Functional analysis of tocopherol biosynthesis in plants
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1. Summary / Zusammenfassung

1.1. Summary

Tocopherols are lipophilic antioxidants, which are synthesized exclusively in plants and some photosynthetic microorganisms. Although several functions have been shown for tocopherol in mammalian cells, there is little knowledge concerning tocopherol function in plants. In vitro experiments showed that tocopherol can be responsible for scavenging reactive oxygen species, thereby preventing the oxidative degradation of fatty acids in membranes. Because tocopherol is particularly enriched in chloroplast membranes, it was proposed to be involved in the protection of chloroplast lipids and of chlorophyll against oxidative damage.

In order to study tocopherol function in plants, several transgenic plants with different levels and composition of tocopherol were created. To obtain transgenic tocopherol deficient plants, tocopherol cyclase (TC) and homogentisate phytyl transferase (HPT) were silenced in tobacco plants using a dsRNAi strategy. Silencing of HPT resulted in creation of severe tocopherol deficient lines, which contained less than 5% of wild type tocopherol content, whereas silencing of tocopherol cyclase created transgenic lines, which contain between 10% - 120% of wild type tocopherol level. Sever tocopherol deficiency (more than 95% reduction) in transgenic HPT: RNAi tobacco plants increased membrane damage and lipid peroxidation, which was paralleled by reduction in photosynthetic capacity, flower initiation and seed yield.

In most plants, \(\alpha\)-tocopherol accumulates predominantly in photosynthetic tissue, while \(\gamma\)-tocopherol is dominant in seeds. Tocopherol analysis in tobacco wild type plants showed a specific distribution for \(\alpha\)- and \(\gamma\)-tocopherol, which implies that there might be also specific function for \(\alpha\)- and \(\gamma\)-tocopherol. To elucidate the potential function of \(\alpha\)-tocopherol in plants, \(\gamma\)-tocopherol methyl transferase (\(\gamma\)TMT) was silenced in tobacco plants following a dsRNAi strategy. Silencing of \(\gamma\)-TMT resulted in an up to 95% reduction of \(\alpha\)-tocopherol in leaves of transgenic tobacco plants. Alpha-tocopherol deficiency was paralleled by an increased level of \(\gamma\)-tocopherol and about 30% seed yield reduction compared to wild type. Except for tocopherol composition and seed yield, transgenic \(\gamma\)TMT: RNAi tobacco plants were indistinguishable from wild type plants under optimal growth conditions. To unravel the specific function for \(\alpha\)- and \(\gamma\)-
tocopherol, wild type, transgenic HPT:RNAi and γ-TMT:RNAi tobacco plants were subjected to salt and sorbitol stress. Salt stress was imposed to additionally disturb cellular physiology primarily by exacerbating ion homeostasis at the vacuole, remote from the oxidative stress caused in chloroplasts. In contrast, sorbitol was picked to additionally impose desiccation to chloroplasts where oxidative stress occurs and tocopherols are predominantly localized. As expected, decreased total tocopherol content lead to a higher sensitivity of transgenic plants towards both, salt and sorbitol stress. Surprisingly, γ-TMT silenced plants showed an improved growth performance under sorbitol stress, while the salt tolerance was strongly decreased. This was paralleled by reduced membrane damage as evident by less lipid peroxidation and electrolyte leakage of leaf discs following osmotic stress treatment of γ-TMT transgenic plants. These results suggest specific roles for α- and γ-tocopherol.
1.2. Zusammenfassung


wurden γTMT-RNAi, HPT-RNAi transgene Tabakpflanzen neben Kontrollpflanzen sowohl Salz- als auch Sorbitolstress ausgesetzt. Salzstress diente zur Änderung der Ionenhomeostase, vornehmlich in der Vakuole, und damit entfernt vom in Plastiden verursachten oxidativen Stress. Sorbitol wurde gewählt, da diese Substanz die osmotische Balance der Plastiden stört und damit direkt am wahrscheinlichen Wirkort von Tocopherol angreift. Die Analysen zeigten, dass eine Verringerung des Gesamt-
Tocopherolgehaltes zu einer erhöhten Sensitivität der transgenen Pflanzen gegenüber sowohl Salz- als auch Sorbitolstress führte. Demgegenüber zeigten γTMT-RNAi Pflanzen eine erhöhte Resistenz gegenüber Sorbitolstress, wobei allerdings keine Salzstressresistenz zu beobachten war. Die Toleranz der γTMT-RNAi transgenen Tabakpflanzen gegenüber Sorbitolstress war von einer verringerten Membranschädigung begleitet, was sich in einer verringerten Lipidperoxidation bzw. Ionenaustrom aus Blattscheiben dieser Pflanzen äußerte. Diese Ergebnisse deuten auf spezifische Funktionen von α- und γ-Tocopherol bei der Adaptation an verschiedene Stresssituatio
nen hin.
2. Introduction

2.1. Vitamin E

Plants produce numerous 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 different derivatives, which are collectively known as vitamin E. Vitamin E is a lipid-soluble molecule that covers a family of eight structurally related derivative with different biological activity. The biological activity of each vitamin E derivatives is the measure of the potency or functional use in the body (Traber and Packer, 1995; Lichtenthaler et al., 1997). The IUPAC-IUB commission on Biochemical Nomenclature recommended that the term vitamin E should be used as a generic description for all derivatives, which qualitatively exhibit the biological activity of \( \alpha \)-tocopherol.

2.2. Discovery of vitamin E

In 1922 at the University of California in Berkeley, Herbert M. Evans, a research physician, and his assistant, Katherine S. Bishop "discovered" vitamin E. They were feeding rats a special semi-purified diet (Evans, 1992). The rats would grow very well, but for all female pregnancies, the whelps would die in the womb. When the rat's diet was supplemented with lettuce, then later with wheat germ, healthy whelps were born. Something must be missing from the diet, Evans and Bishop initially decided to call it "Factor X". Continuing the research, Evans and Bishop found Factor X was in the lipid extract of lettuce, so they knew that the mysterious substance must be fat-soluble. For a time, the unknown component, factor X, was termed the "anti-sterility factor". In 1925, however, Evans decided that, since vitamin D had recently been discovered, the new factor should be known as vitamin E. Some time later, he also proposed the name tocopherol for this unknown compound from two Greek words (In Greek “Tocos” means to bear a child and “phero” means provide a person with power roughly meaning childbirth). Evans and Emerson isolated vitamin E from wheat germ oil, corn oil and cotton seed oil in 1936 and its chemical synthesis was achieved by Paul Karrer (1889-
1971) and his co-workers in 1938 (actually, their chemical synthesis was achieved since the vitamin was found to consist of four very similar compounds).

2.3. Chemical structure of Vitamin E

Eight different amphipathic molecules make up the vitamin E family and all of them are found in plants and in the human body. The basic structural unit of vitamin E is a polar chromanol ring (2-methyl-6-hydroxychromanol) and a hydrophobic side chain of 16 carbon atoms, derived from a prenyl group. The structure of vitamin E homologues are shown in Table 1-1. The substrates for vitamin E biosynthesis are derived from two different metabolic pathways, the chromonal head group is derived from the shikimate pathway and the prenyl group is derived from the plastid-localized non-mevalonate pathway. Some vitamins consist of a single compound, while others contain more than one compound. Vitamin E is a family of different compounds, all working together to protect the body against free radicals. This family is divided into two sub groups: tocopherols and tocotrienols. The name of each member of the two sub-groups starts with one of the first four letters in the Greek alphabet. The individual vitamin E members (tocopherol and tocotrienol) are named alph, beta, gamma, and delta. The main difference between these compounds within one sub-group is the number and position of the methyl group on the chromonal head group.

Based on the number and position of the methyl groups on the chromonal head group four different forms of tocopherol and tocotrienols in plants have been found respectively. As indicated in Table 2-1, the vitamin E compound with 3 methyl groups on the aromatic head group (R1, R2 and R3 = CH3), has been named α-, or 5, 7, 8-trimethyltocol (methyltocol describes both tocopherol and tocotrienols with the identical patterns of methyl groups on the chromonal ring). Compounds with two methyl groups (R2 and R3 = CH3 whereas R1 = H) on position R2 and R3 are known as γ- or 5, 8-dimethyltocol. Whereas the compound with the same number of methyl groups but in different positions on the chromonal ring (R1 and R3 = CH3 but R2 = H) is known as β- or 7, 8-dimethyltocol. Finally, the compound with only one methyl group on the head group (R3 = CH3 whereas R1 and R2 = H) is designated as δ- or 8-methyltocol.
Table 2-1)-Number and position of the methyl group in different forms of vitamin A

<table>
<thead>
<tr>
<th>Group</th>
<th>Name</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃</td>
<td>Alpha- (α) or 5,7,8-Trimethyl tocopherol</td>
<td>CH₃</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>CH₃</td>
<td>Beta- (β) or 5,8-Dimethyl tocopherol</td>
<td>CH₃</td>
<td>H</td>
<td>CH₃</td>
</tr>
<tr>
<td>H</td>
<td>Gamma- (γ) or 7,8-Dimethyl tocopherol</td>
<td>H</td>
<td>CH₃</td>
<td>CH₃</td>
</tr>
<tr>
<td>H</td>
<td>Delta - (δ) or 8-Methyl tocopherol</td>
<td>H</td>
<td>H</td>
<td>CH₃</td>
</tr>
</tbody>
</table>

Table 2-1) - Chemical structure of vitamin E

Vitamin E is composed of two major groups, tocopherols (A) and tocotrienols (B), they differ in the degree of saturation in the prenyl tail, the chromonal head group is the common part for all vitamin E derivatives (tocopherols and tocotrienols).

2.4. Lipid peroxidation

Lipids are hydrophobic molecules like fats and oils, which are soluble in organic solvents and insoluble in aqueous solutions. Fats are made from a type of alcohol with a hydroxyl group on each of its three carbons (glycerol) and three fatty acids, which have the general structure of CH₃(CH₂)n COOH, joined by dehydration synthesis and they are known as triglycerides. They can contain either saturated fatty acids or unsaturated fatty acids (with one or more double bonds in the carbon chain). Unsaturated fatty acids with
more than one double bond in the carbon chain are named polyunsaturated fatty acids (PUFA). Lipid peroxidation is a natural metabolic process under aerobic conditions and it is one of the most investigated consequences of reactive oxygen species (ROS) action on membrane structure and function. PUFAs, which are the main components of thylakoid membrane lipids, are susceptible to peroxidation. Lipid peroxidation is the oxidative deterioration of unsaturated lipids, which results in lipid hydroperoxide (LOOH) formation.

A \[ \text{PUFA-H} + \text{X}^* \rightarrow \text{PUFA}^* + \text{X-H} \]
\[ \text{PUFA}^* + \text{O}_2 \rightarrow \text{PUFA-OO}^* \]

B \[ \text{O}_2 \]
\[ \text{PUFA}^* \]
\[ \text{PUFA-H} \]
\[ \text{PUFA-OO}^* + \text{PUFA-OO}^* \rightarrow \text{Non-Radical products} \]
\[ \text{PUFA-OO}^* + \text{PUFA}^* \rightarrow \text{Non-Radical products} \]
\[ \text{PUFA}^* + \text{PUFA}^* \rightarrow \text{Non-Radical products} \]

Lipid preoxidation is a free radical-related process and this process proceeds in three phases: initiation, propagation and termination phase. (see Figure 2-1) The initiation phase of lipid peroxidation process begins when an initiating radical X\(^{*}\) (such as hydroxyl radical OH\(^{*}\)) removes an hydrogen atom from polyunsaturated fatty acids (PUFA) substrate to form a lipid radical (PUFA\(^{*}\)). The hydrogen atom located between two double bonds of a PUFA is preferred because this C-H bond is the weakest in the molecule. A lipid radical formed during initiation immediately reacts with molecular oxygen to form lipid peroxyl radical (PUFA-OO\(^{*}\)), a more stable lipid radical. As lipid peroxyl radicals are able to abstract hydrogen atoms from lipid molecules, especially in the presence of metals, this results in formation of lipid hydroproxide (PUFA-OOH) and on the other hand a second lipid radical (PUFA\(^{*}\)). This second lipid radical can proceed
through the same reactions as the first, generating additional lipid hydroperoxides. In the absence of chain-breaking antioxidant such as tocopherol, lipid peroxidation eventually terminates via bimolecular processes (Figure 2-1C). The termination event is achieved by reaction of any kind of alkyl radicals (lipid free radical) to form non-initiating and non-propagating species. PUFA° can react with lipid peroxide (PUFA-OO°) to form stable dimers and can be the result of any reaction with another radical, or compound that acts as a free radical trap, forming a stable end product.

2.5. Biological function of Vitamin E

Although only photosynthetic organisms synthesize vitamin E, most of our understanding of its chemistry and function comes from studies in artificial membranes and animal systems. Vitamin E performs numerous critical functions including physical quenching of singlet oxygen, chemical scavenging of various reactive oxygen species (ROS) and free radicals, as well as being a chain breaking antioxidant to protect polyunsaturated fatty acids from lipid peroxidation (Fryer, 1993; Bramley et al., 2000) or being a physiochemical stabilizer of membranes. Because of these and other activities, dietary tocopherols are thought to play an important role in improving the immune function and in limiting the incidence and progression of several degenerative human diseases including certain types of cancer, cataracts, neurological disorders, and cardiovascular diseases (Pryor et al., 1976; Traber and Packer, 1995; Brigelius-Flohe and Traber, 1999; Bramley et al., 2000; Schneider, 2005). In plants, vitamin E performs antioxidant and radical quenching functions similar to those in animals (Fryer, 1992). There is growing evidence that tocopherols in human play other important roles in signalling and gene regulation, which are not related to their antioxidant function (Ricciarelli et al., 2002). Nevertheless, there is little evidence for signal transduction-related or other non-antioxidant roles of vitamin E in plants to date.

2.5.1. Antioxidant function of vitamin E

The antioxidant activity of vitamin E is largely correlated with its ability to donate its phenolic hydrogen to lipid free radicals. The ability of hydrogen donation is favored by (i) the number of methyl groups on the chromonal head (α > β ≥ γ > δ), (ii) the size of the heterocyclic rings, (iii) the stereochemistry at position two and the length of the side chain. Alpha-tocopherol, which contains three methyl groups, has the highest
antioxidant activity of tocopherol (Dillard et al., 1983; Kamal-Eldin et al., 1995; Kamal-Eldin and Appelqvist, 1996). The antioxidant activity of other tocopherols in vivo is in the following descending order (α > β ≥ γ > δ) (Burton et al., 1986; Burton and Ingold, 1989; Traber et al., 1990; Evans et al., 2002). Two main protective mechanisms are assumed from in vitro studies: (i) the main protective mechanism in membranes is assumed to be the removal the polyunsaturated fatty acid radical species which are generated during lipid peroxidation (Foyer, 1992; Munne-Bosch, 2002). (ii) The other protective mechanism of vitamin E is quenching of reactive oxygen species which are generated by metabolic processes (Munne-Bosch, 2002). It has been postulated that altered levels of vitamin E may influence membrane fluidity and stability (Munne-Bosch, 2002).

Figure 2-2)- The schematic position of vitamin E in membranes according to Singer-Nicholson fluid mosaic (1972)
The picture represents the fluid mosaic model (Singer and Nicholson, 1972), the circle is the negatively charged phosphate group and the two tails are the two highly hydrophobic hydrocarbon chains of the phospholipid. The prenyl tails of vitamin E orient towards a hydrophobic environment within the membrane.
This assumption is based, on one hand, on the main role of vitamin E as an antioxidant in biological membranes to protect the polyunsaturated fatty acids in the membrane (Burton et al., 1986; Tesoriere et al., 1996; Wagner and Heinecke, 1997) and on the other hand, on the structure and location of the α-tocopherol in the membrane. There is clear evidence that the chromonal head group of vitamin E is located in the hydrophilic (exterior) side of membranes whereas its prenyl tail is oriented towards the hydrophobic core of the membrane (Quinn, 2004) (Figure 2-2).

There is emerging evidence that α-tocopherol is not randomly distributed throughout the lipid bilayer matrix in biological membranes but instead forms complexes with specific membrane constituents. Vitamin E is believed to act as membrane stabilizer. The stabilization process is caused by the interaction between the chromonal hydroxyl group of α-tocopherol with the carboxyl group of the carbonyl group of the ester carbonyl bond of the phospholipid molecule which increases the rigidity of the membrane (Wang and Quinn, 1999, 2000; Qian et al., 2005).

