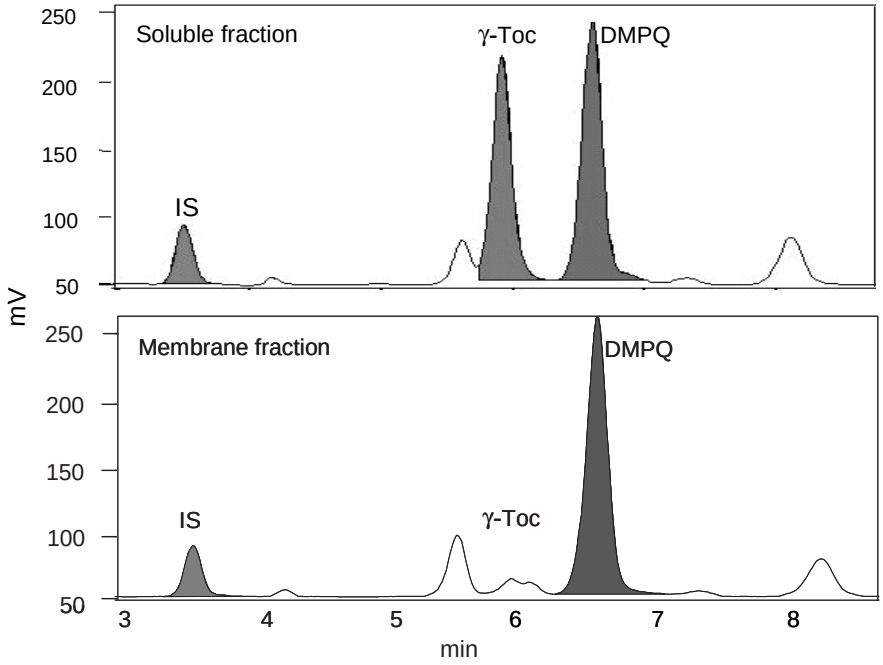


Figure 3.6: TC activity in soluble and membrane fractions of *E. coli* cells overexpressing the recombinant Δ76TC from *Arabidopsis thaliana*. (IS, internal standard α-tocopherol; γ-Toc, γ-Tocopherol; DMPQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol).

Arango and Heise, 1998). In addition, the TC from *Anabaena variabilis* was found to behave like an integral membrane protein, because solubilization with detergents was required in the course of the purification procedure (Stocker et al., 1996). To investigate the behavior of the recombinant Δ76TC protein from *Arabidopsis* in *E. coli*, enzyme assays were performed using total soluble and membrane fractions of *E. coli*. The soluble fractions showed high TC activity whereas no TC activity was detectable in the membrane fractions (Fig. 3.6). These data were obtained regardless whether the recombinant TC protein from *Arabidopsis* was expressed with or without a C-terminal His-tag showing that this tag neither interferes with the enzymic activity nor with the subcellular localization in *E. coli* cells. Hence, the recombinant TC protein from *Arabidopsis* was found to behave like a soluble protein. These results are in accordance with the hydrophobicity profile of the TCs that display no typical transmembrane domains (http://www.cbs.dtu.dk/ services/TMHMM).

3.3 Optimization of the expression conditions for recombinant tocopherol cyclases in *E. coli*

In dependance on the choice of host strains, expression vectors, and growth conditions, most recombinant proteins can be expressed at high levels in *E. coli*. In order to achieve such high expression levels of recombinant TCs from *Arabidopsis* and maize, the T7lac promoter

based expression vector (pET28a) in the BL21(DE3) star was used. Before proceeding to large-scale production of the recombinant TCs, shake flask cultures (100 ml-200 ml medium) were used for the optimization of the expression conditions. The induction of *Arabidopsis* and maize TC gene expression in BL21(DE3) star cells at the exponential growth phase between OD 0.4 to 0.6 with 1mM IPTG at 37°C resulted in high levels of the TC proteins from *Arabidopsis* and maize, but the majority of the proteins accumulated as insoluble aggregates in the cell extract. Therefore, attempts were made to optimize the production of soluble recombinant protein by varying parameters affecting *E. coli* cell growth and protein solubility. The soluble protein expression was estimated by quantification of total cell protein and signal intensity in soluble protein fractions and inclusion bodies on Western blots.

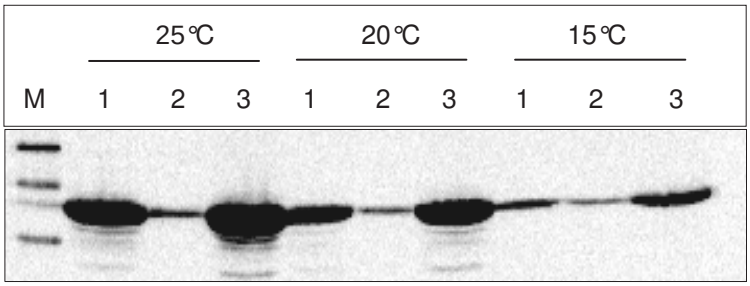


Figure 3.7: Western blot analysis of TC induction from *Arabidopsis* at 25°C, 20°C and 15°C in *E. coli*. 10 μg of each fraction was separated on SDS-PAGE and analyzed by Western blotting. (M, BOA His-tagged protein ladder; 1, clear lysate; 2, soluble fraction; 3, inclusion bodies).

Schein and Noteborn (1988) reported that cell growth and induction at 30°C affects the ratio of soluble and insoluble enzyme forms upon heterologous expression and that a longer induction time at lower temperatures (15°- 20°C) might be helpful to improve the yield of soluble protein. The decrease in the amount of biomass due to inhibition of the cellular metabolism (Shaw and Ingraham, 1967) at lower cultivation temperature is anticipated but this favors the increased solubility of recombinant proteins in the bacterial host. Therefore, the expression of the TC constructs was induced during the exponential growth phase of the transformed *E. coli* cells at different temperatures (25°, 20° and 15°C) for different time periods (2 h to 20 h). Preliminary experiments showed that induction at lower temperatures resulted in production of soluble recombinant TC to a certain degree. Furthermore, induction for short periods (2 to 4 h) regardless of the temperature were not suitable to produce sufficient amount of recombinant TC protein whereas induction for prolonged periods (20 h) resulted in relatively higher amount of protein production. Therefore, induction at lower temperatures (25°, 20° and 15 °C) for 20h were compared to yield high recombinant TC in soluble form. As shown in Fig. 3.7, the level of TC protein from *Arabidopsis* decreased with decreasing cultivation temperature but regardless of the temperature the majority of the re-

combinant *Arabidopsis* TC protein was accumulated as insoluble aggregates. With respect to the recombinant maize protein even lower levels were detected in the soluble fractions (data not shown). As a consequence, there was a need to determine optimal conditions for high expression levels of both TC proteins from *Arabidopsis* and maize in a soluble form in *E. coli*.

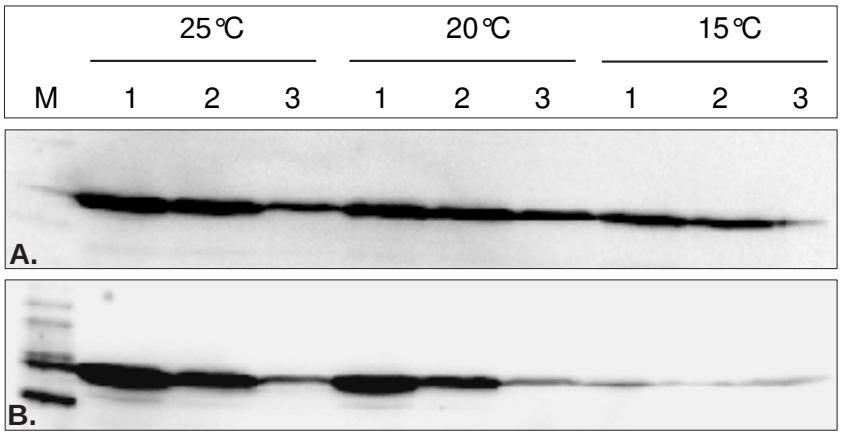


Figure 3.8: Western blot analysis of 2 h induction at high cell density for expression of TC from *Arabidopsis* (A.) and maize (B.) in *E. coli*. 10 μg per lane for each sample was analyzed by Western blotting. (M, BOA His-tagged protein ladder; 1, inclusion bodies; 2, clear lysate; 3, soluble fraction).

Studies on the heterologous expression in *E. coli* have shown that the induction at high cell density (>1) and lower temperature (30°C) produces soluble recombinant proteins which are otherwise expressed as insoluble aggregates upon induction at the exponential phase (Shin et al., 1997). To obtain high levels of soluble recombinant TC proteins, the transgenic *E. coli* cells harboring either the pAtΔ76TC or pZmΔ65TC construct were grown to a high cell density (OD of 2) and induced with 1 mM IPTG for different time periods (1 to 3 h) at 25°, 20°C and 15°C. Under these conditions the highest levels of soluble TC from *Arabidopsis* and maize were obtained when cells were induced at 20 to 25°C for 2 h (Fig. 3.8). The influence of the induction time on the expression levels of the recombinant TCs from maize is shown in Fig. 3.9. An increase in the induction period from 2 to 3 h resulted in a significant decrease in the signal intensity of the soluble fraction and a concomitant increase in the signal intensity of the fraction from the inclusion bodies. In addition, pronounced degradation of recombinant TC was observed at induction for 3 h. These experiments demonstrate that high cell density induction for 2 h resulted in the highest levels of soluble recombinant TC proteins from both *Arabidopsis* and maize.