### 2.5.2. Pro-oxidant function

Although vitamin E is a powerful antioxidant it has to be considered that vitamin E like every redox-active compound, may exert anti and pro-oxidative effects depending on the reaction partners present. Human low-density lipoprotein (LDL) contains various lipophilic antioxidants, including α-tocopherol and ubiquinol-10 (the reduced form of coenzyme Q), among these antioxidants, α–tocopherol is the most abundant antioxidant in low-density lipoprotein (LDL), and has been believed to be effective in protecting LDL from oxidative stress. Pro-oxidant function of vitamin E has been demonstrated in LDL isolated from healthy people and a patient with defect in the α-tocopherol transfer protein (α-TTP) gene (Kontush et al., 1996). This effect of tocopherol has been termed tocopherol-mediated peroxidation (TMP), and this function cannot be explained by the classical mode of action of α–tocopherol as a chain-breaking antioxidant. The tocopherol-mediated peroxidation (TMP) model has been developed to explain the pro-oxidant activity of α-tocopherol during lipoprotein lipid peroxidation (Bowry and Stocker, 1993). TMP proposes that α-tocopherol facilitates the transfer of aqueous radicals into LDL and then subsequent α-tocopheroxyl radical, acts as a peroxidation chain transfer agent, causing the formation of lipid hydroperoxides in lipoproteins (Thomas and Stocker, 2000). Recent studies have shown that under mild antioxidative conditions and in the absence of any co-antioxidants, the antioxidant activity of α–tocopherol is
converted into pro-oxidative activity and could oxidize lipoprotein lipids under these conditions (Brigelius-Flohe and Traber, 1999; van Dam et al., 2003).

2.5.3. Non antioxidant function

Over the last few years, several properties were found for different tocopherol derivatives, which are unrelated to their known antioxidant or pro-oxidant functions. In animal cells, protein kinase C inhibition is caused by \( \alpha \)-tocopherol; this inhibition takes place at the optimal concentration of \( \alpha \)-tocopherol in human beings. In endothelial cells, thrombin-induced PKC activation and endothelin secretion are inhibited by \( \alpha \)-tocopherol but not by \( \beta \)-tocopherol (Azzi et al., 2002). Inhibition of protein kinase C activity by \( \alpha \)-tocopherol at the cellular level was reported to be due to dephosphorylation of protein Protein phosphatase 2A (PP2A) by \( \alpha \)-tocopherol. This activation results in dephosphorylation of PKC, which leads to inhibition of PKC activity (Clement et al., 1997; Ricciarelli et al., 1998). Alpha- tocopherol can also modulate the membrane physical properties and some studies have examined its effects on different membrane-interacting enzymes, including phospholipase A2 (PLA2) activity, showing that some inhibition occurs both \emph{in vitro} and \emph{in vivo} when the concentration of vitamin E is increased (Douglas et al., 1986; Cao et al., 1987; Pentland and Herbert, 1992; Cao et al., 1993). This effect on membrane properties suggests that tocopherols have an inhibitory effect on phospholipase A2 activity (Grau and Ortiz, 1998). Among the tocopherols, Grau et al. showed that \( \alpha \)-tocopherol showed the strongest inhibitory effect on phospholipase A2 activity. The difference in PLA2 inhibition is due to the chemical structure of the tocopherol derivatives. Because of their different structure, they are located progressively deeper within the membrane compared to \( \alpha \)-tocopherol.

2.6. Proposed function of vitamin E in plants

Despite that, the main source for dietary uptake of vitamin E is plant food (vegetables, fruits, seeds and seed oils), most of our knowledge about the function of vitamin E comes from studies on non-plant systems. The physiological roles of vitamin E in plants are far from being clear, but there are some proposed functions for vitamin E in plants. The first proposed function of tocopherol in plants is the protection of the photosynthetic apparatus against photo-oxidative damage (Foyer, 1992; Fryer, 1993; Munné-Bosch and Alegre, 2002). The other proposed function for vitamin E is the protection of
polyunsaturated fatty acids from lipid peroxidation in the chloroplast membrane (Hugly and Somerville, 1992) and in seeds during storage, germination and early development (Munné-Bosch and Alegre, 2002; Munne-Bosch and Falk, 2004; Sattler et al., 2004).

2.6.1. Protection of the photosynthetic apparatus from photo-oxidative damage

Although photosynthetic organisms need to absorb light to perform photosynthesis, they need to be protected against excessive light. Photosynthetic organisms have several mechanisms to protect their photosynthetic apparatus against excessive light. These mechanisms include the movement of the leaves, cells or chloroplasts (to control light absorption) and regulation of photosynthetic light harvesting, thermal dissipation and electron transport (to balance the absorption and utilization of the light energy).

During photosynthesis, several potentially damaging molecules are generated which are called reactive oxygen species. As described by Elster (1991), there are different forms of activated oxygen (singlet oxygen and superoxide) which are produced either by physical or chemical mechanisms. The principal mechanism for activation of oxygen in most biological systems is the reduction of molecular oxygen by chemical processes to create superoxide and its reaction products like hydrogen peroxide and hydroxyl radicals. Molecular oxygen can also be converted to singlet oxygen by photodynamic processes in plants. (Elstner, 1991). As depicted in Figure 2-3, reactive oxygen species are generated at three major sites in the photosynthetic apparatus. In the light harvesting complex (LHC) associated with photosystem II, the PS II reaction center and the PS I acceptor side.

By light chlorophyll (chl) is activated converted to the singlet-excited state of chlorophyll ($^{1}\text{chl}$), which through intersystem crossing can be further converted to triplet chlorophyll ($^{3}\text{chl}$). In contrast to the singlet excited state of chlorophyll ($^{1}\text{chl}$), triplet chlorophyll ($^{3}\text{chl}$) is relatively long lived (ms time scale) and can interact with molecular oxygen to produce singlet oxygen ($^{1}\text{O}_2$). At the oxidizing side of PS II, four single electrons are released from $\text{H}_2\text{O}$ to the PS II reaction centers, which lead to the creation of superoxide and molecular oxygen. The acceptor side of PSI is capable to reduce molecular oxygen to the superoxide anion radical, which can be further converted to hydrogen peroxide or hydroxyl radicals.
Figure 2-3) - Schematic representation of the production and detoxification of reactive oxygen species and the antioxidant network in the chloroplast.

There are three possible sites for creation of the reactive oxygen species (ROS) in electron transport system. Reactive oxygen species (ROS) may be produced (i) from triplet chlorophyll in the light harvesting complex, (ii) from superoxide and hydrogen peroxide in the oxidizing side of PSII or (iii) from reduction of triplet oxygen by ferredoxin on the reducing side of PSI. The created ROS could be detoxified by antioxidant network, which consist of tocopherol, ascorbate and glutathione.
These are highly toxic reactive oxygen species. Generation of singlet oxygen in the PS II reaction center causes damage to lipids, critical pigment cofactors and the D1 subunit of PS II. Damage to these molecules could finally result in the inactivation of the entire reaction center. For example, high light stress in plants results in increased degradation of D1 and inactivation of PSII (Barber and Andersson, 1992; Aro et al., 1993; Trebst et al., 2002; Andersson et al., 2003). Generation of the reactive oxygen on the acceptor side of the PS I cause damage to the key enzymes of photosynthetic carbon metabolism such as phosphoribulokinase, fructose-1, 6- bisphosphatse and NADP-glyceraldehyde-3-phosphate dehydrogenase (Demmig-Adams and Adams, 1992; Asada, 1994).

As mentioned above, light absorption by both photosystems (PSI and PS II) during the electron transport results in the formation of a number of reactive oxygen species (Foyer et al., 2003). The amount of ROS formation is increased under high light (Melis, 1999; Hideg et al., 2000), temperature stress (Larkindale and Knight, 2002) and drought stress situation (Munne-Bosch and Penuelas, 2003).

Because of the potential hazard of ROS to the different components of the photosynthetic apparatus in response to environmental conditions, the amount of these molecules has to be regulated in plants. Photosynthetic organisms have enzymatic and non-enzymatic mechanisms to detoxify ROS. The enzymatic scavenging mechanisms in plants include superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and catalase (CAT) and nonenzymatic mechanisms include antioxidant molecules such as tocopherols, tocotrienols, carotenoids, ascorbate and glutathione (GSH) (Asada, 1994; Roxas et al., 1997; Willekens et al., 1997; Mittler et al., 1999).

To protect the photosynthetic apparatus against photo-oxidative damage, vitamin E (especially α-tocopherol) can physically quench or chemically scavenge singlet molecular oxygen, which is produced during photosynthesis. By quenching, an electron is lost from tocopherol and is donated to singlet oxygen, which leads to the formation of charge transfer exciplex. This charge transfer undergoes intersystem crossing and then dissociates into α-tocopherol and molecular oxygen and finally leads to deactivation of singlet oxygen (Foote et al., 1974). It has been estimated that one molecule of α-tocopherol can deactivate up to 120 singlet oxygen molecules by resonance energy transfer before being degraded (Fahrenholtz et al., 1974). Tocopherol also can chemically scavenge singlet oxygen. During the chemical scavenging process singlet oxygen is deactivated and α-tocopherol is converted to the 8-hydroperoxy tocopheron which is either converted to the α-tocopheryl quinone or at the presence of the ascorbate
is recycled back to tocopherol (Neely et al., 1988). The other reactive oxygen species such as superoxide, hydroxyl radical are scavenged in a similar type of reaction in vitro, if they are not completely detoxified through the water-water cycle in chloroplast, (Nishikimi et al., 1980; Asada, 1999).

2.6.2. Protection of the chloroplast membrane from lipid peroxidation

Biomembranes are made up of a lipid bilayer, which consist of two hydrophobic fatty acid chains linked to a hydrophilic head group. The hydrophobic groups constitute the interior of the membrane and the hydrophilic polar head groups are placed into the aqueous environment. The physical properties of the biomembrane are highly determined by chain length, the polarity and the degree of unsaturation of the fatty acids. Each membrane in the cell consists of a characteristic set of lipids. For example within the chloroplast, thylakoid membranes, which separate the aqueous stromal compartment from the aqueous lumen, have one of the highest contents of unsaturated fatty acids. Hugly and Somerville showed that leaves of *Arabidopsis* wild type contain 86-88% and 11-14% unsaturated and saturated fatty acid, respectively (Hugly and Somerville, 1992). Later on Miller et al. showed that 85 - 90% of the unsaturated fatty acids are monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG) in leaves of *Arabidopsis* (Millar et al., 1998). In tobacco plants lipid composition of the thylakoid membrane shows 85 - 95% unsaturated fatty acids for MGDG and DGDG (Moon et al., 1995). These observations suggest that the high proportion of unsaturated fatty acids in chloroplast might be required to maintain membrane fluidity. Because of the abundance of the polyunsaturated acid side chains in the thylakoid membrane, they are especially susceptible to damage by reactive oxygen species.

As depicted in Figure 2-3, reactive oxygen is able to initiate the oxidation of thylakoid membrane lipids by formation of the lipid radical, which is further converted to the lipid peroxyl radical by molecular oxygen. Vitamin E as a powerful antioxidant acts as a chain-breaking antioxidant by donating its phenolic hydrogen to the chain-propagating lipidperoxyl radical (LOO•), or alternatively, may react directly with the initiating radical to prevent LOO• formation (Figure 2-4B), (Serbinova and Packer, 1994). Vitamin E can also inhibit lipid peroxidation by imparting a hydrogen atom to lipid peroxyl radicals before the propagation reaction. This reaction results in formation of fatty acid hydroperoxide and tocopheroxyl radicals, which are less reactive (Figure 2-4A). Lipid
radicals (alkoxy radical (LO\(^\circ\)), lipid peroxyl radicals (LOO\(^\circ\)) and alkyl radicals (L\(^\circ\))) derived from PUFA oxidation may also be eliminated via a radical–radical reaction with tocopheroxyl radicals to create non radical products (Figure 2-4C), (Kamal-Eldin and Appelqvist, 1996). The reaction between vitamin E and lipid radicals occurs in the membrane-water inter-phase where vitamin E donates a hydrogen atom to lipid radicals with subsequent tocopheroxyl radical (T\(^\circ\)) formation (Buettner, 1993). Tocopheroxyl radicals can be recycled back to their reduced form by the antioxidant network (Figure 2-3) which consists of vitamin C (ascorbate) and reduced glutathione (Foyer, 1992). Ascorbic acid reduces the tocopheroxyl radical back to tocopherol resulting in dehydro ascorbate (DHA) formation. Glutathione reduces the dehydro ascorbate back to ascorbate by donating electrons which results in GSSG formation (Azzi and Stocker, 2000; Kagan et al., 2000). Alternatively tocopheroxyl radicals can be recycled back to tocopherol by coenzyme Q (Wang and Quinn, 1999).

\[ \text{A: } \text{TOH} + \text{LOO}^\bullet \rightarrow \text{TO}^\bullet + \text{LOOH} \]
\[ \text{B: } \text{TOH} + \text{radical oxidant} \rightarrow \text{Inactive oxidant} + \text{TO}^\bullet \]
\[ \text{C: } \text{TO}^\bullet + \text{LOO}^\bullet \rightarrow \text{Non-Radical product} \]

**Figure 2-4)** Prevention of lipid peroxidation by tocopherol

This scheme shows the reaction of tocopherol to prevent the propagation of lipid peroxidation. A and B show the inhibition of the lipid peroxidation process and C shows the termination of the lipid peroxidation process by tocopherol. Fatty acid peroxyl radical, LOO\(^\circ\); Lipid hydroperoxide, LOOH; Tocopheroxyl radical, TO\(^\circ\); Tocopherol TOH

### 2.6.3. Proposed function of tocopherol in stress signalling

Direct evidence for the involvement of tocopherols in signal transduction or gene expression has not been provided so far. Oxidative stress in plants includes intracellular signaling. Because reactive oxygen species, which occur during oxidative stress, can be detoxified by \(\alpha\)-tocopherol it might be speculated that, tocopherol participates in intercellular signaling. Depending on the amount of ROS and the severity of lipid peroxidation, plant cells might induce the antioxidant synthesis (Girotti, 1998). \(\alpha\)-tocopherol breaks the chain reaction of lipid peroxidation by scavenging the lipid radical (Figure 2-4). This effect of tocopherol on lipid peroxidation alters the concentration of the
secondary oxidation product such as jasmonic acid (JA) which participates in intracellular signaling. Silencing of the tocopherol cyclase gene in potato plants resulted in callose accumulation and deformation of the plasmodesmata which further led to a carbohydrate export block in source leaves of transgenic potato plants (Hofius et al., 2004). Yamamoto et al. showed a strong correlation between callose formation, which is often attributed to ROS-derived signaling, and oxidative damage to membrane lipids (Yamamoto et al., 2001). These observations indicate a mechanistic link between reactive oxygen species, lipid peroxidation and callose synthesis. By controlling ROS levels and the extent of lipid peroxidation, and therefore the hydroperoxide content in chloroplasts, tocopherols may indirectly regulate the amounts of jasmonic acid in leaves and may affect jasmonic acid dependent gene expression (Munne-Bosch, 2005). Later on Meunne Bosch et al., showed that JA content of 11 and 13 week-old tocopherol deficient Arabidopsis is 1.6 and 2.4 fold higher than in wild type plants, respectively. Mutant plants with altered JA content showed reduced growth and increased antocyanin accumulation (Munne-Bosch et al., 2006). With these observations, the authors concluded that by controlling the hydroperoxide content in chloroplasts, tocopherols may indirectly regulate the amount of endogenous phytohormone levels such as jasmonic acid in leaves and may affect jasmonic acid dependent gene expression (Munne-Bosch, 2005) and therefore would influence cell signaling in the plants.

2.7. Occurrence and subcellular localization of vitamin E

Vitamin E is only synthesized by plants and certain photosynthetic microorganisms, but it is also found in fungi, algae, and animals, although they cannot synthesize vitamin E themselves (Lichtenthaler, 1968; Singh et al., 1997; Grusak and DellaPenna, 1999; Grusak et al., 1999). Lichtenthaler (1968) reported the occurrence of vitamin E 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 (sepal, petal, stamen and carpel) of higher plants. Alpha-tocopherol is the most abundant form of tocopherol in green leaf tissue and in all parts of plants except seeds.
### Table 2-2) Vitamin E content and composition in selected plant tissues and oils

<table>
<thead>
<tr>
<th>Plant and organ</th>
<th>Total –Toc µg/g FW</th>
<th>% of α-T</th>
<th>% of γ-T</th>
<th>Others</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Leaf</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>10-20</td>
<td>90</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Potato</td>
<td>60-70</td>
<td>95.5</td>
<td>4.5</td>
<td>-</td>
</tr>
<tr>
<td>Tobacco Mature leaf**</td>
<td>30-40</td>
<td>95.6</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td>Tobacco Senescence*</td>
<td>30-40</td>
<td>94.4</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>Lettuce</td>
<td>7.5</td>
<td>55</td>
<td>45</td>
<td>-</td>
</tr>
<tr>
<td>Cabbage</td>
<td>17</td>
<td>100</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td>Spinach</td>
<td>30</td>
<td>63</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>Palm oil</td>
<td>300-500</td>
<td>100</td>
<td>0</td>
<td>-</td>
</tr>
<tr>
<td><strong>Seed</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tobacco</td>
<td>300-400</td>
<td>1</td>
<td>99</td>
<td>-</td>
</tr>
<tr>
<td>Arabidopsis</td>
<td>200-300</td>
<td>1</td>
<td>95</td>
<td>4</td>
</tr>
<tr>
<td>Corn</td>
<td>85-110</td>
<td>4.6</td>
<td>94.4</td>
<td>-</td>
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<tr>
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<td>3.8</td>
<td>96.2</td>
<td>-</td>
</tr>
<tr>
<td><strong>Seed oil</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>75</td>
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<tr>
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<td>28</td>
<td>72</td>
<td>-</td>
</tr>
<tr>
<td>Corn</td>
<td>1000</td>
<td>21</td>
<td>71</td>
<td>8</td>
</tr>
<tr>
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<td>1200</td>
<td>7</td>
<td>70</td>
<td>23</td>
</tr>
<tr>
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<td>47</td>
<td>11</td>
<td>42</td>
</tr>
<tr>
<td>Peanut</td>
<td>340-370</td>
<td>35</td>
<td>58</td>
<td>7</td>
</tr>
<tr>
<td><strong>Fruit</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green pepper</td>
<td>40-60</td>
<td>99.1</td>
<td>0.89</td>
<td>-</td>
</tr>
<tr>
<td>Yellow pepper</td>
<td>105-125</td>
<td>94.4</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>Red pepper</td>
<td>140-270</td>
<td>95.4</td>
<td>4.4</td>
<td>-</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potato Tuber</td>
<td>0.7</td>
<td>90</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>Rice (white grain)</td>
<td>17</td>
<td>19</td>
<td>14</td>
<td>67</td>
</tr>
<tr>
<td>Synechocystis</td>
<td>10</td>
<td>95</td>
<td>5</td>
<td>-</td>
</tr>
</tbody>
</table>

α-T, γ-T are α- and γ- tocopherols respectively, Others represent β and δ tocopherol and α, γ, δ and β-Tocotrienols.

* and ** indicate that samples were taken from leaf number 12 and number 3 of 7 week old tobacco plant (at this time plant has 14 leaves)

¹: (Barnes and Taylor, 1980), (Grusak and DellaPenna, 1999; Dellapenna and Pogson, 2006)

²: This study
With few exceptions, γ-tocopherol predominates in seeds (Franzen et al., 1991; Bartoli et al., 1997; Grusak et al., 1999). The relative content of total vitamin E (tocopherol and tocotrienol) content and composition considerably varies from plant to plant and from tissue to tissue (Table 2-2). Tocopherol contents range from extremely low levels (less than 1 ng per mg fresh weight) in potato to very high levels (500-1200 ng per mg fresh weight) in oil palm leaves and oil seeds (McLaughlin and Weihrauch, 1979; Taylor P and P., 1981; Hess, 1993) (Table 2-2). The occurrence of vitamin E in roots, seeds and etiolated tissues as well as in green leaf tissue indicates that vitamin E can also be synthesized in non chlorophyll-containing tissues. Within the plant cells, tocopherols and tocotrienols have been localized in either amyloplasts of seeds and tubers, chloroplasts of photosynthetic tissue, leucoplasts of petals or chromoplasts of fruits (Lichtenthaler et al., 1981, Fryer, 1992). Rautenkranz et al. showed that although chloroplasts of barley contain the major fraction of α-tocopherol (48 and 57 % of total α-tocopherol under low and high light condition, respectively), vacuoles of barley leaves contain 18.6 and 18.8 % of α-tocopherol under low and high light conditions, respectively. Later, Caro and Pauntarulo showed that microsomal membranes of soybean roots also contain small fractions of α-tocopherol (Caro and Puntarulo, 1996). In plastids α-tocopherol is mainly detected in the inner envelope membrane, where vitamin E is synthesized, (Soll et al., 1980; Soll and Schultz, 1981; Arango and Heise, 1998; Arango and Heise, 1998), in plastoglobuli (Vidi et al., 2006) and in thylakoid membranes (Fryer, 1993; Havaux, 1998). It is assumed that most of the α-tocopherol is partitioned between the plastid envelope and the thylakoids and in some cases it is stored in plastoglobuli (Munne-Bosch and Alegre, 2002). Although tocotrienols are not usually found in the green parts of higher plants, they are the predominant from of vitamin E in seeds of most monocot plants and some dicot plants.

2.8. The vitamin E biosynthetic pathway

The precursors of vitamin E are derived from two different metabolic pathways. The chromonal head group is derived from the shikimate pathway. P-hydroxylphenylpyruvate is converted to homogentisate, which is the precursor for all kinds of vitamin E derivatives and plastoquinone, by p-hydroxylphenylpyruvate dioxygenase (HPPD). The prenyl side chain of tocopherol, which is derived from the non-mevalonate pathway, is attached to the homogentisic acid to yield 2-methyl-6-phytyl-1, 4-benzoquinone (MPBQ) by homogentisate phytol transferase.
Figure 2-6)- Biosynthetic pathway of tocopherol, tocotrienol and plastoquinone (PQ) in plants.

This figure represents the enzymatic reactions and intermediates that are involved in the biosynthesis of tocopherol, tocotrienol and plastoquinone. *Arabidopsis* mutants of the corresponding pathway genes are given in parentheses behind the enzymes.

Abbreviations: DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; HGA, homogentisic acid; HPP, \( \gamma \)-hydroxyphenylpyruvate; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; MSBQ, 2-methyl-6-solanyl-1,4-benzoquinone; phytyl-DP, phytyl-diphosphate; solanesyl-DP, solanesyl diphosphate; HPPD, HPP dioxygenase; HPT (vte2), homogentisate phytyltransferase; HST, homogentisate solanyltransferase; MPBQMT (vte3), MPBQ/MSBQ methyltransferase; TC (vte1), tocopherol cyclase; \( \gamma \)-TMT (vte4), \( \gamma \)-tocopherol methyltransferase; PK (vte5), phytol kinase; PPK, phytyl phosphate kinase.
MPBQ is the first intermediate and common precursor of all four forms of tocopherol (Soll et al., 1980; Shinati et al., 2002; Collakova and DellaPenna, 2001). MPBQ can be further methylated at the C-3 position to yield 2,3-dimethyl-6-phytyl-1,4-benzoquinone (DMPBQ), through MPBQ methyltransferase (Marshall et al., 1985; Soll, 1987; Norris et al., 1995; Shintani et al., 2002; Van Eenennaam et al., 2003). The second ring in the tocopherol structure is formed in MPBQ and DMPBQ by tocopherol cyclase (TC) to yield β- or γ- tocopherol, respectively (Porfirova et al., 2002; Sattler et al., 2003). Alpha- and δ- tocopherol are finally produced by either second methylation of γ- tocopherol or first methylation of β- tocopherol by γ- tocopherol methyltransferase (γ-TMT) (Soll et al., 1980; Shintani and DellaPenna, 1998), respectively. The biosynthesis of tocotrienols is assumed to arise from the condensation of homogentisate acid (HGA, the common precursor for all vitamin E derivatives) and geranyl geranyl pyrophosphate(GGPP) to yield 2-methyl-6-geranylgeranyl-1,4-benzoquinone (MGGBQ) by homogentisate geranyl geranyl transferase (HGGT) (Cahoon et al., 2003). Although HGGT seems to be specific for tocotrienol biosynthesis, the same methyltransferase (MT), tocopherol cyclase (TC) and γ- tocopherol methyl transferase (γ-TMT) enzymes, which are involved in tocopherol biosynthesis, are involved in tocotrienol biosynthesis. Theses enzymes are able to metabolize intermediates to form all kinds of tocotrienols (Soll and Schultz, 1979; Porfirova et al., 2002; Cahoon et al., 2003; Herbers, 2003).

2.9. Genes, function and mutants

The enzymatic steps of vitamin E biosynthesis in plants were biochemically elucidated several years ago, and all enzymes in this pathway were localized to the inner chloroplast envelope (Soll and Schultz, 1979; Soll et al., 1980; Soll and Schultz, 1981). By combination of genetic and genomic approaches and the availability of complete genome sequences, in particular from Arabidopsis and Synechocystis sp. PCC6803, all biosynthetic genes in tocopherol biosynthesis have been cloned to date (Herbers, 2003).
2.9.1. Homogentisate phytyl transferase (HPT)

The HPT gene encodes homogentisate phytyl transferase (HPT) which is the first enzyme involved in the tocopherol biosynthetic pathway. The committed step in the synthesis of all prenylquinones is the condensation of various aromatic precursors and prenyl-diphosphate (DP). Sequence comparisons of many polyprenyl synthases showed a conserved domain, which has been found in a broad range of proteins. These proteins catalyze the condensation of a polyprenyl group to a variety of substrates. These domains have been suggested to be the binding site of the polyprenyl-DP (Lopez et al., 1996). The condensation of various aromatic precursors and prenyl-diphosphate (DP) substrates is catalyzed by a small family of related polyprenyl transferases (Lopez et al., 1996). Based on these observations, Collakova et al. (2001) hypothesized that the homogentisate polyprenyltransferase would show some similarity to polyprenyl transferases which have been previously characterized from cyanobacteria and plants that utilize similar prenyl-DPs as substrates (Collakova and DellaPenna, 2001). As chlorophyll synthase is one of the prenyltransferases, which condensate PDP or GGDP to chlorophyllide in chlorophyll synthesis (Lopez et al. 1996, Oster et al. 1997), the chlorophyll synthase open reading frame (ORF) of synechocystis was selected to search in the Cyanocystis data Base (Kaneko et al., 1996). Among several ORFs, which showed varying degree of similarity, SLR1736, which showed approximately 20%
identity to *Synechocystis* sp. PCC 6803 ChlG has been selected as a putative HPT (Collakova and DellaPenna, 2001; DellaPenna, 2005). The function of SLR1736 has been tested using homologous recombination of the kanamycin cassette-disrupted SLR1736 allele into the wild-type SLR1736 locus (Collakova et al., 2001). This mutation resulted in loss of tocopherols and phytol transferase activity (Schledz et al., 2001; Savidge et al., 2002). Based on the homology to the Escherichia coli 4-hydroxybenzoate-octaprenyltransferase (ubiA, Gen-Bank accession no.1790473) four putative prenyltransferase appeared to be potential *Arabidopsis* orthologs of genes identified in the *Synechocystis* sp. PCC 6803 search. Based on the similarity to the synHPT protein sequence, a single gene on chromosome 2 (VTE2, gene AT2g18950) of the *Arabidopsis* genome has been identified and characterized on the biochemical level (Collakova et al. 2001). The *Arabidopsis* protein has been named AtHPT for *Arabidopsis* homogentisate phytol transferase.

SynHPT and AtHPT enzymes were expressed in Escherichia coli and both showed HPT enzyme activity. SynHPT could utilize PDP and GGDP as substrates to yield 2-methyl-6-phytyl benzoquinone and 2-methyl-6-geranylgeranyl benzoquinone for the tocopherol and tocotrienol pathway, respectively, whereas the AtHPT enzyme could only utilize PDP to yield 2-methyl-6-phytyl benzoquinone (Collakova and DellaPenna, 2001) in tocopherol pathway.

Different *Arabidopsis* mutants for HPT have been described so far, *vte2-1* and *vte2-2*. The *vte2-1* *Arabidopsis* mutants were isolated in an HPLC–based screen for tocopherol mutants (Sattler et al., 2003) and *vte2-2* mutants were isolated through a PCR-based reverse genetics screen for T-DNA insertions in homogentisatephytyl transferase (Sattler et al., 2004). Complementation test indicated that *vte2-1* and *vte2-2* were allelic to each other. *Arabidopsis vte2* mutants show reduction in tocopherol content, while they do not accumulate any tocopherol pathway intermediates. Under normal growth conditions, the germination of *vte2* mutants was comparable to the wild type but *vte2* seedlings were generally smaller than wild type and they exhibited a wide range of defects in one or both cotyledons (Sattler et al., 2004). Although the mutants showed a severe phenotype at an early seedling stage, *vte2* plants, which survived germination and early seedling development, were indistinguishable from wild type. Sattler et al. (2004) have tested the roles of tocopherols in seed longevity. In response to an accelerated aging treatment, the authors showed that germination of wild type was reduced 20 to 30% relative to untreated controls whereas the same treatment of *vte1-1*, *vte2-1*, and *vte2-2* seeds resulted in more than 90% reduction in germination (Sattler et al., 2004). Wild type and
Arabidopsis vte2-1 mutants had identical carbohydrate contents under permissive growth conditions while Arabidopsis vte2-1 mutants accumulate carbohydrate under low temperature conditions (Maeda et al., 2006).

2.9.2. Tocopherol cyclase (TC)

The isolation of the tocopherol cyclase (TC) gene has been achieved by screening Arabidopsis EMS mutants for tocopherol deficiency (Porfirova et al., 2002). Lipid contents of the individual Arabidopsis M2 mutant plants were separated by thin layer chromatography (TLC). After screening the mutants for changes in lipid composition two vitamin E deficient lines (vte1) were identified that lacked all four forms of tocopherol. This mutation results in accumulation of the intermediate pathway DMPBQ (Dormann, 2003). Later on, numerous additional mutants have been obtained by Sattler et al. (2003). Among these mutants, two mutants (vte1-1 and vte1-2) were devoid of tocopherols in leaf tissue.

The gene At4g32770 has 52.2 and 41.1 % homology and similarity to DNA and amino acid level of the Synechocystis ORFslr1737, respectively (Sattler et al., 2003). Therefore, At4g32770 represented a candidate gene for VTE1 which encodes a functional protein able to convert 2,3-dimethyl-5-phytylhydroquinone(DMPBQ) and 2,3-dimethyl-5-geranylgeranylhydroquinone(DMGBQ) into \( \gamma \)-tocopherol and \( \gamma \)-tocotrienol, respectively, or to convert MPBQ and MGBQ into \( \beta \)-tocopherol and \( \beta \)-tocotrienol, respectively. Interestingly, the VTE1 protein turned out to be homologous to SXD1 from maize which was previously identified by analysis of the sucrose export defective 1 (sxd1) maize mutant (Provencher et al., 2001). Arabidopsis vte1 and wild type plants have very similar chlorophyll content, photosynthetic yield and overall growth under optimal growth condition whereas under oxidative stress condition, the mutants show slight decrease in chlorophyll content and photosynthetic quantum yield as compared to wild type. Mutation of tocopherol cyclase in maize results in carbohydrate, anthocyanin and callose accumulation and also lead to ultrastructural changes of plasmodesmata between bundle sheath and vascular parenchyma cells under normal growth condition (Russin et al., 1996). Silencing of tocopherol cyclase in potato plants also resulted in callose accumulation and ultrastructural changes of plasmodesmata between bundle sheath and vascular parenchyma cells. Silenced plants showed carbohydrate accumulation in their lower source leaves under normal growth conditions (Hofius et al.,
2004). Arabidopsis vte1 mutants do not show carbohydrate accumulation under optimal growth condition, but Maeda et al., (2006) reported that Arabidopsis vte1 mutants accumulate carbohydrate under low temperature conditions (Maeda et al., 2006).