Piatak et al. (1988) have reported that media composition can also affect protein production and solubility. Striking differences between the levels of expression in different media are often noted. Therefore, LB medium was compared with TB medium that has been shown

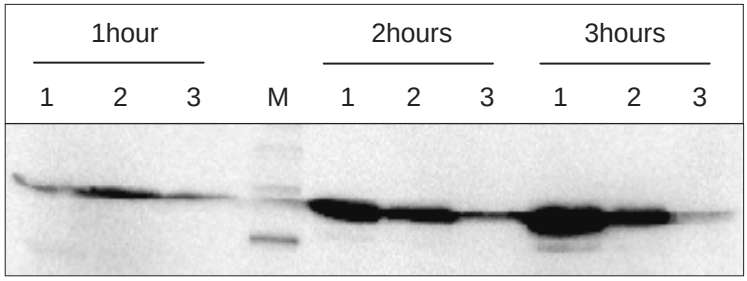


Figure 3.9: Optimization of induction period for the expression of the maize TC in *E. coli*. Western blot of different fractions (10 μg) of transgenic *E. coli* in which expression of the maize TC construct was induced at a high cell density with 1 mM IPTG for 1 to 3 h at 25°C. (M, BOA His-tagged protein ladder; 1, inclusion bodies; 2, clear lysate; 3, soluble fraction).

to improve bacterial growth in high cell density cultures. Under optimal conditions, the induction of *Arabidopsis* TC expression in transgenic *E. coli* cells harboring pAtΔ76TC grown in TB medium resulted in about 2 times higher total protein amount than that obtained after induction in LB media (Fig. 3.10). As judged from Western blot analysis, the band intensity for TC was about 2 to 3 times higher upon induction in TB medium. This yield of recombinant TC protein was significantly high for protein purification.

In summary, the concentration of soluble TC proteins from *Arabidopsis* and maize depends

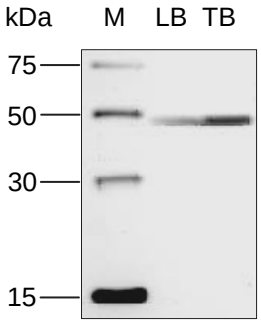


Figure 3.10: Effect of media on the level of soluble *Arabidopsis* TC expressed in *E. coli*. Cells overexpressing pAtΔ76TC were induced in LB and TB media at a high cell density and 20°C for 2 h. 10 μg of clear lysates were analyzed. (M, Qiagen His-tagged protein ladder; LB, Luria broth; TB, Terrific broth).

mainly on the cell growth conditions, temperature, induction period and media composition. Slowing down the rate of expression favors the accumulation of the soluble product, while the prolonged induction time at high cell density led to the formation of insoluble aggregates and to the pronounced degradation of the expressed recombinant protein.

3.4 Purification of recombinant tocopherol cyclases by affinity chromatography

To facilitate the development of an appropriate purification protocol, mature protein sequences of TC from *Arabidopsis* and maize were engineered to introduce six histidine residues at the carboxyl-termini (pAtΔ76TC and pZmΔ65TC) and expressed in *E. coli* cells as fusion protein. The His-tagged TC protein was purified by immobilized metal ion affinity chromatography. This affinity technique is highly specific and based on the binding action of imidazole rings in the His residues to the nickel ions immobilized by the nitrilotriacetic acid (NTA) groups.

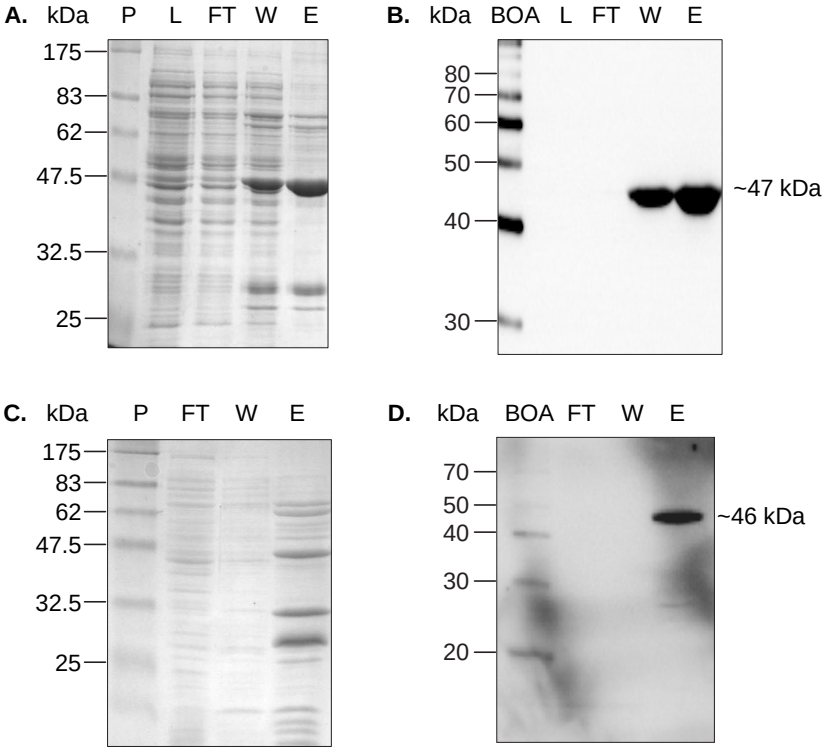


Figure 3.11: Purification of recombinant TC from *Arabidopsis* and maize by Nickel-NTA column. 10 μg of the protein fractions obtained during protein purification were analyzed by SDS-PAGE (A and C) and Western blots (B and D). (P, prestained protein standards; BOA, His-tagged protein ladder; L, total soluble lysate; FT, unbound proteins; proteins eluted with 20 mM (W) and 250 mM imidazole (E)).

The purification procedure consisted of batch adsorption of His tagged proteins on Ni-NTA agarose followed by elution at a high imidazole concentration. Cell lysis was performed as described in materials and methods using protein lysis buffer (Table 2.2). In order to limit non-specific protein adsorption on Ni-NTA agarose 300 mM NaCl and 10 mM imidazole were included in the lysis buffer. 1 mM PMSF was used in lysis buffer to avoid the proteolytic

degradation. The non-specifically bound proteins were washed from the column with lysis buffer containing 20 mM imidazole and elution of His-tagged TC proteins was performed with 250 mM imidazole in lysis buffer. The typical elution patterns of the His-tagged TC from *A. thaliana* and *Zea mays* are presented in Fig. 3.11. Under optimized expression conditions, the yield of purified TC was estimated up to 1.5 mg protein per liter culture grown in TB medium. A small amount of TC was present in wash fractions, which could be explained by excessive washing at the adsorption limit of His-tagged TC. Although, analysis of the elution fractions by SDS-PAGE revealed a band of ∼47 kDa as an enriched recombinant TC but the elution procedure resulted in weak contamination with other proteins, the most prominent ones being approximately ∼70 kDa, ∼60 kDa and ∼28 kDa in mass (Fig. 3.11 A and C). The Western blot analysis of the various elution fractions revealed only a single band at the expected sizes (Figure 3.11 B and D). None of the other proteins that co-eluted with the TCs were detected in the Western blot showing that the contaminants were not multimeric or degraded forms of the TC, but unrelated bacterial proteins. Their molecular masses correspond to those of bacterial chaperonins that are involved in the proper folding of nascent proteins in *E. coli* (Gottesman and Hendrickson, 2000).

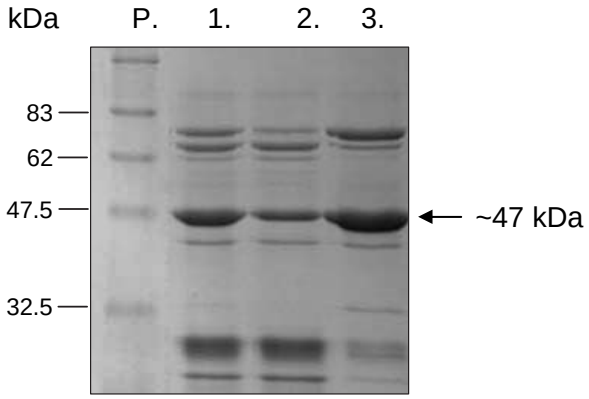


Figure 3.12: SDS-PAGE analysis of enriched TC from *Arabidopsis*. The partially purified TC from *Arabidopsis* was precipitated at 45% ammonium sulphate saturation. Each fraction (10 μg protein) was analyzed by SDS-PAGE. (P, prestained protein standards; 1, enriched TC protein eluted from Ni-NTA column; 2, supernatant after ammonium sulphate precipitation; 3, proteins precipitated by 45% ammonium sulphate saturation).