2.9.3. Gamma- tocopherol methyltransferase (γTMT)

The γ-tocopherol methyltransferase (γTMT) catalyzes the last enzymatic step of tocopherol and tocotrienol biosynthetics, to yield either α- or δ- tocopherol and tocotrienol, which are the final products of the vitamin E biosynthesis. This gene has been cloned from Synechocystis sp. PCC6803 and Arabidopsis (Shintani and DellaPenna, 1998). The Arabidopsis HPPD gene was used to identify the putative tocopherol biosynthethic operon in Synechocystis sp., and a candidate γ-TMT ORF (SLR0089) was isolated based on its sequence similarity to plant sterol-C24-methyl transferases and the presence of S-adenosylmethionine (SAM)-binding domains. Disruption of SLR0089 resulted in γ-tocopherol (the biosynthetic precursor) accumulation and α- tocopherol deficiency. Based on the homology to the Syn.γ-TMT protein one γ-TMT cDNA clone was identified in Arabidopsis. Vte4-1 null mutant was isolated in a HPLC–based screen for altered tocopherol content and composition in Arabidopsis EMS mutants. A second mutant allele (vte4-2) was identified using a T-DNA tagged mutant population (Bergmuller et al., 2003). Both Arabidopsis mutant accumulated high amounts of γ-tocopherol whereas the predominant form of tocopherol in Arabidopsis leaves (α-tocopherol) was absent in both mutants. Vte4 mutants were indistinguishable from wild type plants under optimal growth conditions. During oxidative stress such as high light, high temperature, cold stress there was no difference in the chlorophyll content and photosynthetic quantum yield of the vte4 mutants and wild type plants. Fatty acid and lipid composition were also very similar in wild type and vte4 mutants (Bergmuller et al., 2003).

2.9.4. 2-Methyl-6-phytylbenzoquinone methyltransferase (MPBQ MT, MT1)

The MPBQ MT1 gene encodes an enzyme, which methylates MPBQ to yield 2, 3-dimethyl-6-phytyl benzoquinone (DMPBQ) in tocopherol biosynthetic pathway. MPBQ MT was cloned and identified in Synechocystis sp. based on its sequence similarity to previously cloned and characterized Synechocystis γ-tocopherol methyltransferases.
Among several related gene sequences, ORF SLR0418 shared significant similarity to *Synechocystis* and *Arabidopsis* γ-TMT protein sequences (Shintani and DellaPenna, 1998; Shintani et al., 2002). To prove that SLR0418 gene encoded a MPBQ MT, a targeted mutation in this gene was created. Differences in tocopherol composition of the mutant compared to the wild type indicated that disruption of SLR0418 results in a decrease in MPBQ methyltransferase activity (Shintani and DellaPenna 2002). The initial attempt to identify the plant homolog of *Synechocystis sp.* MT1 was not successful. This indicates that plant and cyanobacterial MT1 proteins have only a low degree of similarity. Therefore, a HPLC-based screen for mutation was used to identify mutants of *Arabidopsis* with altered tocopherol profiles in leaf tissues (Cheng et al., 2003; Sattler et al., 2003; Sattler et al., 2004). This yielded a mutant line, which has been named *vte3-1*. *Vte3-1* showed reduced levels of γ- and α- tocopherol and increased levels of δ- and β-tocopherol. The VTE3 locus was identified by map-based cloning and encoded the MPBQ MT1 in *Arabidopsis* as confirmed by in vitro enzyme assay. The *vte3-2* *Arabidopsis* mutant was isolated from the SALK T-DNA insertion line collection. A T-DNA insertion in the first exon of At3g63410 at nucleotide 163 of the ORF resulted in a complete loss of MPBQ MT enzyme activity. The null, *vte3-2*, mutant is deficient in plastoquinone, α- and γ- tocopherol (Cheng et al., 2003).

2.10. Scientific aims of the work

The aim of this PhD thesis is to unravel the biological role of tocopherol in higher plants. To achieve the objectives two strategies were followed:

(i) In planta investigation of protein-protein networks in the tocopherol biosynthetic pathway:

Using the yeast two-hybrid system, a number of proteins interacting with potato tocopherol cyclase could be identified. This observation indicates that tocopherol cyclase could be a member of a multi-protein complex, which might be important for the regulation of its enzymatic activity.

To elucidate whether these interactions take place *in planta*, *Arabidopsis vte1* knockout mutants were transformed with tocopherol cyclase fused to a TAP-Tag. Protein complexes were affinity purified from *Arabidopsis vte1* mutant, which complemented with TC fused to TAP-Tag. Purified complex protein was separated on SDS-PAGE and the visualized bands were cut and analyzed by MALDI-TOF and LC-ESI-MS/MS.
(ii) Biological roles of vitamin E in plants:

Although several functions have been shown for tocopherol in mammalian cells, there is little knowledge concerning tocopherol function in plants. *In vitro* experiments showed that tocopherol can be responsible for scavenging reactive oxygen species, thereby preventing the oxidative degradation of fatty acids in membranes. Because tocopherol is particularly enriched in chloroplast membranes, it was proposed to be involved in the protection of chloroplast lipids and of chlorophyll against oxidative damage.

To increase the knowledge about tocopherol function in plants, creation of transgenic plants with different levels and different composition of tocopherol would be very helpful. Therefore, in order to obtain transgenic tocopherol deficient lines, tocopherol cyclase (TC) and homogentisate phytyl transferase (HPT) were silenced in tobacco plants using a dsRNAi strategy.

In additional, since the exact function of the four different tocopherols in plants is unknown, to unravel the function of α-tocopherol in plants, γ-tocopherol methyl transferase (γTMT) was silenced in tobacco plants following a dsRNAi strategy. To study the consequences of altered tocopherol content and composition on stress responses, transgenic γTMT:RNAi, which lacked α- and δ-tocopherol, HPT:RNAi, which lacked all forms of tocopherol, and wild type tobacco plants were exposed to increasing amount of sorbitol and NaCl (to induce osmotic and oxidative stress). Four weeks treated plants were analyzed on the biochemical levels.
3. Results

3.1. Approaches to identify tocopherol cyclase interacting proteins

Proteins control and mediate many biological activities of the cell. Although some proteins act primarily as single monomeric units, a significant percentage of all proteins are assumed to function in association with partner molecules, or as components of larger protein assemblies. Thus, to obtain a thorough understanding of the cellular function, the function of proteins must be understood in context of other interactive proteins. There are different approaches to identify and characterize protein-protein interactions (Phizicky and Fields, 1995). One of the most popular and powerful approaches is the yeast two-hybrid system which identifies protein-protein interactions solely on a genetic basis using yeast as a host cells (Fields and Song, 1989). Due to the intrinsic limitations of the yeast two-hybrid system, complementary approaches have been developed over the past years. On such approach in protein-complex affinity purification coupled to mass spectrometry (AP-MS) (Bauer and Kuster, 2003). Here, a protein of interest is translationally fused to an affinity tag and subsequently ectopically expressed in a host cell. The protein is then purified from cell extracts and co-purifying proteins are separated on an SDS-gel and can be identified using MS. One of the most widespread used affinity tags for AP-MS is the tandem-affinity-purification tag (tap tag) (Rigaut et al., 1999) which allows affinity purification of fusion proteins in a two-step protocol. This increases purity and reduces binding of unspecifically interacting proteins. The aim of the experiments described below was to identify proteins interacting with the tocopherol cyclase. To this end, the full length cDNA of Arabidopsis tocopherol cyclase was (i) fused C-terminally with GFP to investigate its localization in the cell and (ii) fused with a tap-tag to allow for protein complex purification from plant cells. The Arabidopsis vte1 mutant was used as a genetic background to separately transform both constructs. After confirming functionality of the fusion proteins by complementing the vte1 mutant the tap-tagged cyclase was purified from transgenic plants and copurifying proteins were separated by one or two dimensional gel electrophoresis. Visual protein bands on the gel were further analyzed by Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS).
3.1.1. Creation of *Arabidopsis* transgenic plants to investigate the localization of tocopherol cyclase

Analyzing the sequence of the tocopherol cyclase protein using ChloroP ([http://www.cbs.dtu.dk/services/ChloroP](http://www.cbs.dtu.dk/services/ChloroP)) indicated the presence of a transit peptide at the N-terminal region of the tocopherol cyclase protein (1–98 amino acids) with high probability (probability score 0.56). This is in accordance with previous studies that also showed plastidic localization of mature tocopherol cyclase (Hofius et al., 2004, Porfirova et al. 2003). To confirm the sub-cellular localization of the *Arabidopsis* tocopherol
cyclase, a GFP fusion protein was expressed in transgenic *Arabidopsis* plants and its localization was investigated by CLSM.

### 3.1.1.1. Construction of tocopherol cyclase (TC) fused to green fluorescence protein (GFP)

The GFP vector (Figure 3-2B), which had been already constructed by inserting the GFP protein into the pBin vector (Figure 3-2A) between 35S promoter and *ocs* terminator, was used in this work (Giese et al., 2005). The complete coding region of tocopherol cyclase was PCR amplified from the full-length cDNA *Arabidopsis* clone (1485 bp, At.TC cDNA; accession no. NM119430, gene number AT4g32770) by using specific oligonucleotides (5’-GGTACCGCATATTTCTTCTTCTTTTCCATTATGG-3’) and (5’-GGATCCAGACC CGGTGGCTTGAAGAAAGG-3’). The RT-PCR amplified fragment was sub-cloned into the PCR-blunt vector.

![Figure 3-2](image)

**Figure 3-2** Schematic drawing of pBin-TC-GFP construct

The full length GFP fragment had been inserted into the binary vector pBin (A) between 35S promoter and *ocs* terminator to yield PBin-GFP (B). The full-length cDNA of *Arabidopsis* tocopherol cyclase fragment, (1485 bp, accession no.NM119430), was inserted into the binary vector pBin-GFP between 35S promoter and GFP fragment to yield pBin:TC:GFP construct( C).
The positive clone which containing the tocopherol cyclase fragment was digested with Asp718 and BamHI restriction enzymes. The Asp718/BamHI-digested PCR fragment was inserted into the Asp718/BamHI sites of pBin-GFP between 35S promoter and GFP fragment to yield pBin-TC-GFP expression vector (Figure 3-2-C). This plasmid DNA was transferred into the Agrobacterium to allow for the generation of transgenic plants.

3.1.1.2. Transformation of Arabidopsis vte1 mutant and pre-screening of the transgenic plants

Before stable transformation of Arabidopsis, the functionality of the construct and the subcellular localization of tocopherol cyclase were tested. Therefore, the TC::GFP fusion protein was transiently expressed in Nicotiana benthamiana plants using Agro-infiltration (Voinnet et al., 2003).

Figure 3-3) Chloroplast localization of the Arabidopsis tocopherol cyclase (At.TC) gene fused with GFP protein

Agrobacterium tumefaciens carrying TC: GFP (A, B and C) was mixed with the same amount of Agrobacterium which carrying P19 protein of Tomato bushy stunt virus (TBSV); the solution was infiltrated into young, fully expanded leaves of 4-week-old tobacco plants. Samples were taken 2-day post infiltration (dpi) and GFP signals and chlorophyll auto fluorescence were observed with a confocal laser-scanning microscope. (A-C) Tobacco infiltrated leaves were investigated for GFP localization under confocal microscopy 2 days post infiltration. A; shows fluorescence image of TC fused with GFP in tobacco leaves (green), B; shows fluorescence image of chlorophyll (red ) and C; shows the merge image of GFP and chlorophyll . Bars represent 1μm.
Because of the ability of P19 protein, which is driven from *Tomato bushy stunt virus* (TBSV), to suppress the post transcriptional gene silencing (PTGS) (Qiu et al., 2002), *Nicotiana benthamiana* plants were inoculated with mixture of *Agrobacterium* carrying pBinTC::GFP and *Agrobacterium* which carrying P19 protein of *Tomato bushy stunt virus* (1:1). Two days after infiltration, localization of the fusion protein was analyzed by CLSM. As depicted in Figure 3-3, A to C, the fluorescence of the TC::GFP fusion protein (Figure 3-3 A) is colocalizing in the merged picture (Figure 3-3 C) with the red autofluorescence of chlorophyll (Figure 3-3 B). This observation indicates that *Arabidopsis* tocopherol cyclase is targeted into the chloroplasts. After having confirmed expression of the TC::GFP fusion protein in transient assays, the construct was used to generate stably transformed *Arabidopsis* plants using the floral dip method (Bechthold et al., 1993; Clough and Bent, 1998). Seeds were harvested from transformed plants and sown on MS-Kanamycin medium to select transformed plants. Altogether 40 kanamycin resistant lines were transferred to the greenhouse for further analysis.

### 3.1.1.3. *Arabidopsis* tocopherol cyclase is located into the chloroplast

All the kanmycin resistant lines, following transformation of *vte1* mutant by pBin: TC: GFP, were transferred to soil, two to three weeks after transfer, the transgenic plants were primarily screened by Axiovert microscope. Following the Axiovert microscopy analysis 14 lines showed the green fluorescence of GFP (data not shown), from these 14 transgenic lines four lines (5, 15, 10, 35) were selected for further analysis. The sub-cellular localization of tocopherol cyclase (TC) in *Arabidopsis* transgenic *vte1* plants were determined by laser scan microscopy, leaves of 2-3 weeks old plants (after the transferr to the soil), were checked for GFP and chlorophyll fluorescence.

Although GFP and chlorophyll are excited at 488 nm, the green fluorescence of the GFP is emitted at the wavelengths between 510 and 525 nm, whereas the red fluorescence of chlorophyll is emitted at the wavelengths between 645 and 700 nm. Because of the differences in their emission wavelength, we are able to monitor their fluorescence using microscopy analysis.

By merging of these two pictures, we can observe the localization for both GFP and chlorophyll as shown in Figure 3-4 A-D. As GFP is able to emit the green fluorescence, the green fluorescence in Figure 3-4a represents the functionality of the construct in the stable transformed plants. As the chlorophyll is located into the chloroplast and it is able
to emit the red fluorescence then the red fluorescence, which is observed in the Figure 3-4b represents the chlorophyll protein and the chloroplast location. To investigate the localization of the green fluorescence in the selected transgenic lines, green fluorescence, which is derived from tocopherol cyclase fused GFP protein, and red fluorescence, which is emitted from chlorophyll protein, were merged.

Figure-3-4) GFP fusion protein of full-length AtTC (pBin TC:GFP) was transformed into *Arabidopsis* vte1 mutant by *agrobacterium* and putative stable transformed plants were analyzed by confocal laser scan microscopy (a-d) and HPLC analysis (e). Green colour indicates GFP fluorescence and red color reveals chlorophyll (Chl) fluorescence. Yellow colour indicates the overlay of both chlorophyll and GFP fluorescence.

(a)- Intracellular localization of AtTC:GFP in stomata cell. b)- Chlorophyll fluorescence indicates chloroplast distribution. c) - indicates merged image a and image b. d) - represent cells, Bars represent 10 μm.

(e)- Tocopherol content of *Arabidopsis* plants was determined by HPLC analysis. Black bar represents wild-type plants, grey bars represent transgenic plants and green bar represents the vte1 mutant. Data are given as the mean of four replicates (independent transgenic plants) ± SD.
As depicted in Figure 3-4c, which represents the merged picture, the green fluorescence of the TC fused GFP protein is colocalized with the red chlorophyll fluorescence. This observation indicates that Arabidopsis tocopherol cyclase is targeted into chloroplasts of stably transformed Arabidopsis plants. Tocopherol contents of wild type, vte1 mutant and selected transgenic lines was determined in leaf extracts from 3-weeks old plants by HPLC. As indicated in picture 3-4e, transgenic plants have 15.1-19.3 ng tocopherol per mg fresh weight which is comparable to wild type having 18.2-24.1 ng tocopherol per mg fresh weight. They both have significantly more tocopherol as compared to the non-transformed Arabidopsis vte1 mutant. Base on these data it can be concluded that vte1 Arabidopsis mutants is complemented with tocopherol cyclase fused GFP.

3.1.2. Creation of Arabidopsis transgenic plants to study protein-protein interaction of tocopherol cyclase

Using the yeast two-hybrid system a number of proteins interacting with potato TC could be identified (Table 3-1, Daniel Hofius, personal communication). This observation indicated that TC might be a component of a multi-protein complex, which might be important for the regulation of its enzymatic activity.

To identify protein-protein interactions of TC in planta an AP-MS strategy using a TC::TAP-Tag fusion protein was followed. The vte1 Arabidopsis mutant was used as a genetic background in this work. This has several advantages as compared to using a wild-type background for these kinds of studies: (i) there is no competition for binding with the endogenous TC and (ii) the functionality of the fusion protein can be shown by restoring tocopherol levels in transformed plants.