High concentrations of imidazole were found to inhibit TC activity (data not shown). Therefore, eluted fractions were subjected for an ultrafiltration step using viva spin concentrator tubes in order to remove imidazole and to concentrate the proteins in storage buffer (Table 2.2). The enriched TC fractions could be stored for 4 to 6 months at -80°C without significant loss of activity.

To improve the purity of enriched *Arabidopsis* TC further, ammonium sulphate precipitation was chosen as a next step of purification. The partially purified TC from *Arabidopsis* was precipitated at 45% saturation of ammonium sulphate and resuspended in storage buffer. The residual ammonium sulphate was removed using viva spin concentrators that were washed with an excess of storage buffer. This procedure resulted in a significant reduction of the abundance of the lower molecular mass proteins of about 28 kDa (Fig. 3.12). The resultant enriched fraction was further utilized to characterize TC properties.

3.5 Size exclusion chromatography of purified tocopherol cyclase

To determine the native molecular mass of the *Arabidopsis* TC and to improve the purity of the enriched TC fractions, size exclusion chromatography was performed. A calibration curve was obtained by plotting the logarithm of the standard proteins of known molecular mass versus the corresponding K_{av} values calculated from the measured elution volumes. The partially purified TC from *Arabidopsis* obtained after affinity purification was applied

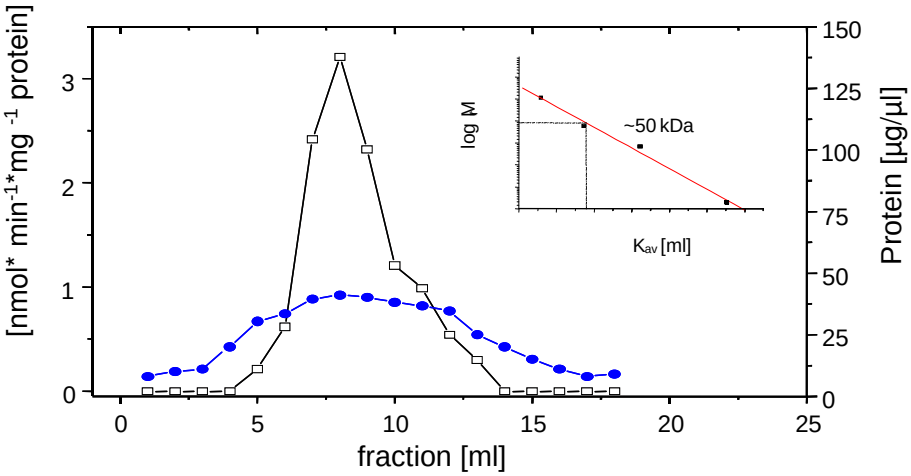


Figure 3.13: Elution profile of the *Arabidopsis* TC by gel filtration chromatography under non-denaturing conditions. (□, specific TC activity; •, protein concentration; Inset, standard curve K_{av} versus the log of the molecular mass was derived from the elution profiles of standards. The peak position of the *Arabidopsis* TC is indicated by a line).

to a bonded silica SEC 125-5 gel filtration column and the TC activity was eluted in the 50 kDa molecular mass fraction (Fig. 3.13), which corresponds well to the monomeric form of the enzyme (calculated molecular mass 46.5 kDa). The UV absorbance at 280 nm showed that the TC from *Arabidopsis* eluted from the column as a large single peak with a small shoulder as reflected in the TC activity profile.

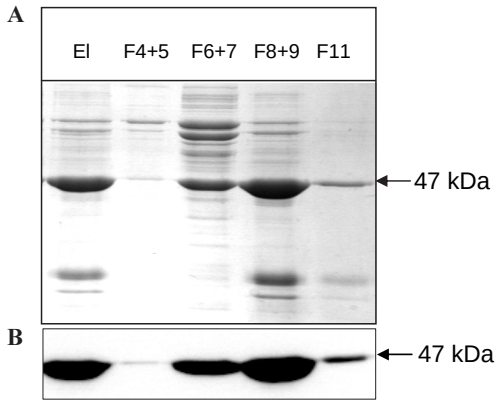


Figure 3.14: SDS-PAGE (A) and Western blot (B) analysis of fractions obtained during purification of the recombinant His-tagged TC from *Arabidopsis* by gel filtration chromatography.(El, eluate after affinity chromatogrphy; F, fractions eluted from gel filtration columnns).

As depicted in Fig. 3.13 the gel filtration chromatography did not improve TC purification apart from minor effects but provided evidence that the TC from *Arabidopsis* is catalytically active in its monomeric state. Hence, the *Arabidopsis* TC does not have tendency to make multimeric aggregates such as those that have been reported for a cyclase involved in the synthesis of the isoprene β-carotene (Candau et al., 1991).

3.6 Characterization of tocopherol cyclases from plants

In order to analyze the enzymatic properties of the recombinant TCs from *Arabidopsis* and maize, *in vitro* TC assay conditions were optimized using enriched *Arabidopsis* and maize TC protein fractions obtained after Ni-NTA affinity purification.

The enzyme TC plays a key role in the formation of chromanol ring of the different tocopherol isoforms. In the tocopherol biosynthetic pathway, it utilizes two naturally occurring substrates, 2,3-dimethyl-6-phytyl-1,4-benzoquinol (DMPBQ) and 2-methyl-6-phytyl-1,4-benzoquinol (MPBQ) and converts them into γ-tocopherol and δ-tocopherol, respectively. Due to the highly hydrophobic nature of DMPBQ and MPBQ, their solubility in an aqueous phase limits the activity of TCs in *in vitro* assays. The formulation of poorly water-soluble compounds with a complex forming agent such as cyclodextrin is a common practice in drug industry to increases their solubility, bioavailability and stability (Loftsson and Brewster, 1996). In this study, methyl-β-cyclodextrin was used for the formation of substrate-cyclodextrin inclusion complexes as described under materials and methods. The formulation with cyclodextrin was presumed to mask the hydrophobic tail of DMPBQ and

MPBQ (Stocker et al., 1993). To achieve a quantitative conversion of DMPBQ and MPBQ

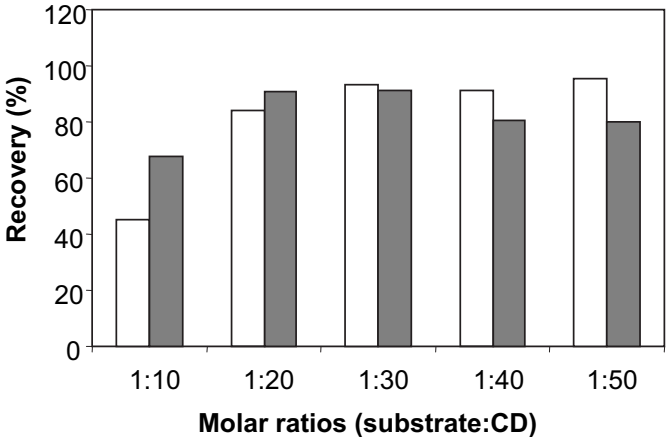


Figure 3.15: Formulation of TC substrates with methyl-β-cyclodextrin. 2 mg of either DMPBQ or MPBQ were formulated with different concentration of methyl-β-cyclodextrin in a total volume of 2 ml. The recovery of substrate converted into water soluble inclusion complex was calculated by HPLC analysis. The white and gray bars show recovery from DMPBQ and MPBO, respectively. (DMPBQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; CD, methyl-β-cyclodextrin).

into their water-soluble complexes, the formulation was performed with various molar ratios of substrate and cyclodextrin from 1:10 to 1:50, respectively. The recovery of the formulated substrates was measured by HPLC after extraction of the formulated inclusion complex with n-hexane. As shown in Fig. 3.15, a molar ratio of 1:20 between substrate and methyl-β-cyclodextrin turned out to yield an almost complete recovery of the substrates ($>$ 90%) in the water-soluble complex. The formulations at lower concentrations than 1:20 of substrate and methyl-β-cyclodextrin resulted in more than 40 % loss of substrate, indicating that the concentration of cyclodextrin is not sufficient for formulation. On the other hand, the formulation with cyclodextrin at molar ratios higher than 1:20 did not improve the recovery of substrate significantly. Hence, a 1:20 ratio was optimal for extracting the substrate into the respective cyclodextrin inclusion complex.

Furthermore, the redox state of both, the substrate and the reaction centre of the TC protein, are important for optimal turnover rates (Arango and Heise, 1998; Stocker et al., 1993). The addition of reducing agents such as dithiothreitol and ascorbate to a final concentration of 4 mM and 75 mM, respectively, gave maximal formation rates of γ-tocopherol (data not shown). On the other hand, divalent cations like Mg^{2+} are not imperative for TC activity, as depletion of divalent cations from the TC assay had no effect on the activity of the TCs from both *Arabidopsis* and maize.

3.6.1 pH and temperature optimum

The activity of the recombinant TC from *Arabidopsis* and maize was evaluated as a function of pH using DMPBQ as substrate. The recombinant TC from *Arabidopsis* showed highest cyclization rates of DMPBQ at pH 7.0 (Fig. 3.16). On the other hand, the recombinant TC from maize had a broad pH optimum between 7.5 and 9.0 (Fig. 3.16). The TC activities of both *Arabidopsis* and maize enzyme varied in dependence on the buffer components. Maximal activities for both enzymes were measured in phosphate buffer, while MOPS buffer or a buffer mix containing MOPS, MES and tricine gave 2- to 3-fold lower activities. The analysis of the cyclization rates as a function of the buffer concentrations revealed that the TCs from both *Arabidopsis* and maize were most active in 200 mM phosphate buffer.

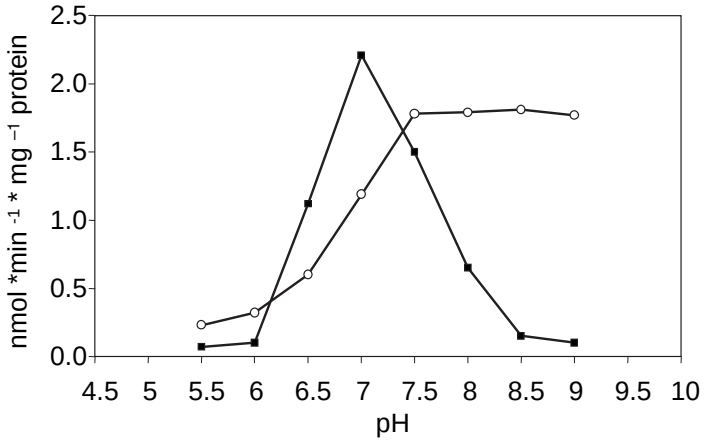


Figure 3.16: pH dependence of TC activities from *Arabidopsis* (■) and maize (○). Enzyme assays were carried out in a mixture with each 50 mM MES, 50 mM MOPS and 50 mM tricine buffer using DMPBQ as substrate.

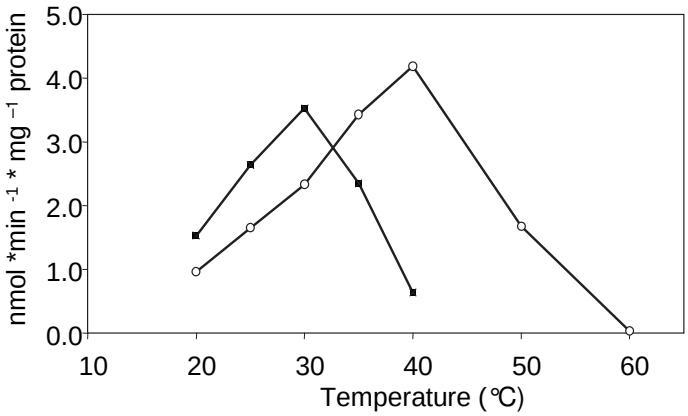


Figure 3.17: Temperature dependence of TC activity from *Arabidopsis* (■) and maize (○). Cyclization rates were measured as function of temperature using DMPBQ and enriched fractions of TC protein from *Arabidopsis* and maize at temperatures shown.

The TC activities from both *Arabidopsis* and maize as a function of the temperature are shown in Fig. 3.17. Like the pH optima, the temperature optima for both TCs varied clearly. The TC from *Arabidopsis* was found to have the highest cyclization rate at 30°C, while higher temperatures inhibited the enzyme. In contrast to the *Arabidopsis* TC, the enzyme from maize showed maximal activities at 40°C. The activity was sharply decreased above this temperature and approached to almost zero at 60°C, probably due to protein denaturation.

3.6.2 Kinetic parameters

The properties of both TC proteins were further examined by determining kinetic parameters. Both TCs from *Arabidopsis* and maize exhibited regular Michaelis-Menten kinetics (Fig. 3.18). The maximum activities of both TCs were obtained at 350 μM DMPBQ and apparent K_m values of 90 and 180 μM were measured for the *Arabidopsis* and maize TC, respectively. The maize TC showed a 2-fold higher V_{max} than that of the TC from *Arabidopsis* at 40°C. Although TCs from *Arabidopsis* and maize share a significant degree of sequence similarity

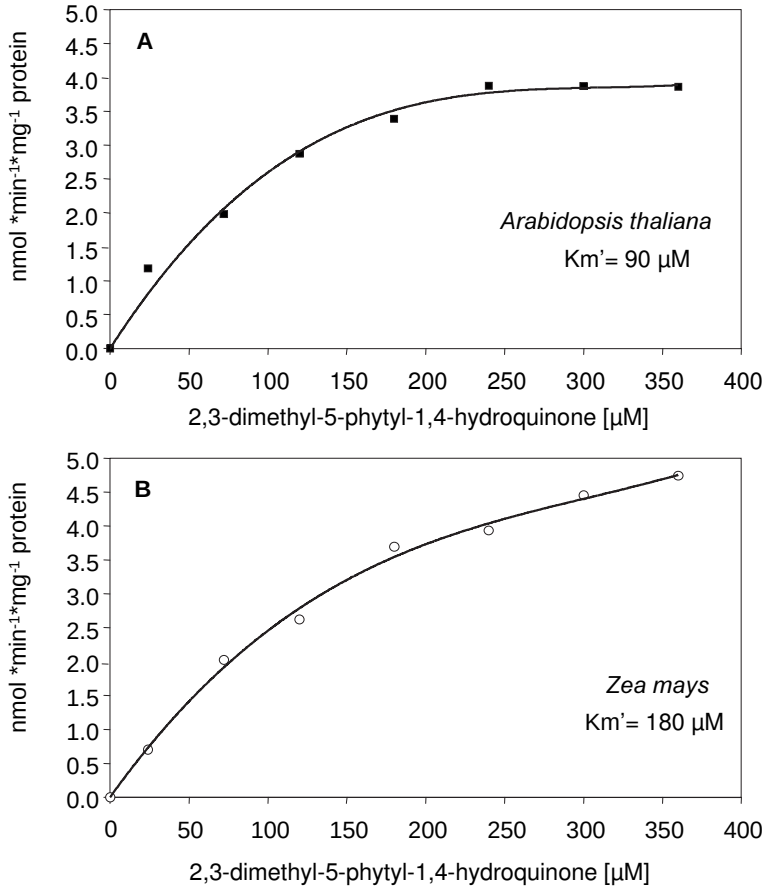


Figure 3.18: Michaelis–Menten plot of TC activity from *Arabidopsis* (A) and maize (B) at 30°C. The maize TC has a two fold higher Vmax at 40°C.

and are functional orthologs, both TCs showed remarkable differences in their enzymatic

properties with respect to pH and temperature optima as well as their kinetic properties. These differences are likely due to the diverse origins of the assayed enzymes because we compare the properties of a protein from a C_3 dicot plant with those from a C_4 monocot one, therefore meeting other demands concerning adaptation to environmental conditions.

3.6.3 Protein and time linearity

Under these *in vitro* conditions, protein linearity experiments were determined with enriched recombinant TC fractions from *Arabidopsis* and maize over a range from 250 ng to 5 μg protein. The formation of cyclization product as a function of increasing protein concentration is shown in Fig. 3.19. The rates of γ-tocopherol formation with recombinant *Arabidopsis*

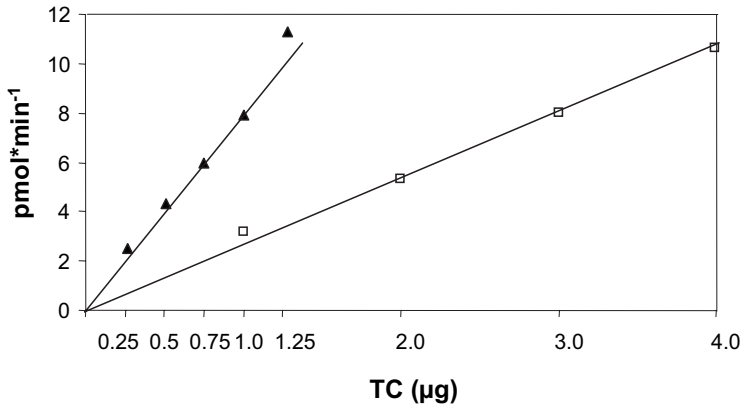


Figure 3.19: Protein linearity conditions of TC activity from *Arabidopsis* (▲) and maize (□). The TC activities as a function of protein concentration of TC from maize and *Arabidopsis* is shown.

TC protein were found to be linear up to at least 1 μg protein, whereas recombinant TC from maize showed the cyclization reaction in linear progression up to 4 μg protein. Similar to the protein linear conditions, time linearity experiments were performed with both recombinant TCs from *Arabidopsis* and maize over a period of 2 h. Although the cyclization rates of DMPBQ were constant up to 2 h for both the recombinant TCs, but 30 min assays were adopted already giving a sufficient amount of γ-tocopherol to be distinctly identified by HPLC analysis (data not shown).