3.1.2.1. Construction of tocopherol cyclase (TC) fused to tandem affinity purification tag protein (TAP-Tag) expression vector

To obtain the TAP-Tag construct, which contains 2 protein A parts, TEV cleavage site and one calmodulin binding peptide (CBP) part (Figure 3-5 and 3-9), the complete coding region of TAP-Tag was PCR amplified from the synthetic construct (575 bp, CTAP-Tag DNA; accession no. AY436346) by using specific oligo-nucleotides (5'-GGTACCGCATATTTCCTTCTTCTTCCATTATGG-3') and (5'-GGATCCAGAGCACCGTTGGCTTGAAGAAAGG-3').
### Table 3-1) Proposed protein partners for tocopherol cyclase using yeast two hybrid screening

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<tr>
<td>XIP2</td>
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<td>X95933</td>
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<td>NM_130225</td>
<td>33 / 43    (76%)</td>
<td>Calcium-binding protein</td>
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The tocopherol cyclase (sxd1) from potato plant was used as a bait protein to screen a yeast two-hybrid cDNA library prepared from potato leaf material. Yeast two-hybrid screening has been already performed by Hofius et al., (unpublished data). XIP is sxd1 interacting protein.

The amplified fragment was sub-cloned into the PCR- blunt vector. A positive clone containing the TAP-Tag fragment was digested with BamHI and XbaI. The BamHI/XbaI-digested PCR fragment was inserted into the BamHI/XbaI sites of pBin vector between 35S promoter and ocs terminator to yield pBin-TAP-Tag. Then the same tocopherol cyclase fragment, which was used for the GFP fusion construct, was inserted into the Asp718/BamHI sites of pBin-TAP-Tag vector between 35S promoter and TAP-Tag to create pBin-TC-TAP-Tag expression vector (Figure 2 B). This plasmid DNA was transferred into Agrobacterium for plant transformation.
3.1.2.2. *Arabidopsis* vte1 mutant was complemented by tocopherol cyclase fused to TAP-Tag (pBin: TC: TAP-Tag)

To investigate expression of the TC::TAP-Tag fusion proteins, *Nicotiana benthamiana* leaves were Agro-infiltrated with the pBin:TC-TAP-Tag vector. The tobacco leaves were infiltrated with a mixture between an *Agrobacterium* strain harboring the TC-TAP-Tag construct and another one containing a vector to express the viral silencing suppressor p19. *Agrobacterium* containing P19 alone were infiltrated to serve as a control. Samples were taken immediately after infiltration (day 0) and every day after infiltration until 4 days after infiltration. Total protein, which was extracted from the samples, was loaded on a 12.5% SDS-PAGE gel and analyzed by western blot.

![Diagram A](image1)

![Diagram B](image2)

![Diagram C](image3)

**Figure 3-5) Schematic drawing of pBin-TC-TAP-Tag**

The full length TAP-Tag fragment was inserted into the binary vector pBin (A) between 35S promoter and *ocs* terminator to yield PBin-TAP-Tag construct (B). Tocopherol cyclase full-length cDNA fragment (1485 bp, At.TC cDNA; accession no.NM119430), was inserted into the binary vector pBin-TAP-Tag between 35S promoter and TAP-Tag fragment to yield pBin:TC:TAP-Tag construct (C).
As indicated in Figure 3-6 no protein was detected at day zero whereas a protein of about 66 KDa was detected two days after infiltration in samples which were infiltrated with the TC-TAP-Tag construct. The molecular weight of the band is in good accordance with the predicted molecular weight of the TC::TAP-tag fusion protein. The signal of tocopherol cyclase fused TAP-Tag protein was weaker in samples, which were taken four days after infiltration compared to the second day after infiltration. No protein was detected in the samples, which were infiltrated with P19 alone at all time points analyzed. These results confirm expression of the TC::TAP-tag fusion protein plant cells.

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</tbody>
</table>

Following the confirmation of the functionality of the TAP-Tag construct and the localization of the tocopherol cyclase protein, *Arabidopsis vte1* mutants were transformed with the pBin-TC-TAP-Tag construct. Three to four weeks after transformation, dry seeds of transformed plants were collected. Harvested seeds were plated on selective media (MS-Kanamycin). Following germination, 55 putative transgenic lines were obtained for the TC-TAP-Tag construct. All the 55 kanamycin resistant lines were transferred to the soil. Two to three weeks after transfer, the transformants were screened for tocopherol accumulation and by western blot analysis. Following the western blot analysis using PAP antibody against protein A, among 55 putative transgenic lines, 32 lines showed a positive signal at the size (66 KDa) expected for the TC-TAP-tag fusion protein (data not shown). Subsequently, all 55 putative transgenic lines were further analyzed for their tocopherol content by HPLC.
Figure 3-7) Transformation of the vte1 mutant by TC-TAP-Tag results in accumulation of total tocopherol in leaves of transgenic plants

The cDNA (under control of the cauliflower mosaic virus 35S promoter), was introduced into vte1 mutant plants. All the pre-screened transgenic plants were transferred to the soil and the amounts of different forms of tocopherol (α, γ, and δ) were determined by fluorescence HPLC. A, HPLC chromatogram of tocopherols extracted from leaves of wild type (black), non-transformed vte1 mutants (pink) and transformed vte1 mutants (blue) and the tocopherol standard (brown). B, Percentage of the total tocopherol content in leaves of transgenic plants compare to the wild type. The amounts of total tocopherol (α, γ and δ) in leaves of transgenic plants were determined by fluorescence HPLC and the percentage of the total tocopherol in leaves of transgenic plants were calculated compare to the wild type content. Due to the percentage of the tocopherol content, transgenic plants were categorized into different group.

As showed in picture 3-7B, putative TC::TAP-Tag expressing transgenic lines have different amounts of tocopherol ranging between wild-type levels and those found in the vte1 background. Eight lines had the same amount as wild type (80-110 % of wild type), ten lines have 60-80 %, three lines have 40-60 %, fourteen lines have 10-20 %, nine lines have 8-10% of tocopherol compared to the wild type and eleven lines have 5-8 % of wild type tocopherol (nearly the same amount of tocopherol in vte1 mutants). Based on tocopherol analysis, four transgenic lines (TC-TAP-42, 54, 49 and 15) with nearly the same amount of tocopherol as wild type were selected for further experiments.
As all subsequent experiments were supposed to be carried out on material homozygous for the TC::TAP-tag transgene, segregation of the kanamycin resistance gene was analyzed following germination of seeds on selective medium. Seeds of the wild type and selected transgenic lines were placed on MS medium containing kanamycin. Following this test, green seedlings were counted as resistant plants containing the resistance gene which is always inherited together with the TC::TAP-tag transgene. These resistant seedlings will be either homozygous or heterozygous. White-yellowish seedlings were counted as susceptible lines, which have lost the transgene through segregation.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number of the seed</th>
<th>Number of the seedling</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
<td>White-yellow</td>
</tr>
<tr>
<td>42</td>
<td>121</td>
<td>88</td>
<td>31</td>
</tr>
<tr>
<td>54</td>
<td>113</td>
<td>85</td>
<td>27</td>
</tr>
<tr>
<td>49</td>
<td>104</td>
<td>73</td>
<td>26</td>
</tr>
<tr>
<td>15</td>
<td>130</td>
<td>96</td>
<td>33</td>
</tr>
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<td>13</td>
<td>122</td>
<td>90</td>
<td>31</td>
</tr>
<tr>
<td>7</td>
<td>129</td>
<td>97</td>
<td>30</td>
</tr>
</tbody>
</table>

Table 3-2) Segregation patterns of resistance to kanamycin in the T2 generation of Arabidopsis vte1 transformed with pBin TC:TAP-Tag construct

As indicated in table 3-2, in all of the six selected transgenic lines a 3:1 Mendelian segregation pattern of kanamycin resistance was observed. This observation indicates that the transgene is inherited as single recessive nuclear gene. Resistant plants were transferred to the greenhouse. Western blot analysis was performed from the leaf samples of 4-week old transgenic, vte1 mutant and wild type plants. The results of western blot analysis (Figure 3-8A), shows that a protein about the 66 KDa was detected in all transgenic lines (42, 54, 49 and 15) and no protein was detected by western blot analysis for wild type and non-transformed vte1 mutant.
Figure 3-8) Characterization of vte1 Arabidopsis mutant complemented with tocopherol cyclase TAP-Tag

A, Western-blot analysis of selected transgenic lines. Protein was extracted from leaves of 4-week old wild type, mutant and transgenic lines, extracted protein was separated by SDS-PAGE and western blot analysis was performed using a proxidase anti proxidase (PAP) antibody.

B, tocopherol analysis of selected plants. Tocopherol was analyzed from leaves of 4-week old wild type, non transformed vte1 mutant and vte1 mutant which was complemented with TC-TAP-Tag, Arabidopsis plants by HPLC method. Lines 42, 54, 49 and 15 were analyzed in this experiment. Results are means of five wild type and vte1 mutant plants and five T2 progenies of each independent transgenic line ± standard deviation.
Tocopherol content of the selected transgenic lines, \textit{vte1} mutant and wild type plants, was measured by HPLC. As indicated in Figure 3-8B, wild type plants have 24.8 and transgenic lines 42, 54, 49 and 15 have 23.2, 22.7, 22.1 and 21.1 ng tocopherol per mg fresh weight, respectively. Whereas the tocopherol content of non-transformed \textit{vte1} mutant was about 1.42 ng tocopherol per mg fresh weight. The results of western blot and tocopherol analysis indicated that the transgene is stably inherited in transgenic lines and that the mutant phenotype was complemented.

To obtain homozygous lines and to do further analysis, transgenic lines (TC-42, 49, 54, and 15) were placed on MS/Kanamycin medium. During the germination on kanamycin resistance, segregation patterns were analyzed in the T3 generation of these selected transgenic lines. Transgenic line TC-54 did not exhibit any segregation, which indicated that this line was homozygous for the transgene, whereas transgenic lines TC-42, 49, and 15 still exhibited a 3:1 Mendelian segregation pattern indicating that these transgenic lines were heterozygous (Table 3-3).

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number of the seed</th>
<th>Number of the seedling</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
<td>White-yellow</td>
</tr>
<tr>
<td>42</td>
<td>82</td>
<td>61</td>
<td>19</td>
</tr>
<tr>
<td>54</td>
<td>89</td>
<td>86</td>
<td>0</td>
</tr>
<tr>
<td>49</td>
<td>108</td>
<td>78</td>
<td>27</td>
</tr>
<tr>
<td>15</td>
<td>96</td>
<td>71</td>
<td>22</td>
</tr>
</tbody>
</table>

### 3.1.3. Protein complex purification from transformed TC-TAP-Tag plants

Since its first description in 1999 (Rigaut et al., 1999) TAP has been successfully used in the identification of binding partners of various proteins (Puig et al., 2001; Gingras et al., 2005). The TAP-tag consists of two IgG binding domains of the \textit{S. aureus} protein A and a calmodulin binding peptide separated by a TEV protease cleavage site. To identify possible interacting partners of tocopherol cyclase, the TAP-tag was placed at the C-terminus of \textit{Arabidopsis} tocopherol cyclase and the \textit{Arabidopsis} \textit{vte1} mutant was transformed with the respective expression vector (see above). To purify the tocopherol...
cyclase interacting partners ten gram leaf material from transgenic line (TC-54, the homozygote line) was used for tandem affinity purification of the tagged protein according to the scheme depicted in Figure 3-9. Tissue samples were homogenized with three volumes of extraction buffer, the supernatant (step1) of the homogenized material was separated by centrifugation. An aliquot of both supernatants (vte1 mutant and transgenic plants) were load on 12.5 % SDS-PAGE gel. As indicated in the Figure 3-10, a protein of about 66 kDa was detected in the supernatant of transformed plants (lane 2a and 2b ) with both antibody, whereas no protein was detected at this height in the vte1 mutants (lane 1a and 1b ).

Figure 3-9) The schematic structure of the different step in the purification of the tocopherol cyclase fused TAP-Tag

1; crude extract from transgenic plants, 2; crude extract incubated with IgG resin following the washing steps (bound protein), 3; supernatant (unbound protein), 4; the supernatant of incubated protein with IgG resin which is eluted by TEV protease (protein eluted from the resin), 5; IgG resin which is eluted by TEV (protein remaining on resin), 6; protein complex incubated with calmodulin resin following the washing steps (bound protein), 7; Supernatant after the incubation with calmodulin resin (unbound protein), 8; calmodulin resin after elution by EGTA (protein remaining on resin, step 8 ), 9 and 10; purified protein eluted by EGTA-eluted, (purified protein pool 1-3 and 4-8)
Figure 3-10) Purification of the complex protein from *vte1* *Arabidopsis* mutants that were transformed by TC fused TAP-Tag

Protein complex were purified to isolate the protein partner of the tocopherol cyclase from the leaves of four-weeks old *Arabidopsis vte1* mutant which was complemented with TC-TAP-Tag. The different fractions collected during purification of AtTC using TAP were separated by SDS-PAGE. The protein were analysed by western blot using PAP (a) or CBP (b) antibody. Molecular masses of marker proteins are indicated in kDa. An amount of 40µl of each fraction was applied per lane. Note that TEV protease cleavage leads to about 13-kDa reduction in protein size.

M, Protein marker; Lane 1, crude extract of non transformed* vte1* mutant; Lane 2, crude extract of transgenic plants before the addition of IgG resin (step 1); lane 3, supernatant after the incubation with IgG resin (unbound protein, step 3); lane 4, IgG resin after washes, before elution (bound protein, step 2); lane 5, IgG resin after elution by TEV (protein remaining on resin step 5); lane 6, calmodulin resin after elution (protein remaining on resin, step 8); lane 7, EGTA-eluted protein, pool 1-3 (purified protein, step 9); lane 8, supernatant after the incubation with calmodulin resin (unbound protein, step 7); lane 9, eluted protein, pool 4-8 (purified protein, step 10).

To perform the first purification step the supernatant of the crude extract was incubated with IgG beads. Then the IgG beads were separated from the supernatant by
centrifugation. As indicated in Figure 3-10, one protein at the same height as in the crude extract is observed as an IgG column bound protein (lane 4a and lane 4b). However, no protein was detected in the supernatant after incubation with IgG (lane 3a and lane 3b). This result indicates that all the tocopherol cyclase in the supernatant can be bound to the IgG sepharose. The observation in lane 5a and lane 5b indicates that TEV protease partially digested the protein A from the column and results in a protein of about 52 kDa (14 kDa will be removed from the original protein because of the cleaved two protein A domains). After incubation of the eluate with calmodulin beads, the pellet (step 6) was separated from supernatant (step 7) by centrifugation. As seen in lane 8b, there is no protein left in the supernatant after incubation with calmodulin beads and lane 6b indicates that, there is a trace of protein remaining in the calmodulin bead after elution by EGTA. Lane 7b and 9b represent the final elution from the calmodulin beads, which was carried out by incubation with EGTA. Elution step by EGTA was performed several times and the eluates 1-3 were collected together (step 9) and eluates 4-8 was collected together (step 10), all the eluates were finally collected and concentrated. Lane 7 and 9 indicate that, tocopherol cyclase protein could be purified by this method and detected by CBP antibody.

3.1.4. Visualization of the protein complex using one or two dimensional gel electrophoresis

After having established purification of the tocopherol cyclase, large-scale complex protein purification was performed. The final purified complex protein, which was eluted from the column, was subjected to one-dimensional polyacrylamide gel electrophoresis (SDS-PAGE). The proteins, which were separated by electrophoresis, were visualized by colloidal Coomassie brilliant blue (3-11 A). The bands, which were observed after staining, were excised from the gel and analyzed by mass spectrometry after trypsin digestion. As indicated in Figure 3-11A, three areas at about 70, 52 and 30 KD were stained by colloidal Coomassie brilliant blue. All single bands, which were visualized by colloidal Coomassie brilliant blue staining in one dimensional gel electrophoresis, were then cut off from the gel and digested with trypsin and analyzed by Matrix-assisted laser desorption ionization-time of the flight (MALDI-TOF analysis was performed by Hans-Peter Mock, IPK Gatersleben).
Figure 3-11) The visualization of the purified protein
Protein complex was purified from the leaves of 4-weeks old *Arabidopsis vte1* mutant, which was complemented with TC-TAP-Tag construct. The purified complex protein was separated by one or two-dimensional gel electrophoresis. (A), Protein complex was separated on 12.5% one dimensional SDS-PAGE and the separated proteins were visualized by colloidal Coomassie brilliant blue staining. The proteins, which are observed by colloidal Coomassie brilliant blue staining, were then cut from the gel and analyzed by MALDI-TOF (B). The purified complex proteins were first separated by their iso electric focusing point on the strip (the range of the pH was between 3 - 10), the separated protein on the strip (First dimension) then separated by the molecular weight on the 12.5% SDS-PAGE (second dimensional gel). The separated proteins on the second dimensional gel were visualized by siver staining, which is compatible to the mass spectrometer. The proteins, which are observed by the siver staining staining, were then cut from the gel to mass spectrometer analysis. 2-dimentional gel electrophoresis was carried out by by Proffesor Georg Kreimer (University of Erlangen-Nürnberg).