3.6.4 Substrate specificity

In plants δ- and β-tocopherol represent only minor components (Table 1.1) suggesting that plant TCs convert MPBQ less effectively than DMPBQ or that MPBQ is rarely available to the TCs because the MPBQ methyltransferase rapidly convert MPBQ to DMPBQ. To

investigate whether the low levels of δ- and β-tocopherol are primarily due to the properties of the plant TCs, the substrate specificities of the recombinant TC from *Arabidopsis* and maize were analyzed. When formulated MPBQ was used as substrate its cyclization product δ-tocopherol was undetectable by HPLC analysis regardless of the MPBQ concentration and the incubation time (Fig 3.20). Even under conditions under which DMPBQ was almost completely converted to γ-tocopherol, MPBQ was not converted (Fig 3.20). In order to analyze the effect of the additional C-terminal His-tag on substrate specificity, assays were performed using the TC from *Arabidopsis* without fused His-tag. Nevertheless, it did not alter the results indicating that the presence or absence of a C-terminal His-tag did not affect the cyclization activity of the TC from *Arabidopsis* with respect to MPBQ. Stocker et al. (1996) provided evidence that for instance the *E*-configuration of the double bond in the precursor and the chirality of the phytyl substructure are critical determinants for the cyclization reaction. To exclude that an inoperative stereoisomer of MPBQ was selected during the chemical synthesis of the substrates, the right confirmation of MPBQ was confirmed by mass spectroscopy and NMR.

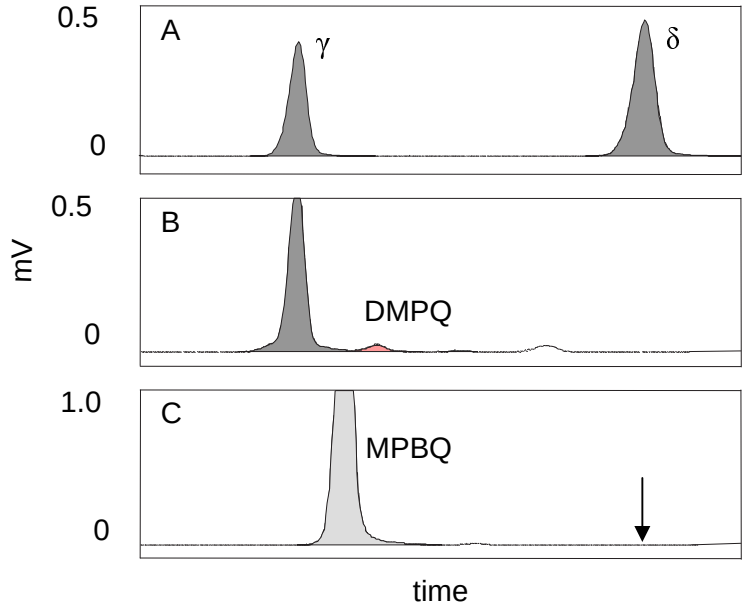


Figure 3.20: Determination of the substrate specificity of the TC from maize. Normal-phase HPLC analysis of *in vitro* TC assays with Ni-NTA purified recombinant maize TC as described in material and methods. A, Separation of γ- and δ-tocopherol product standards; B & C, *in vitro* assays with the substrates DMPQ and MPBQ, respectively. (MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; γ, γ-tocopherol; δ, δ-tocopherol; DMPQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol).

These data clearly show that the recombinant plant TCs possess a higher specificity for DMPBQ at least under *in vitro* conditions. Hence, the recombinant *Arabidopsis* and maize TC activities differ from the TC from *A. variabilis* that possesses no pronounced specificity

with regard to degree and position of methylation at the aromatic ring of the substrate under *in vitro* conditions (Stocker et al., 1996). On the other hand, the analysis of tocopherol compositions of *Arabidopsis* mutants deficient MPBQ methyltransferase activity (Cheng et al., 2003) provided strong evidence that the *Arabidopsis* TC can utilize not only DMPBQ but also MPBQ as substrate at least when MPBQ accumulates in high levels in the mutant plants. In view of the fact that the TC is encoded by a single copy gene (Porfirova et al., 2002), these data suggest that the substrate specificity of the *Arabidopsis* TC is less pronounced under *in vivo* than *in vitro* conditions. Perhaps the interaction with the envelope membrane of plastids or with other plastidial proteins are required for a conversion of MPBQ by a plant TC.

3.7 Transgene expression of plant Tocopherol cyclases in seeds of *Brassica napus*

Due to high economic value of vitamin E and its benefits for human health, the generation of plants with elevated tocopherol content is highly desirable by overexpressing one of the structural genes of the tocopherol biosynthetic pathway. With the objectives to better understand the regulatory mechanism of TC in the biosynthetic pathway and to improve tocopherol content of rapeseed oil, transgene expression of TC genes from *Arabidopsis* and maize in developing seeds of *Brassica napus* was carried out in cooperation with Prof. Friedt´s group at the Institute of Crop Science and Plant Breeding I, Justus-Liebig-University, Giessen.

To achieve this goal, TC genes from *Arabidopsis* and maize were cloned into a seed specific napin-nos cassette under the control of a strong napin promoter . The chimeric TC genes in the plant expression cassette were ligated into the binary vector carrying the NptII gene conferring kanamycin resistance and the resultant constructs were transformed into *Agrobacterium*. For the generation of trangenic plants, hypocotyl segments of spring rapeseed (cv. 'Drakkar') were transformed by infection of *A. tumefaciens*, harboring either a chimeric TC gene construct from maize or *Arabidopsis*. Regenerated plants which functionally expressed the NptII gene were considered as transgenic and their seed oils were analyzed by HPLC with regard to tocochromanol content and composition. The total tocochromanol content was calculated as the sum of α-, γ- and δ-tocopherol as well as plastochromanol-8 (P8).

The results for the analysis of T_1 and T_2 populations and selected individual plants are summarized in Table 3.3. T_1 and T_2 plants were grown in different environments, hence the absolute values for the respective wild-type plants differed. In the T_1 population, the total tocochromanol levels of 25 and 36 individual lines overexpressing TC from maize and

Arabidopsis were examined, respectively. Plants overexpressing chimeric TC from maize and *Arabidopsis* showed a significant increase of 18% and 28% in the average total tocochromanol content relative to wild type plants, respectively. The maximum increase in tocochromanol content by 30% was recorded for ZmTC/677 line, harboring the maize TC gene and AtTC/684 line overexpressing TC from *Arabidopsis*. Analysis of the progeny of these two transgenic lines revealed that the T_2 plants contained 20% to 55% higher total tocochromanol levels than the respective wild type plants. Hence, these data confirmed the trend of significant enhancement of total tocochromanols in the offspring of the T_1 plants.

Table 3.3: Tocochromanol content of transgenic rapeseed plants overexpressing chimeric TC genes. Mean values ± SE and individual values of selected plants are given. (N, number of analyzed plants; ZmTC, AtTC, and transgenic *Brassica napus* populations expressing the TC from maize and *Arabidopsis*, respectively).

		Tocochromanol content (mg kg^{-1} oil)				
	N	α	γ	δ	P8	Total
T1 population						
Wild-type	8	199 ± 15.9	466 ± 15.2	10 ± 1.7	8 ± 1.6	683 ± 26.9
ZmTC	25	241 ± 9.6	538 ± 12.3*	16± 0.8	14 ± 1.1	809 ± 11.9*
AtTC	36	277 ± 12.7*	552 ± 7.7*	27 ± 2.0*	19 ± 1.6*	875 ± 16.2*
Selected T1 plants						
ZmTC/677	1	199	650	24	22	895
AtTC/684	1	235	616	36	23	909
T2 population						
Wild-type	29	311 ± 9.0	417 ± 9.3	10 ± 0.2	10 ± 0.9	748 ± 12.0
ZmTC/677	17	385 ± 17.1*	553 ± 16.4*	18 ± 1.3*	14 ± 1.1	970 ± 25.5*
AtTC/684	40	318 ± 9.2	476 ± 8.8*	36 ± 2.0*	24 ± 1.7*	855 ± 8.4*
Selected T2 plants						
ZmTC/677-36	1	386	720	**32**	21	**1159**
ZmTC/677-5	1	404	511	16	**23**	954
AtTC/684-16	1	507	333	34	**76**	951
AtTC/684-5	1	303	513	**67**	34	916
AtTC/684-37	1	436	510	36	37	**1018**