Although peptide analysis using MALDI-TOF could detect several proteins such as putative glycine-rich (29Kda), DNA binding (29Kda), sulfotransferase (37Kda), unknown protein (60Kda) and an unnamed protein product (64Kda) from *Arabidopsis* were
detected (Table 3-4A), following MALDI-TOF analysis, no peptide related to tocopherol cyclase was detected. Some IgG proteins were also detected when resulting peptide of the digested complex protein were analyzed by MALDI, which is related to the leak of IgG from the sepharose column. Some peptides were also detected, which were related to human ceratins that arises as a contamination during handling of the gel (Table 3-4A). Since we failed to detect tocopherol cyclase in protein complex, which was separated by one-dimensional SDS-PAGE, it was decided to repeat the protein purification to separate the isolated protein complex by two-dimensional gel electrophoresis. Large-scale complex protein purification from 10-gram leaves of 4-week-old transgenic plants was done independently at different times and the purified protein was separated independently on 2-D gel electrophoresis (2-D gel electrophoresis was performed by Prof. Georg Kreimer, University Erlangen-Nürnberg). The results were reproducible, as judged from the spot pattern of the silver stained gels from independent experiments. As indicated in Figure 3-11B, about nineteen spots was visualized on two dimensional gel electrophoresis, which was stained with silver nitrate. About twelve spots at about 45 to 66 Kda, 7 spots at about 40 to 35 Kda and 11 spots at about 20 to 30 Kda were visualized by silver staining. Some of the strongest bands, which were observed on the gel, were cut and identified by ISI/MS/MS analysis (ISI/MS/MS analysis was performed by Prof. M, Mittag from University of Jena - Germany). Peptide analysis using ISI/MS/MS could detect several proteins such as putative lectin, probable H+ transporing ATPase, vacuolar ATP synthase subunit B, and β-glucosidase and glycosyl hydrolase family from Arabidopsis (Table 3-4B). Although several strongest spots from the 2-D gel of about tocopherol cyclase size (45-60kD) were cut and analyzed by ISI/MS/MS, no peptide related to tocopherol cyclase was identified by mass spectrometer analysis (Table 3-4B).
Table 3-4 A) The proteins with high homology to the peptides which were obtained from the MALDI analysis

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Score</th>
<th>Peptide</th>
<th>Sequence coverage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>putative glycine-rich, zinc-finger DNA-binding protein [Arabidopsis thaliana]</td>
<td>44</td>
<td>5</td>
<td>24</td>
</tr>
<tr>
<td>F3</td>
<td>DNA binding / nucleic acid binding [Arabidopsis thaliana]</td>
<td>43</td>
<td>5</td>
<td>23</td>
</tr>
<tr>
<td>D1</td>
<td>ST; sulfotransferase [Arabidopsis thaliana]</td>
<td>45</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>E1</td>
<td>unknown protein [Arabidopsis thaliana]</td>
<td>43</td>
<td>4</td>
<td>11</td>
</tr>
<tr>
<td>E2</td>
<td>unnamed protein product [Arabidopsis thaliana]</td>
<td>42</td>
<td>4</td>
<td>10</td>
</tr>
</tbody>
</table>

All single bands, which were visualized by colloidal Coomassie brilliant blue staining in one dimensional gel electrophoresis, were cut off from the gel and digested with trypsin and analyzed by Matrix-assisted laser desorption ionization-time of the flight (MALDI-TOF analysis was performed by Hans-Peter Mock, IPK Gatersleben).

Table 3-4 B) The proteins with high homology to the peptides which were obtained from the ISI/MS/MS analysis

<table>
<thead>
<tr>
<th>Spot</th>
<th>Protein</th>
<th>Score</th>
<th>Peptide</th>
<th>Sequence coverage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>Myrosinase-binding protein-like</td>
<td>160.31</td>
<td>32</td>
<td>10.36</td>
</tr>
<tr>
<td>5</td>
<td>putative lectin</td>
<td>110.25</td>
<td>26</td>
<td>12.23</td>
</tr>
<tr>
<td>4</td>
<td>acalin-like lectin domain</td>
<td>38.31</td>
<td>5</td>
<td>2.35</td>
</tr>
<tr>
<td>3</td>
<td>probable H+-transporting ATPase</td>
<td>42.23</td>
<td>5</td>
<td>11.29</td>
</tr>
<tr>
<td>3</td>
<td>vacuolar ATP synthase subunit B</td>
<td>30.23</td>
<td>3</td>
<td>1.23</td>
</tr>
<tr>
<td>3</td>
<td>ATP synthase α-ß- family, ß-barrel domain</td>
<td>40.23</td>
<td>6</td>
<td>10.58</td>
</tr>
<tr>
<td>2</td>
<td>ß-glucosidase, putative</td>
<td>60.30</td>
<td>12</td>
<td>10.32</td>
</tr>
<tr>
<td>2</td>
<td>Glycosyl hydrolase family 1</td>
<td>20.32</td>
<td>4</td>
<td>1.23</td>
</tr>
<tr>
<td>1</td>
<td>putative ß-glucosidase</td>
<td>134.36</td>
<td>31</td>
<td>26.5</td>
</tr>
</tbody>
</table>

The complex protein were separated by two dimensional gel electrophoresis, separated protein were visualized by siver staining on the second dimensional gel electrophoresis, 19 single spots were visualized by siver staining, several single spots were cut off from the gel (2-D gel was performed by prof G.Kreimer, University of Erlangen-Nureenberg). The spots, which were cut from the gel, were digested with trypsin and analyzed by mass spectrometer (ISI/MS/MS analysis was performed by Prof.M, Mittag from University of Jena - Germany).
3.2. **Study the biological function of tocopherol in tobacco plants under optimal growth conditions**

Under natural conditions, plants are exposed to a variety of biotic and abiotic stress, which results in accumulation of reactive oxygen species (ROS) in leaves. Accumulation of ROS causes the oxidation of cellular components, including proteins, chlorophyll and lipids. To cope with oxidative stress plants have evolved two general protective mechanisms, enzymatic and non-enzymatic detoxification. Non-enzymatic mechanisms are based on antioxidants including vitamin E, ascorbate, glutathione, carotenoids and flavonoids (Dat, 2000; Alscher, 2002). Tocopherol, which is a powerful antioxidant, is considered as a group of four structurally related compounds that differ in the degree of methylation of their chromonal ring.

Although tocopherols are synthesized exclusively in plants and some photosynthetic microorganisms, there is little knowledge concerning tocopherol function in plants. Instead several functions have been shown for tocopherol in mammalian cells, for instance *in vitro* experiments showed that tocopherol can be responsible for scavenging reactive oxygen species, thereby preventing the oxidative degradation of fatty acids in membranes. In order to unravel the tocopherol function in plants, study the distribution of the tocopherol throughout the plants and creation of the transgenic plants with different levels and composition of tocopherol would be very helpful.

### 3.2.1. **Tissue specific distribution of tocopherol derivatives in tobacco plants**

As indicated in Table 1-2 and Table 3-6, tocopherol composition in plants differs between different species and different tissues within one species. Leaves commonly accumulate α-tocopherol whereas seeds usually are rich in γ-tocopherol. Therefore, α-tocopherol has been proposed to participate in the detoxification of reactive oxygen species together with glutathione and ascorbate (Foyer and Noctor, 2003). In contrast to leaves, γ-tocopherol is the predominant tocopherol derivative in seeds, where total tocopherol content is 10 to 100 times higher than in leaves (Table 3-6). In oil-storing seeds like *Arabidopsis*, γ-tocopherol has been shown to protect polyunsaturated fatty acids (PUFAs) from oxidation, thereby increasing seed longevity (Sattler et al., 2004). Likewise, γ-tocopherol might also be involved in desiccation tolerance of seeds.

To investigate the plasticity of tocopherol biosynthesis in tobacco, we examined tocopherol composition in a variety of vegetative and reproductive organs in wild type tobacco plant. In this attempt, we used pure methanol for tocopherol extraction (see
material and method section 5-12-1), the validity of the method was testified by recovery of the standard tocopherol from the samples. As shown in Table 3-5, the value of the recovery was about 89-96% for α-, γ-, and δ-tocopherol in Dionex-HPLC using C\textsubscript{18} column. Since we had no standard for β- tocopherol and we were interested to quantify β- tocopherol especially in transgenic γTMT tobacco plants, therefore the reliability of the pure tocopherol extraction method was also evaluated by Waters-HPLC using C\textsubscript{30} column (Mohammad-Reza Hajirezaie, IPK-Gatersleben). Alpha-, γ-, δ- and β-tocopherol was recovered by value about of 87%, 98%, 99% and 106%, respectively.

<table>
<thead>
<tr>
<th>Tocopherol</th>
<th>Dionex- HPLC (C\textsubscript{18} column )</th>
<th>Waters- HPLC (C\textsubscript{30} column )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>Alpha</td>
<td>91.7</td>
<td>10.7</td>
</tr>
<tr>
<td>Gamma</td>
<td>89.1</td>
<td>12.2</td>
</tr>
<tr>
<td>Beta</td>
<td>-----</td>
<td>-----</td>
</tr>
<tr>
<td>Delta</td>
<td>95.5</td>
<td>16.23</td>
</tr>
</tbody>
</table>

The validity of the methanol extraction method was tested by measuring of the tocopherol standards (α-, γ-, and δ-tocopherol) which were added to the sample before the extraction, in different concentration. Recovery of the tocopherols (α-, γ- and δ- tocopherol) was measured by Dionex HPLC using C\textsubscript{18} column. As we had no standard for β- tocopherol the recovery of the tocopherol (α-, γ-, δ- and β- tocopherol) was also performed by Waters-HPLC using C\textsubscript{30} column (Mohammad-Reza Hajirezaei, IPK Gatersleben).

When the validity of the tocopherol method was confirmed by tocopherol recovery, tocopherol was measured from different tissues (Table 3-6). The tocopherol pool in seedlings, leaves, roots and stigma consisted more than 90% of α-tocopherol, female flower organs contained between 70% and 80% α-tocopherol, sepals and petals contained roughly equal amounts of α- and γ-tocopherol, while stamina (80% γ-tocopherol) and seeds (99.8% γ-tocopherol) were dominated by γ-tocopherol. Interestingly, the fraction of γ-tocopherol and total tocopherol content increased progressively with seed maturation. Thus, α-tocopherol accumulates in vegetative organs, while reproductive tissues were found to be enriched for γ-tocopherol, especially
when they are desiccation tolerant like stamina or seeds. Interestingly, seed imbibition did not result in a reduction of \( \gamma \)-tocopherol contents. These data confirm a tissue specific accumulation of \( \alpha \)- and \( \gamma \)-tocopherol, which might indicate the specific function for tocopherol derivatives in tobacco plants.

<table>
<thead>
<tr>
<th>Plant tissue</th>
<th>Total –Toc µg/g FW</th>
<th>% of ( \alpha )-T</th>
<th>% of ( \gamma )-T</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vegetative tissues</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seedling</td>
<td>27.4 ± 4.3</td>
<td>96.1 ± 3.9</td>
<td>3.9 ± 3.1</td>
</tr>
<tr>
<td>Mature leaf</td>
<td>32.4 ± 7.3</td>
<td>94.6 ± 4.5</td>
<td>5.4 ± 4.1</td>
</tr>
<tr>
<td>Senescent leaf</td>
<td>39.3 ± 6.5</td>
<td>92.4 ± 3.6</td>
<td>7.6 ± 3.1</td>
</tr>
<tr>
<td>Root</td>
<td>20.7 ± 2.1</td>
<td>93.2 ± 4.2</td>
<td>6.7 ± 4.1</td>
</tr>
<tr>
<td><strong>Flower organs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sepal</td>
<td>28.5 ± 6.4</td>
<td>51.7 ± 3.4</td>
<td>48.3 ± 3.1</td>
</tr>
<tr>
<td>Petal</td>
<td>15.1 ± 8.7</td>
<td>37.4 ± 3.1</td>
<td>62.6 ± 3.2</td>
</tr>
<tr>
<td>Stamen</td>
<td>45.7 ± 10.7</td>
<td>20.6 ± 4.2</td>
<td>79.4 ± 4.4</td>
</tr>
<tr>
<td>Stigma</td>
<td>38.6 ± 6.3</td>
<td>90.1 ± 7.2</td>
<td>9.9 ± 7.1</td>
</tr>
<tr>
<td>Carpel</td>
<td>53.6 ± 15.2</td>
<td>73.6 ± 6.5</td>
<td>22.4 ± 6.1</td>
</tr>
<tr>
<td><strong>Seed</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8-DAP</td>
<td>101.4 ± 20.3</td>
<td>82.2 ± 5.7</td>
<td>17.7 ± 5.2</td>
</tr>
<tr>
<td>14-DAP</td>
<td>141.1 ± 30.3</td>
<td>69.9 ± 6.6</td>
<td>30.1 ± 6.1</td>
</tr>
<tr>
<td>Mature seed</td>
<td>280.3 ± 35.1</td>
<td>1.2 ± 1.1</td>
<td>98.8 ± 1.8</td>
</tr>
<tr>
<td>Imbibed seed</td>
<td>170.9 ± 25.3</td>
<td>2.8 ± 2.1</td>
<td>97.1 ± 2.7</td>
</tr>
</tbody>
</table>

Tocopherols were extracted from the indicated tobacco tissues with HPLC method. \( \alpha \)-T, \( \gamma \)-T are \( \alpha \)- and \( \gamma \)-tocopherol respectively; \( \beta \)- and \( \delta \)-tocopherol jointly represented less than 5% to the tocopherol pool and were omitted from the representation for clarity reasons. Thus, the percentage distribution of \( \alpha \)- versus \( \gamma \)-tocopherol is given in the table, being mean and standard deviation of four independent samples.
3.2.2. Generation of transgenic plants with altered content and composition of tocopherol using dsRNAi

Reverse genetics approaches is a powerful method in the identification of metabolic control steps. In this approaches a set of plants with progressively decreased expression of the enzyme in question must be created. One of the reverse genetic approach is down-regulation of the gene using antisense or co-suppression technologies (van der Krol et al., 1988; Napoli et al., 1990; Smith et al., 1990), or the recently developed RNA-interference (RNAi) technology.

Because of the advantage of the dsRNAi approaches, we decided to this system to obtain transgenic tobacco plants with altered content and composition of tocopherol. In order to obtain transgenic tocopherol deficient lines, tocopherol cyclase (TC) and homogentisate phytyl transferase (HPT) were silenced in tobacco plants using dsRNAi strategy. In addition, to create the α-tocopherol deficient plants, which also contain high amount of γ-tocopherol, γ-tocopherol methyl transferase (γTMT) was silenced in tobacco plants following a dsRNAi strategy.

3.2.2.1. Generation of tocopherol deficient plants with constitutively silenced homogentisate phytyl transferase (HPT) using dsRNAi

3.2.2.1.1. Creation of HPT: RNAi construct

To obtain the HPT sequences for the RNA interference construct, a partial potato HPT EST clone (accession no. BI919738) was identified by homology search with tBLASTn (Altschul et al., 1990). A fragment containing the 650 bp of the StHPT ORF, which contains nts 51 to 701 of the potato HPT EST clone, (accession number BI919738) was amplified by RT-PCR using primers AA7 and AA8, which introduced terminal BamHI (5′) and SalI (3′) recognition sites, respectively. To manipulate tocopherol metabolism in transgenic tobacco plants the cDNA fragment of HPT from potato was PCR amplified by flanked BamHI/SalI restriction sites primer. The resulting HPT fragment was cloned into the PCR blunt vector (Table 2-3) and the fragment was confirmed by sequencing. To create the RNAi constructs, the HPT (650bp) fragment was inserted in sense orientation downstream of the GA20 oxidase intron in the pUC-RNAi vector(Chen et al., 2003). The same fragment was subsequently placed in antisense orientation into the Xhol/BglII sites of pUC-RNAi already carrying the HPT sense fragments. Finally, the entire RNAi
cassette containing sense and antisense fragments of HPT interspersed by the GA20 oxidase intron were excised from pUC-RNAi using the flanking PstI sites and inserted into the SbfI site of pBinAR between the cauliflower mosaic virus 35S promoter and OCS terminator to yield pBin-HPT-RNAi construct (Figure 3-12).