*$P < 0.01$, significant

The total content as well as the composition of tocopherols varies in different plant organs. Plant seeds contain predominantly higher amounts of tocopherol than the leaves (Table 1.1). Various studies on identification of limiting steps in tocopherol pathway have been reported by overexpression of one of the structural genes of the pathway such as HPPD or HPT. The

overexpression of HPPD or HPT elevated tocopherol levels in leaves by a factor of 1.4 and 4.4, respectively (Tsegaye et al., 2002; Collakova and DellaPenna, 2003). On the other hand, transgene expression of HPPD and HPT in seeds resulted in 1.3-fold and 1.7-fold increase in total tocopherols, respectively (Tsegaye et al., 2002; Savidge et al., 2002). These studies attributed HPT as limiting enzyme for channelling the carbon flux in tocopherol biosynthesis. Although HPT overexpression resulted in a 4.4-fold accumulation of tocopherol in leaves, but the increase in the absolute tocopherol levels appears less significant than in seed due to distinctly lower tocopherol levels in leaves than in seeds. In the current study, overexpression of a TC in developing seeds of *B. napus* significantly enhanced the tocochromanol content by a factor of 1.6 that is consistent with the previous reports for HPT. In addition, differences with respect to the tocochromanol composition were noticed in the seed oil between wild type and transgenic *B. napus* plants overexpressing the TCs from *Arabidopsis* or maize (Table 3.3). In comparison to wild type plants, the δ-tocopherol content was 3.2-fold and 6.7-fold higher in transgenic lines expressing TC from maize (ZmTC/677-36) and *Arabidopsis* (AtTC/684-5), respectively (Table 3.3). In accordance with the analysis of the *Arabidopsis* mutants, the TCs from *Arabidopsis* and maize are *in planta* able to utilize not only DMPBQ but also MPBQ as a substrate, at least under conditions that provide elevated MPBQ concentrations or TC expression levels (Fig. 3.21). The high accumulation and alteration in

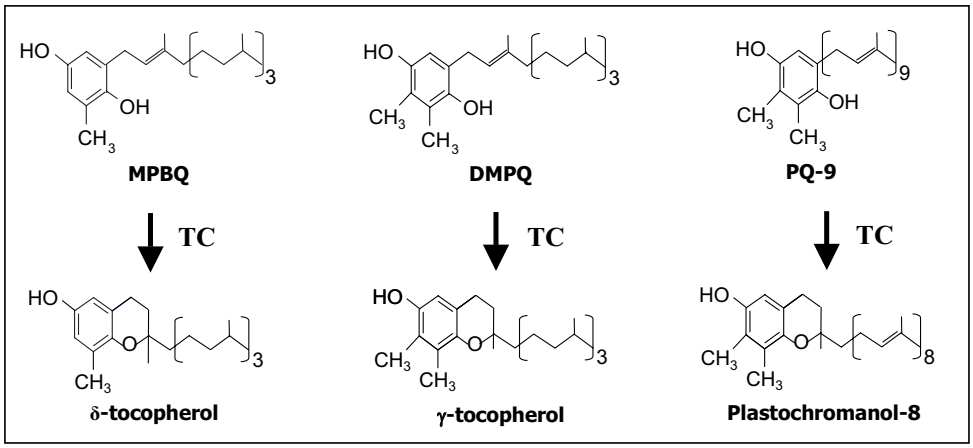


Figure 3.21: Chromanol head group formation catalyzed by TC utilizing MPBQ, DMPQ and PQ-9 as substrates. MPBQ, 2-methyl-6-phytyl-1,4-benzoquinol; DMPQ, 2,3-dimethyl-6-phytyl-1,4-benzoquinol; MT, MPBQ methyltransferase; PQ-9, plastoquinone-9.

composition of tocochromanol levels were directly attributed to the activity of recombinant TC activity in transgenic plants. The present investigation demonstrated that TC activity influences to a certain extent total tocopherol content in plants by effectively channelling the flux of the prenylquinone intermediates towards the different tocopherol end products. This hypothesis is supported by a recent study of Kanwischer et al. (2005). The authors report

that overexpression of TC in *Arabidopsis* leaves induced high levels of tocopherol accumulation and resulted in an alteration of tocopherols composition of leaves to γ-tocopherol as predominant isoform.

Furthermore, transgenic rapeseed plants expressing the TC from *Arabidopsis* or maize showed a noticeable variance regarding the plastochromanol-8 (P8) content (Table 3.3). P8 is a minor tocochromanol component in the seed oils of various plants and predominantly found in the oil of *B. napus*, *Linum usitatissimum* and *Cannabis sativa* seeds but its biosynthetic pathway has not been elucidated yet. In the transgenic plants expressing the maize TC, a slight rise of the average P8 content was observed, whereas the transgenic plants expressing the *Arabidopsis* TC showed a significant 1.9- to 2.4-fold enhancement in the T_1 and T_2 population, respectively. Analyses of individual T_2 plants confirmed the tendency for the maize progeny and substantiated the results for the *Arabidopsis* plants by reaching a 7.6-fold increase (AtTC/684-16) in the P8 content compared to the wild-type plants (Table 3.3). In conclusion, these findings provide for the first time a strong evidence that plant TCs from *Arabidopsis* and maize possess a broad substrate specificity and can cyclize plastoquinone-9 to P8 (Fig. 3.21). In addition, these findings suggest a regulatory role of the TC in prenyllipid metabolism.

Chapter 4

Summary

Tocopherols, collectively known as vitamin E, are amphiphatic molecules consisting of a polar chromanol head ring and a lipophilic isoprenoid tail. Because of the diverse functions, dietary supplements of tocopherols are thought to play an important role in improving immune function and in limiting the incidence and progression of several degenerative human diseases. Tocopherols are synthesized only in photosynthetic organisms. In plants, tocopherol biosynthesis proceeds at the inner envelope membrane of plastids. In the tocopherol biosynthetic pathway, tocopherol cyclase (TC) catalyses the key step in the biosynthesis of the chromanol substructure of the vitamin E family.

To investigate the role of TC in the tocopherol biosynthesis, the present study was aimed at cloning and characterization of the TC genes from *Arabidopsis* and maize. The TC genes from *Arabidopsis* and maize were engineered for functional expression studies in *E. coli*. Firstly, the expression conditions were optimized to achieve the accumulation of high levels of recombinant TC proteins in the bacterial host cells. Subsequently, the recombinant TC proteins, which behaved like soluble proteins and were catalytically active in their monomeric forms, were purified by affinity chromatography and used for the analysis of their enzymatic properties. These experiments revealed that the functional orthologs from the two plant species possess remarkable differences in their enzymatic properties with respect to pH and temperature optima and their kinetic constant.

To gain new insights into the mechanisms regulating the tocopherol biosynthesis, chimeric TC gene constructs were overexpressed in developing seeds of transgenic rapeseed plants. This TC overexpression resulted in a significant increase in total tocopherol content, suggesting that TC activity is the limiting factor of tocopherol biosynthesis. Furthermore, overexpression of the recombinant TCs in developing seeds of *Brassica napus* enhanced the plastochromanol-8 content several folds. These findings show that TCs from *Ara-*

bidopsis and maize possess a broad substrate specificity and can cyclize plastoquinone-9 to plastochromanol-8. In addition, they provided evidence for a regulatory function of the TC in prenyllipid metabolism.