3.2.2.1.2. Plant transformation and pre-screening the transgenic plants

The pBin-HPT-RNAi vector was transformed into Agrobacterium tumefaciens strain C58C1 (pGV2260). Nicotiana tabacum plants were transformed by this construct. Following this transformation, 68 kanamycin-resistant tobacco plantlets and 45 kanamycin-resistant potato plantlets were re-generated and for further analysis, these resistant T₀ plants were transferred to the greenhouse.

![Figure 3-12) Schematic structure of the binary intron-spliced hairpin RNA (RNAi) expression construct used for HPT silencing](image)

PBin:HPT:RNAi construct was created by inserting the fragment of HPT:RNAi, which comprises the sense and antisense HPT fragments (650bp, nts 51 to 701 of partial potato HPT EST; accession number. BI919738) and the GA20 spacer, between the cauliflower mosaic virus 35S promoter and the OCS terminator of the Bin19-derived vector using the indicated restriction sites.

3.2.2.1.3. Screening of the transgenic plants

To screen the primary transformants, all positive lines were transferred to the greenhouse and screened for tocopherol deficiency by HPLC analysis. Tocopherol content in the transgenic lines ranged from more than 130% to less than 2% of wild type (Figures 3-13 A and B), demonstrating efficient silencing of tobacco HPT by a heterologous potato dsRNAi construct. As HPT catalyses the committed step in Vitamin E biosynthesis, the condensation of the precursors homogentisic acid (HGA) and phytol...
Results

diphosphate (PDP), silencing of HPT affected all tocopherols to the same extent leaving the ratio between individual tocopherols constant (data not shown).

Figure 3-13) Screening of the T0 generation of HPT-RNAi transgenic tobacco plants by tocopherol analysis

Tocopherol content in leaves of 4-weeks old transgenic lines (T0) and wild type plants was measured by HPLC. Percentage of the tocopherol in transgenic lines was calculated compared to the wild type plants. A) Tocopherol content of eight representative transgenic plants compared to the wild type are shown.

B) Based on tocopherol deficiency compared to the wild type, transgenic lines were classified into different groups. Number of the plants with varying degree of total tocopherol deficiency (α- plus γ- tocopherol) in transgenic plants compared to the wild type are shown. Data are given as the single measurement of each independent transgenic line. (C) Chromatogram of the tocopherol separation using the HPLC. Tocopherols were eluted from the C<sub>18</sub> column and detected at different retention times (Δ- at 12.5, γ- at 15.2 and α- at 17.5 min). The pink chromatogram is standard (mixture of 20 ng of α-, γ- and δ- tocopherol), the black one is wild type and the blue one represents a chromatogram of the transgenic line HPT-28.
Transgenic lines were categorized in eight different groups. Nineteen lines with more than 90% reduction in tocopherol had the strongest tocopherol deficiency as compared to the wild type. Eight lines had 80-90 %, twelve lines had 60-80 %, five lines had 40-60 % and eight lines had 20-40 % reduction in tocopherol compared to the wild type. Twenty-one lines displayed almost no tocopherol deficiency compared to the wild type and some of these 21 lines even contain more tocopherol compared to the wild type (Figure 3-13A). The four strongest transgenic lines StHPT-RNAi-28, 49, 35 and 44 as well as HPT-RNAi-39 line (with 30 % of wild type tocopherol) and HPT-RNAi-65, which contains about 60% of wild type tocopherol content were selected for further analysis (Figure B). Among these selected lines, StHPT-RNAi-28 and 49 showed the strongest depletion of total tocopherol which is comparable to the residual tocopherol activity in the Arabidopsis vte1 (TC) and vte2 (HPT) null mutants and comparable to StSXD1-RNAi potato plants (Porfirova et al., 2002; Hofius et al., 2004).

3.2.2.1.4. Tocopherol deficiency is inherited in selected HPT: RNAi tobacco transgenic lines

In order to check the heritability of gene silencing, seeds of T₀ transgenic lines H-28, H-49, H-44, H-35, H-39 and H-65 were placed on MS medium containing the antibiotic kanamycin.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number of seeds</th>
<th>Number of seedlings</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of green seeds</td>
<td>Number of white-yellow seeds</td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>77</td>
<td>55</td>
<td>19</td>
</tr>
<tr>
<td>49</td>
<td>73</td>
<td>52</td>
<td>16</td>
</tr>
<tr>
<td>44</td>
<td>68</td>
<td>47</td>
<td>16</td>
</tr>
<tr>
<td>35</td>
<td>83</td>
<td>60</td>
<td>19</td>
</tr>
<tr>
<td>39</td>
<td>79</td>
<td>57</td>
<td>20</td>
</tr>
<tr>
<td>65</td>
<td>82</td>
<td>60</td>
<td>19</td>
</tr>
</tbody>
</table>

As indicated in table 3-7A, the selected transgenic lines showed a 3:1 Mendelian segregation of resistance to kanamycin which indicates that, the silencing of HPT is
inherited as single recessive nuclear gene. To obtain homozygous transgenic lines, the seeds of the T2 progeny were placed on selective media and as indicated in Table3-7B. Lines H-28, H-49 and H-35 seem to be homozygous in the T3 generation while the other lines H-44, H-39 and H-65 still show 3:1 segregation that means these lines are not homozygous.

**Table 3-7B) Segregation patterns of resistance to kanamycin in T3 generation of the transgenic HPT:RNAi tobacco seedlings**

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number of seeds</th>
<th>Number of seedlings</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Green</td>
<td>White-yellow</td>
</tr>
<tr>
<td>28</td>
<td>98</td>
<td>95</td>
<td>0</td>
</tr>
<tr>
<td>49</td>
<td>87</td>
<td>84</td>
<td>0</td>
</tr>
<tr>
<td>44</td>
<td>49</td>
<td>32</td>
<td>11</td>
</tr>
<tr>
<td>35</td>
<td>78</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td>39</td>
<td>54</td>
<td>38</td>
<td>12</td>
</tr>
<tr>
<td>65</td>
<td>58</td>
<td>37</td>
<td>13</td>
</tr>
</tbody>
</table>

3.2.2.1.5. Biochemical and physiological characterization of HPT: RNAi tobacco plants under ambient growth condition

3.2.2.1.5.1. Silencing of HPT resulted in severe tocopherol deficiency in transgenic plants

In order to assess the degree of inhibition of HPT expression and to select the best transgenic lines, T3 progeny of transgenic lines H-28, 49, 35, 44, 39 and 65 were selected in T2 generation. The T3 seedlings from T2 progeny were transferred to the greenhouse and 4 weeks after transferring to the greenhouse, tocopherol content and composition was measured from fully expanded leaves of this T3 progeny of HPT: RNAi plants. To accurately quantify tocopherol deficiency, tocopherol contents of the transgenic plants were calculated in percent of wild type tocopherol content. As illustrated in Figure 3-14A, H-28, H-49, H-35 and H-44 have the lowest tocopherol content within the selected lines, (less than 1 ng per gram fresh weight). Within the transgenic lines compared to the wild type plants, H-28, H-49, H-35 and H-44 lines showed the strongest depletion of total tocopherol with less than 5 % of wild type levels,
which is comparable to the residual tocopherol activity in the *Arabidopsis vte1* (TC) and *vte2* (HPT) null mutants and it is also comparable to StSXD1-RNAi potato plants (Porfirova et al., 2002; Hofius et al., 2004).

**Figure 3-14) Tocopherol content and composition in selected transgenic HPT:RNAi tobacco lines**

Tocopherol content of T₃ transgenic lines and wild type plants was measured by HPLC. Percentage of tocopherol in transgenic lines was calculated compared to wild type plants. Tocopherol content was measured in leaves of 4-week old plants. (A)-tocopherol composition of transgenic and wild type tobacco plants (black column represents α-tocopherol grey shows γ-tocopherol content and dark grey indicates total tocopherol content), (B) The percentage of total tocopherol content of transgenic plants compared to the wild type. The data are means of five wild type plants and six individual transgenic plants of each independent transgenic line ± standard deviation within the individual plant lines. (C) The detection limit for tocopherol measurement, less than 1 ng α-tocopherol could not be measured by our HPLC.
Because of the detection limit in our HPLC (less than one ng for $\alpha$-tocopherol) (Figure 3-14C), it is difficult to measure the precise percentage of tocopherol deficiency in the transgenic lines which contain lower than 0.2 ng total tocopherol per mg fresh weight. As an estimate we can approximately say that H-28, H-49, H-35 and H-44 lines have about less than 5%, H-39 has 29-34% and H-65 has 61-69 % of wild type tocopherol content.

3.2.2.1.5.2. Silencing of HPT by dsRNAi leads to phenotypic alteration in source leaves of transgenic plants

After transferring the transgenic and wild type tobacco plants into the greenhouse, the transgenic plants were indistinguishable from wild type plants besides a slow growth of the strongest tocopherol deficient lines. At the beginning the transgenic plants did not show any phenotypic change compared to the wild type, but later on the transgenic lines with high reduction in tocopherol content started to develop a phenotypic alteration in lower source leaves. As depicted in figure 3-15, transgenic lines H-28(a), H-49 (b), H-44(c) and H-35(d) with more than 95% tocopherol deficiency show the strong chlorotic phenotype in lower source leaves whereas the transgenic lines H-39 and H65 which contain 30 and 60 percent of wild type tocopherol content, respectively, did not develop any phenotypic effect on the leaves. The bleached areas in transgenic plants developed in intercostal regions of source leaves.

3.2.2.1.5.3. Growth response of tocopherol deficient tobacco plants

To find the correlation between tocopherol deficiency and some growth parameters, transgenic and wild type plants were grown in the greenhouse under normal growth conditions. Growth parameters were acquired by measuring the fresh weight (FW), plant height and flowering time point. Under controlled conditions (tissue culture condition), the transgenic plants were indistinguishable from wild type. Nevertheless, the transgenic plants showed different growth parameters as compared to the wild type under greenhouse conditions. Shoot growth and leaf number of the wild type and transgenic plants were measured 43 days after the transfer to the greenhouse.
Figure 3-15)- Phenotypic effect and growth response of tobacco HPT: RNAi transgenic plants

Chlorosis and bleaching were observed at flowering time in upper source leaves of transgenic tobacco plants with strong reduction in tocopherol content. (a), (b), (c) and (d) represent lines H-28, H-49, H-44, and H-35, respectively and (e) and (f) are wild-type tobacco plants. Transgenic lines showed strong growth retardation with respect to the control plants. The picture was taken nine weeks after transfer of the T2 offspring of HPT: RNAi transgenic tobacco and wild type tobacco plants to the greenhouse. The white bar is equivalent to 50 cm. Plants from the left to right are HPT-RNAi-28, H-44 and tobacco wild type.

As indicated in Table 3-7 and figure 3-15, shoot growth was reduced by 25-40%, 17-35%, 10-18%, and about 1 to 9% in the H-28, H-49, H-44, and H-39, respectively, as compared to the wild type plants. The leaf number of the transgenic plants at the
same age (43 days) was reduced by 44 - 64% , 30 - 53%, 14 - 41% and -2 to 20% in the transgenic lines H-28, H-49 , H-44 and H-39 , respectively , as compared to the wild type. Flowering time was delayed 12 –22 days, 9-19 days, 6-14 days and 3 - 7 days in H-28, H-49, H-44 and H-39 transgenic plants, respectively (Table 3-8). These results indicate that a 30% reduction in tocopherol deficiency (HPT-39) did not cause growth retardation, whereas severe tocopherol deficiency led to a reduction in biomass of the transgenic plants compare to the wild type.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Shoot length</th>
<th>Leaf number</th>
<th>Flowering time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Height (cm)</td>
<td>Number</td>
<td>% of WT</td>
</tr>
<tr>
<td>WT</td>
<td>92.6 ± 3.76</td>
<td>22.4 ± 3.6</td>
<td>100</td>
</tr>
<tr>
<td>H-39</td>
<td>88.4 ± 4.45</td>
<td>20.4 ± 3.4</td>
<td>95.8 ± 5.1</td>
</tr>
<tr>
<td>H-44</td>
<td>80.4 ± 3.5</td>
<td>16.5 ± 2.7</td>
<td>86.1 ± 3.8</td>
</tr>
<tr>
<td>H-49</td>
<td>68.4 ± 8.3</td>
<td>13.5 ± 2.8</td>
<td>73.8 ± 9.0</td>
</tr>
<tr>
<td>H-28</td>
<td>62.1 ± 6.7</td>
<td>12.6 ± 3.6</td>
<td>66.9 ± 7.2</td>
</tr>
</tbody>
</table>

Vegetative growth parameters (shoot length and leaf number) of HPT: RNAi tobacco plants were determined in 43 days old plants under normal growth conditions in the greenhouse. Results are means of five wild type plant and five T2 offsprings of each independent transgenic line ± standard deviation.

3.2.2.1.5.4. Inhibition of HPT leads to seed yield reduction of transgenic tobacco plants

Silencing of the tobacco HPT by HPT: RNAi construct not only resulted in retardation of flower initiation, but also reduced the number of flowers in the transgenic plants as compared to the wild type (table 3-8). To determine the effect of the tocopherol deficiency on flower initiation, the number of flowers were counted in the T2 progeny of transgenic and wild type plants. As shown in Table 3-8, the number of flowers was reduced by 32%, 28%, 15% and 5 % in transgenic lines H-28, H-49, H-44 and H-39, respectively, as compared to the wild type. As a measure of seed yield, the weight of 100 seeds was determined but transgenic plants exhibited no obvious differences
compared to the wild type (data not shown). As seed weight for wild type and transgenics was similar, the weight of the capsid was quantified as the next factor. The weight of the capsid was reduced by 35%, 31%, 26% and 8% in H-28, H-49, H-44 and H-39 transgenic lines, respectively, in comparison to the wild type. To determine the seed yield of the transgenic and wild type plants, whole capsules of the T2 progeny of transgenic lines and wild type plants were harvested. As indicated in Table 3-9, seed yield was reduced by 45-55%, 40-48 %, 20-35 % and 5 -10% in the transgenic lines H-28, H-49 , H-44 and H-39, respectively, as compared to wild-type plants (Table 3-9)

These data indicate that, in plants with 10-30% reduction in tocopherol content a slight decrease of seed yield was measured with values of 90-95% of wild type plants, whereas more than 95% tocopherol deficiency led to a remarkable reduction of the seed yield as compared to the wild type.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Number of flowers</th>
<th>Seeds per capsid</th>
<th>Seed yield</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number</td>
<td>% of WT</td>
<td>Weight (gram)</td>
</tr>
<tr>
<td>WT</td>
<td>140.8 ± 9.4</td>
<td>100</td>
<td>0.58±0.11</td>
</tr>
<tr>
<td>H-39</td>
<td>130.7 ± 10.8</td>
<td>95.3±10.2</td>
<td>0.53±0.09</td>
</tr>
<tr>
<td>H-44</td>
<td>119.3 ± 11.3</td>
<td>85.1±8.1</td>
<td>0.44±0.07</td>
</tr>
<tr>
<td>H-49</td>
<td>101.2 ± 12.4</td>
<td>72.1±8.7</td>
<td>0.40±0.08</td>
</tr>
<tr>
<td>H-28</td>
<td>96.8 ± 14.7</td>
<td>68.5±10.4</td>
<td>0.37±0.06</td>
</tr>
</tbody>
</table>

Reproductive growth parameters of HPT: RNAi tobacco plants were determined when the whole seed ripped under normal growth condition in greenhouse. Results are means of five wild type plants and six T2 individuals of each independent transgenic line ± standard deviation.

### 3.2.2.1.5.5. Plastoquinone analysis

During the mutant screening, the pds2 Arabidopsis mutant was obtained and proposed to be disrupted in homogentisate phytol transferase (Grusak and DellaPenna, 1999). This mutant shows a reduction in both tocopherol and plastoquinone content. This
observation suggests that HPT might be involved in both tocopherol and plastoquinone biosynthesis (Grusak and DellaPenna, 1999). Later on, the vte2 mutant was isolated based on an HPLC-screening from EMS Arabidopsis mutants (Sattler et al., 2003) and a PCR-based reverse genetics screen for T-DNA insertions in homogentisate phytol transferase, which resulted in tocopherol deficiency. To confirm that silencing of HPT only affects tocopherol biosynthesis but not plastoquinone biosynthesis, plastoquinone content was measured from HPT-silenced tobacco transgenic and wild type plants. The reliability of the plastoquinone extraction method and analysis were tested using internal standard, the recovery of the internal standard was between 65%-75%. As indicated in Figure 3-17, a slight decrease in PQ-9 content was measured in transgenic plants with a severe tocopherol deficiency (H-28, H-49 and H-44) compared to the wild type but this difference is not statistically significant. These data clearly indicate that two different genes must be involved in tocopherol and plastoquinone biosynthetic pathway and that silencing of HPT specifically affects tocopherol biosynthesis.