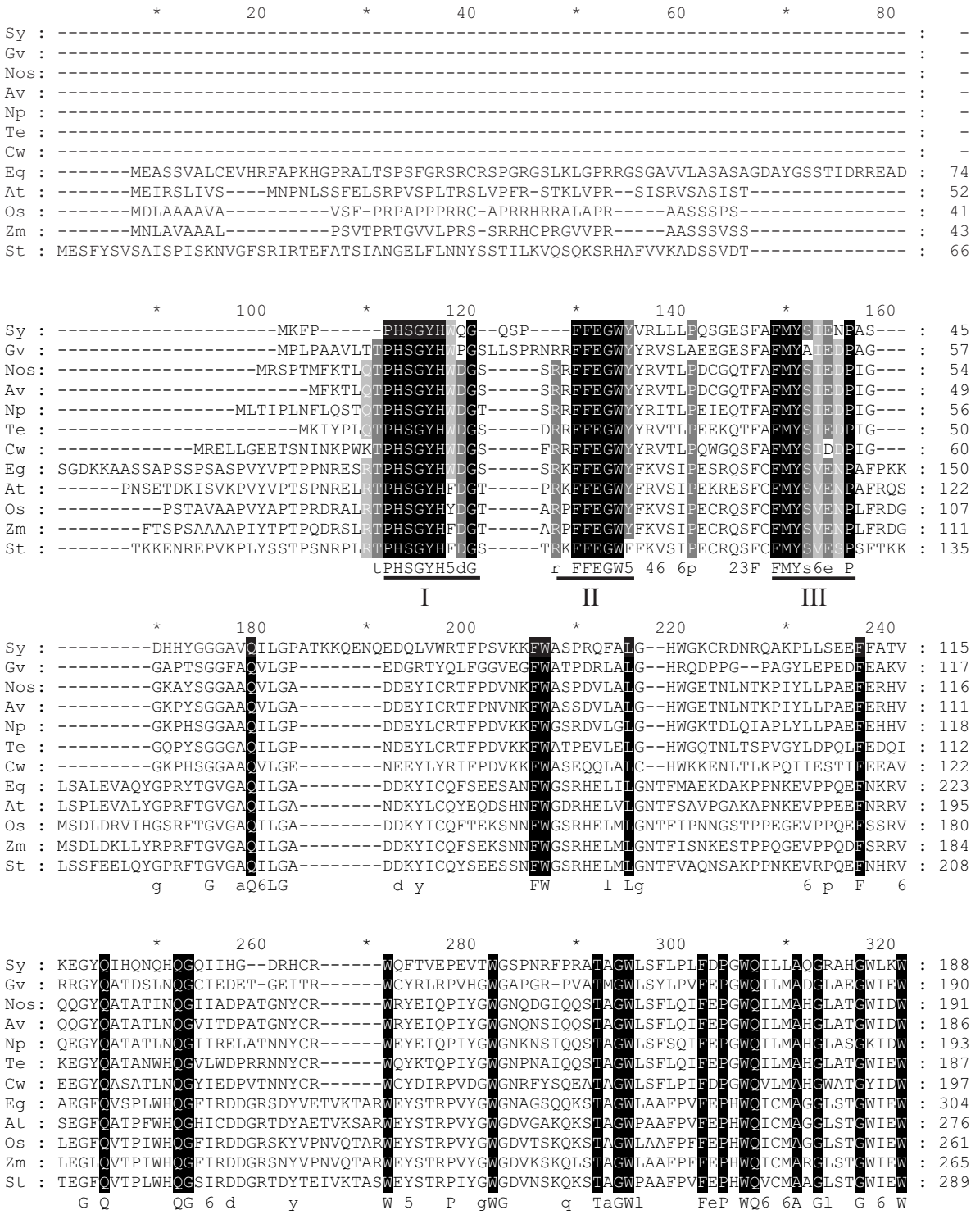
Appendix

A.1 Abbreviations

Abbreviation	Full form
%	Percentage
°C	Degree Celsius
A	Adenine
Amp/Amp^R	Ampicillin/ampicillin resistance
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Bps	Base pairs
BSA	Bovine serum albumin
Carb	Carbenicillin
cDNA	Complementary DNA
CoA	Coenzyme A
cv.	Cultivars
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside Triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
His	Histidine
HPLC	High pressure liquid chromatography
HPP	4-Hydroxy phenyl pyruvate
HPPD	Hydroxyphenylpyruvate dioxygenase
HPT	Homogentisate phaytyltransferase
IMAC	Immobilized metal ion affinity chromatography
IPTG	Isopropyl-β-D-thiogalactoside
Kan/Kan^R	Kanamycin/kanamycin resistance

Abbreviation	Full form
kb	Kilobase pairs
KCl	Potassium chloride
kD	Kilodalton
K_m	Michaelis constant
LB medium	Luria Bertani medium
LDL	Low-density lipoprotein
min	Minute
ml	Milli-liter
mM	Milli molar
MOPS	3-(N-morpholino) propane sulfonic acid
mRNA	Messenger RNA
mV	Milli Volt
MW	Molecular weight
MWCO	Molecular weight cut-off
NaCl	Sodium chloride
NCBI	National center for biotechnology information
Ni-NTA	Nickel charged nitriloacetic acid resin
OD	Optical density
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonylfluoride
PUFA	Poly unsaturated fatty acid
Rif	Rifampicin
RNA	Ribonucleic acid
rpm	Rotation per minute
RT	Room temperature
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
sec	Second
Taq	*Thermus aquaticus*
TC	Tocopherol cyclase
TEMED	N, N, N´, N´-tetramethyl ethylene diamine
Tris	Trishydroxymethylaminomethane

A.2 The alignment of sequences using CLUSTAL X



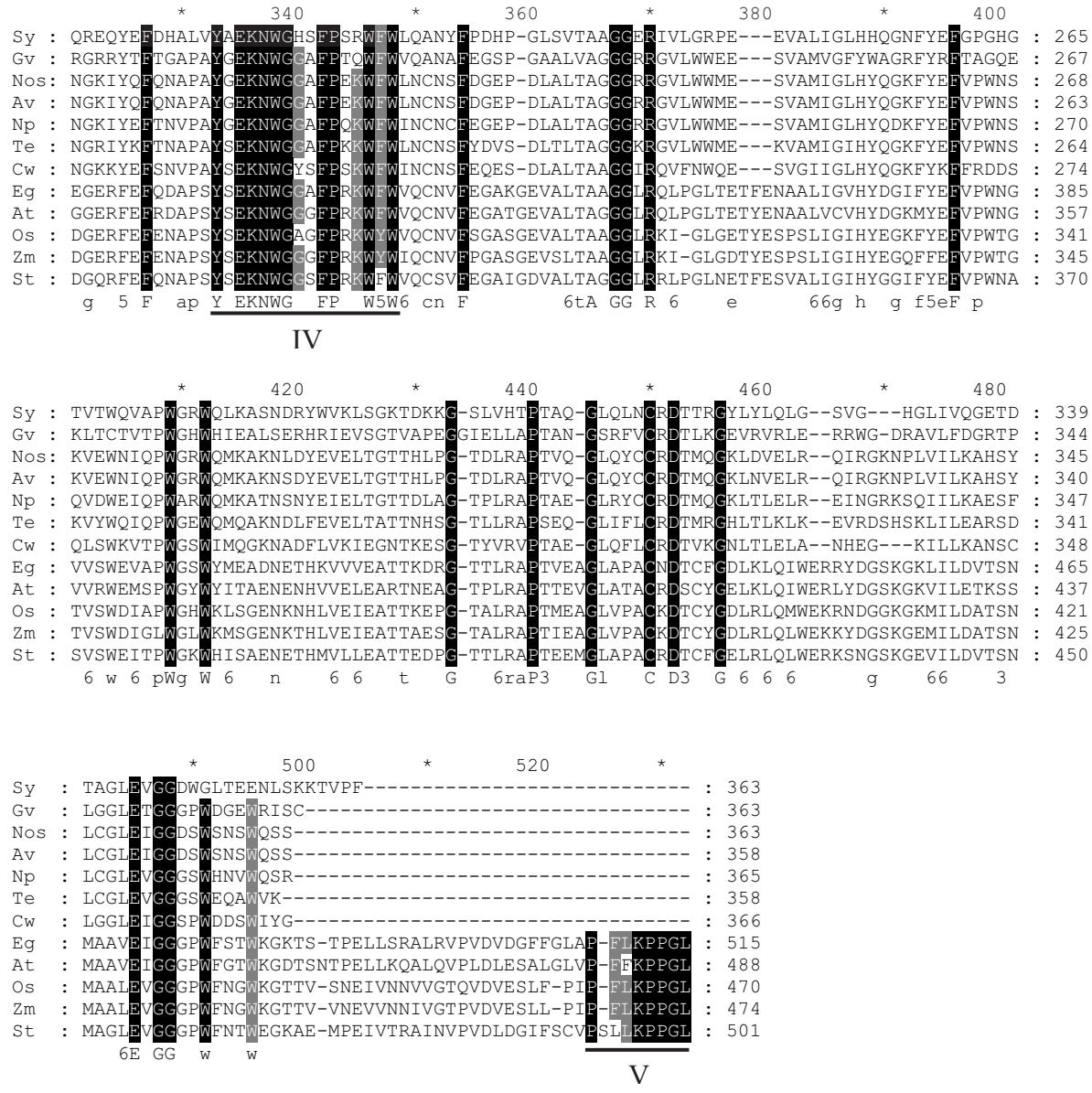


Figure A.1: The alignment of sequences was performed using the clustal X algorithm. Black background show amino acids conserved in all sequences. Regions I, II and III are stretch of amino acids conserved at the N-terminus while region IV is conserved at the C-terminus end of all sequences. Region V is conserved only in plant sequences. [Sy, *Synechocystis* sp. PCC 6803; Gv, *Gloeobacter violaceus* PCC 7421; Nos, *Nostoc* sp. PCC 7120; Av, *Anabaena variabilis* ATCC 29413; Np, *Nostoc punctiforme* PCC 73102; Te, *Trichodesmium erythraeum* IMS101; Cw, *Crocosphaera watsonii* WH 8501; Eg, Eucalyptus *gunnii*; At, *Arabidopsis thaliana*; Os, *Oryza sativa* (japonica cultivar-group); Zm, *Zea mays*; St, *Solanum tuberosum*].

A.3 Vectors maps for cloning and expression in *E. coli*

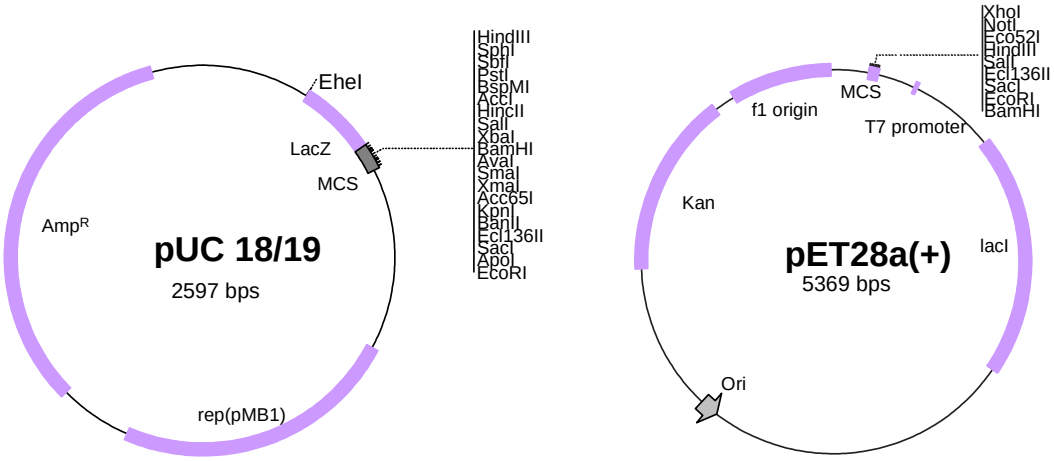


Figure A.2: Vectors used for cloning and expression studies of recombinant TCs in *E. coli*. (pUC 18/19 - Amp^R, gene for β-lactamase; lacZ, gene for β- Galactosidase; rep(pMB1), replication origin; MCS, multiple cloning site; pET28a - T7 promoter, bacteriophage T7 promoter sequence recognize by T7 RNA polymerase of host cells; lacI, lac repressor, Kan^R, gene for kanamycin resistance, MCS, multiple cloning site; f1 origin, origin of replication).

A.4 Chimeric construct maps for *E. coli* expression

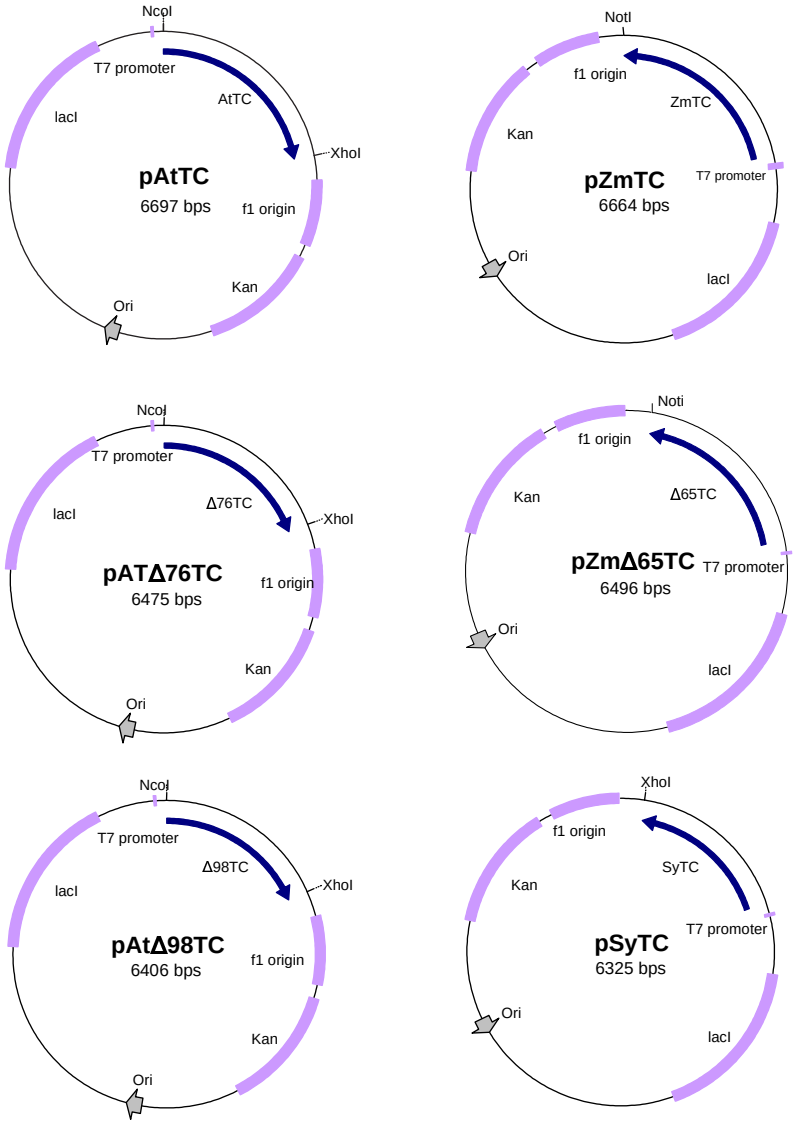


Figure A.3: Chimeric TC constructs for expression studies in *E. coli*. All chimeric genes were cloned under the control of the strong T7 bacteriophage promoter, which was further controlled by the *LacI* gene to reduce the basal level of expression. (*Arabidopsis* constructs - AtTC, *VTE1*; Δ76TC, *VTE1* without 5´end encodes 76 amino acids; Δ-98TC, *VTE1* without 5´end encodes 98 amino acids; maize constructs - ZmTC, *SXD1*; ΔTC - *SXD1* without 5´end encodes 65 amino acids; SyTC - *SLR1737* ORF from *Synechocystis*).

A.5 Map for pPZP111 and chimeric TC construct for *B. napus* transformation

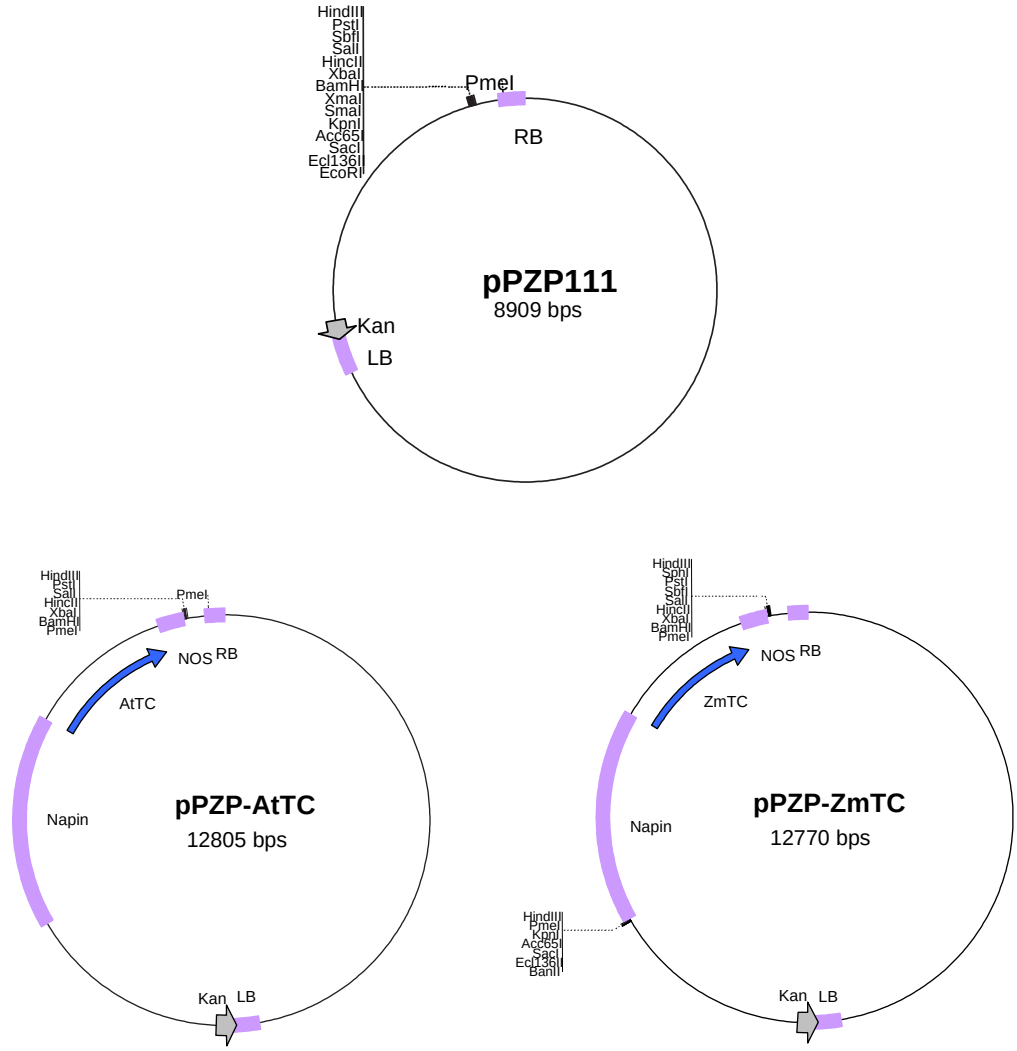


Figure A.4: Vectors and chimeric TC constructs used for overexpression in seeds of *Brassica napus*. (pPZP111 - binary vector for plant transformation carrying genes for NptII and kanamycin resistance; RB, right boarder; LB, left boarder; The chimeric TC constructs from *Arabidopsis* and maize were cloned under control of napin promoter from *Brassica napus*; Nos, termination region of the nopaline synthase gene for *Agrobacterium tumefaciens*.)

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