Figure 3-16) – Plastoquinone content from T2 generation of HPT: RNAi tobacco transgenic plants
Plastoquinone content of T2 transgenic lines and wild type plants was measured by HPLC. Plastoquinone content was measured in leaves of 4-week old plants. Results are means of five wild type plant and six T2 progenies of each independent transgenic line ± standard deviation.
3.2.2.1.5.6. Tocopherol deficiency leads to soluble sugar accumulation in transgenic tobacco plants

Mutation of tocopherol cyclase in maize and silencing of this gene in potato plants resulted in a sucrose export block in both maize and potato plants (Russin et al., 1996; Provencher et al., 2001, Hofius et al. 2004). Mutation of tocopherol cyclase (TC) in Arabidopsis did not alter carbohydrate metabolism under optimal growth conditions (Sattler et al., 2003) whereas vte1 and vte2 Arabidopsis mutants showed a carbohydrate phenotype only under low temperature conditions (Maeda et al., 2006). The steady-state levels of soluble sugars and starch were determined to evaluate whether tocopherol deficiency in HPT: RNAi -silenced tobacco transgenic lines resulted in a sucrose export block similar to the maize sxd1 mutant phenotype and sxd1-silenced potato plants.

Table 3-10) Soluble sugar contents in leaves of HPT- silenced tobacco plants compared to the wild type

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Total soluble sugar</th>
<th>Leaf 12</th>
<th>Leaf 8</th>
<th>Leaf 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Soluble sugar (mmol per m²)</td>
<td>% of WT</td>
<td>Soluble sugar (mmol per m²)</td>
<td>% of WT</td>
</tr>
<tr>
<td>WT</td>
<td>4.07 ± 0.76</td>
<td>100</td>
<td>3.57 ± 0.65</td>
<td>100</td>
</tr>
<tr>
<td>H-39</td>
<td>4.41 ± 0.85</td>
<td>108.8 ± 20.1</td>
<td>4.12 ± 0.62</td>
<td>115.6±17.2</td>
</tr>
<tr>
<td>H-44</td>
<td>7.43 ± 1.1</td>
<td>182.1 ± 27.8</td>
<td>5.13 ± 0.7</td>
<td>143.6±19.4</td>
</tr>
<tr>
<td>H-49</td>
<td>11.44 ± 1.8</td>
<td>281.8 ± 44.2</td>
<td>7.96 ± 1.16</td>
<td>222.6±32.1</td>
</tr>
<tr>
<td>H-28</td>
<td>16.36 ± 2.1</td>
<td>401.9 ± 51.2</td>
<td>8.82 ± 1.23</td>
<td>247.6±34.6</td>
</tr>
</tbody>
</table>

Samples were harvested from different leaves of 56-day-old tobacco plants at the end of the dark period. (12th, 8th, and 3rd leaf as counted from the top) and soluble sugar contents was measured in these leaves. Concentrations are given in mmol per m². Results are means of five wild type plant and five T2 individuals of each independent transgenic line ± standard deviation, total soluble sugars represent the sum of Glucose, Fructose, and Sucrose.

To this end, the set of selected HPT-RNAi transgenic lines, which show tocopherol deficiency, was analyzed in three different leaf stages (leaf 12, leaf 8 and 3 from the top) at flowering time. As indicated in table 3-10, fifty-nine-day-old plants of line H-28, which
showed the strongest reduction in tocopherol content, accumulated massive amounts of carbohydrates in lower source leaves (leaf 12) at the end of the dark period. Soluble sugar levels increased 4.1-fold, compared to wild-type levels.

A considerable accumulation of soluble sugar contents was also detectable for H-49 and H-44 plants (2.8-fold and 1.8 respectively), whereas sugar levels in line H-39 were only slightly, but not significantly, enhanced relative to wild type plants. In upper source leaves (leaf 8), carbohydrate status remained strongly increased in line 28 and 49 (2.4-fold and 2.2 fold respectively) and slightly increased in line 44 (1.4 fold), but the carbohydrate content was not significantly altered in line 39, whereas in young developing leaves (leaf 3) carbohydrate levels did not accumulate in any of the transgenic lines. These data indicate that severe tocopherol deficiency (more than 95% compared to the wild type), results in a paralleled accumulation of total soluble sugars in HPT-silenced tobacco plants which is a leaf age-dependent phenotype in transgenic plants. The accumulation of starch has been reported in parallel with total soluble sugar accumulation in potato and maize.

Figure 3-17) Starch content and callose concentration in lower source leaves of transgenic tobacco plants.
Starch and callose contents were measured in lower source leaves of 8-week-old tobacco wild type and transgenic plants at the end of the photoperiod. The data for callose content (A) are given as β-1, 3-glucan pachyman equivalents, and the data for starch content are given as mmol per m⁻² glucose (B). Data are means of five wild type plant and five T2 progenies of each independent transgenic lines ± standard deviation.
To investigate whether tocopherol deficiency results in starch accumulation in tobacco transgenic plants, the starch content was measured from the same leaves, which showed sugar accumulation. As indicated in Figure 3-21, starch content decreased approximately 2-fold in transgenic H-28 tobacco plants compared to wild type plants. The starch content was 1.71, 2.81 and 3.16 mmol per m² in transgenic lines H-49, H44 and H-39 respectively, whereas the wild type contained 3.55 mmol per m². These data indicate that, the total soluble sugar pool was increased in parallel with tocopherol deficiency in transgenic tobacco plants, but unlike the maize and potato scenario, the amount of starch was decreased in transgenic tobacco plants compared to the wild type. 

To investigate whether the sugar accumulation was caused by callose deposition in plasmodesmata, callose concentrations were analyzed in the same lower source leaves of HPT: RNAi tobacco plants as used for the sugar determination before. As showed in Figure 3-21, callose content is 0.85, 0.82, 0.76 and 0.72 mg per gram fresh weight in transgenic lines H-28, H-49, H-44 and H-39 respectively, whereas the wild type content is 0.71 mg per gram fresh weight. Together with starch content of transgenic plants this data indicates that tocopherol deficiency in tobacco plants does not lead to starch and callose accumulation in lower source leaves of transgenic plants which exhibit sugar accumulation. The composition of the soluble sugar pool was resolved further. As indicated in Figure 3-18, glucose content (A) showed an 5.9-, 3.3-, 1.7- and 0.85-fold increase and fructose content (B) exhibited an 4.5-, 2.7-, 1.7- and 1.4-fold increase in transgenic lines H-28, H-49, H44 and H-39, respectively, as compared to the wild type. Although sucrose content was also increased in transgenic plants, the accumulation of sucrose was not as strong as fructose and glucose. As indicated in Figure 3-18, sucrose content (C) showed a 1.99-, 1.39-, 1.22- and 1.21-fold increase in the transgenic lines H-28, H-49, H44 as compared to the wild type. The ratio of glucose to fructose was calculated in wild type and transgenic plants and as indicated in Figure 3-18 D, the ratio of glucose to fructose was 2.9 in wild type plants and 3.08, 2.7, 2.4 and 1.7 in transgenic lines H-28, H-49, H44 and H-39, respectively. These data indicate that the accumulation of glucose is paralleled by accumulation of fructose and that there is no significant difference in the ratio of glucose to fructose in wild type and transgenic plants.
Results

Figure 3-18) Carbohydrate contents in source leaves of HPT: RNAi transgenic and wild type tobacco plants

Samples were harvested from lower source leaves of 56-day old tobacco plants at the end of the dark period. The content of the soluble sugars, Glucose (A), Fructose (B), Sucrose (C), and the ratio of the glucose to fructose (D), were measured in these leaves. Concentrations are given in mmol per m². Results are means of five wild type and T2 individuals of each independent transgenic line ± standard deviation.

3.2.2.1.5.7. The amino acid content and composition changed in lower source leaves of the tocopherol deficient tobacco plants

To further clarify the effect of HPT silencing in tobacco plants, amino acid contents and composition in phenotypic source leaves, were determined at the end of the photoperiod in the same leaf, which was used for sugar and starch determination. As shown in Figure 3-19 D, total amino acid levels in lower source leaves (leaf 12) increased by 150%, 130% and 117% in HPT-RNAi-28, 49 and 44 transgenic lines, respectively, as compared to wild type plants.
The content of proline, which can serve as an osmoprotectant, was enhanced 2.2- and 1.8-fold in HPT-RNAi-28 and H-49 transgenic lines, respectively, (Figure 3-19C), whereas line H-44 has the same proline content as the wild type. Glutamine content was increased by 386%, 328% and 180% in HPT-RNAi-28, 49 and 44 transgenic lines respectively as compared to the wild type plants (Figure 3-19A). The level of aspargin content was enhanced 4.3-, 3.7- and 2-fold in HPT-RNAi-28, H-49 and H-44 transgenic lines, respectively, as compared to the wild type (Figure 3-19 B).

Figure 3-19) Free amino acid contents in source leaves of transgenic HPT: RNAi and wild-type tobacco plants

The free amino acid contents and composition of wild type and transgenic HPT:RNAi tobacco plants were assayed from lower source leaves of 8-weeks old plants. The A, B, C and D chart represent glutamine, aspargin, proline, and total amino acid, respectively. The data are given as pmol per mg fresh weight. Data are means of five wild type plants and five T3 offspring of each independent transgenic line ± standard deviation.
3.2.1.5.8. Analysis of chlorophyll and carotenoids in transgenic plants

To determine the effect of the inhibition of the tocopherol biosynthetic pathway on primary metabolism, chlorophyll and carotenoid contents in HPT-silenced plants were investigated in source leaves of wild type and transgenic plants in two different stages: (i) flowering and (ii) after flowering. As displayed in Figure 3-15, the transgenic plants with the lowest amount of tocopherol as compared to the wild type exhibited a chlorotic phenotype, which is observed around the flowering stage. As shown in Table 3-11, there is a slight decrease in chlorophyll a, chlorophyll b, total chlorophyll and carotenoid contents of transgenic plants compared to the wild type, but the decrements are not statistically different.

This data indicate that tocopherol deficiency has no strong impact on chlorophyll and carotenoid content of transgenic plants under normal growth conditions. As mentioned above, leaves of HPT-RNAi plants exhibited a bleached phenotype, which is observed during flowering, therefore the chlorophyll content was investigated in leaves of transgenic plants after flowering. To obtain a gradient of chlorophyll reduction through the whole plants chlorophyll content and composition were measured from different leaves of HPT-silenced tobacco plants.

Table 3-11) Chlorophyll and carotenoid contents of transgenic and wild type tobacco plants

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Chlorophyll content</th>
<th>Carotenoids (µg / cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chl-a (µg / cm²)</td>
<td>Chl-b (µg / cm²)</td>
</tr>
<tr>
<td>WT</td>
<td>16.80 ±3.26</td>
<td>5.75 ± 1.01</td>
</tr>
<tr>
<td>H-28</td>
<td>13.73 ± 2.42</td>
<td>5.02 ± 1.03</td>
</tr>
<tr>
<td>H-49</td>
<td>13.62 ± 2.17</td>
<td>5.24 ± 0.94</td>
</tr>
<tr>
<td>H-44</td>
<td>14.38 ± 2.41</td>
<td>5.20 ± 0.84</td>
</tr>
<tr>
<td>H-39</td>
<td>15.36 ± 3.91</td>
<td>5.71 ± 1.11</td>
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</tbody>
</table>

Chlorophyll and carotenoid contents were determined spectrophotometrically in lower source leaves of five-week-old plants under normal growth conditions in the greenhouse. The data shown are means from five replicate samples ± SD from one representative experiment.
As depicted in Figure 3-20A, leaves of wild type plants during the flowering, did not show any chlorosis, even in lower source leaves (leaf number 15), whereas leaves of HPT-silenced plants showed bleaching even in upper source leaves (leaf number 7). Total chlorophyll content was measured from leaf number 2 to 15 of both wild type and transgenic plants. As indicated in Figure 3-20B, chlorophyll content of wild type was 25.3 µg per cm² in the third leaf from the top and was 12.6 µg per cm² in the fifteenth leaf while chlorophyll contents ranged from 27.6 µg per cm² in the third leaves to 6.2 µg per cm² in the fifteenth leaf of both transgenic lines (H-28 and H-49). Chlorophyll content of wild type leaves decreased from top to the bottom, whereas chlorophyll content of the transgenic plants were dramatically decreased even in the upper source leaves. The chlorophyll content of lower source leaves (leaf number 12-15) of wild type plants is comparable with upper source leaves (leaf number 5-8) of HPT-silenced plants.
Figure 3-20) Tocopherol deficiency leads to reduction of total chlorophyll content in upper source leaves of transgenic tobacco plants

Samples were taken from different leaves of 59-day-old tobacco plants grown at normal growth conditions in the greenhouse at the end of the dark period. At this time, transgenic and wild type plants have 18 and 22 leaves, respectively. Chlorophyll content was determined spectrophotometrically in different leaves (starting from third leaf to 15th leaf as counted from the top) of wild type and transgenic plants. A, shows the chlorotic phenotype of leaves of transgenic plants, leaf numbers as counted from the top of the plants are given above the leaf disc. B, the chlorophyll content in different leaves of tobacco plants in µg per cm². The data shown are means from five replicate samples ± SD from one representative experiment.

3.2.2.1.5.9. Silencing of tobacco HPT gene decreased photosynthetic capacity in transgenic plants

Retarded development, the reduced amount of growth and seed yield indicated that photosynthesis was affected in the strongest transgenic lines. Light-dependent photosynthesis at 400 µmol CO₂ per mol was measured on attached fully expanded leaves using an open gas-exchange system in four to five weeks-old wild type and HPT-silenced plants. In the light response curves, the photosynthetic photon CO₂ gas exchange was determined in response to increasing amount of the photon flux density (PPDF from 0 to 2000-µmol photons m⁻² s⁻¹). As displayed in Figure 3-21 A, the transgenic lines H-28, 49 and 44, which show more than 95% reduction in tocopherol compared to the wild type, have less CO₂ uptake at any photon flux density (PPDF 50-2000 µmol photons m⁻² s⁻¹) as compared to the wild type. Although the transgenic line H-39 contains only 30% tocopherol of the wild type, the CO₂ uptake of this transgenic line was not impaired at either low or high PPDF (50-2000 µmol photons s m⁻² s⁻¹) as compared to the wild type.

To measure the net photosynthetic capacity leaves of similar developmental stage and size were clamped with a LI-6400 photosynthetic gas exchange devise. The leaves were subjected to saturating light (2000 mmol photons m⁻² s⁻¹) for 15 min and the rate of CO₂ assimilation was measured using a LICOR-LI6400 portable photosynthesis instrument. As indicated in Figure 3-20B, the maximum CO₂ uptake of the transgenic lines H-28, H-49, H-35, H-44 and H-39 , under these saturated conditions, was inhibited by 76, 61, 33, 15 and 13 %, respectively, as compared to the wild-type (Figure 3-21B). The maximum quantum yield (calculated as the ratio of CO₂ uptake to transported electron) was
decreased by 77%, 70%, 53%, 13% and 2% in HPT-28, HPT-49, HPT-35, HPT-44 and HPT-39, respectively as compared to the control plants (Figure 3-21). As shown in Figure 3-21B, dark respiration measurement was not statistically different between the strongest transgenic lines and the wild type.

Figure 3-21) - Photosynthetic capacity (Light response curves, maximum quantum yield of CO₂ fixation, maximum net CO₂ uptake rate and dark respiration) in tocopherol-deficient tobacco lines.

Photosynthetic rates were measured on attached mature leaves at 400µm per mol CO₂ concentration using a LICOR LI6400 portable photosynthesis instrument. Photosynthetic parameters of HPT-28, 49, 35, 44 and 39 transgenic lines and wild type plants were determined. Light response curves (A), maximum quantum yield of CO₂ fixation (B), and dark respiration (C) and maximum net CO₂ uptake rate (D) were derived from gas exchange measurements in source leaves of transgenic tobacco plants from line 28, 49, 35, 44 and -39 and a wild-type, respectively. Leaf No. 8 as counted from the top was used for the measurement. Values represent the mean (±SD) of five measurements.
As carbohydrate and chlorophyll contents varied with the developmental stages of leaves from the transgenics, chlorophyll fluorescence imaging was employed to examine the dynamics of photosynthesis with leaf age in three HPT:RNAi lines. As indicated in Figure 3-22D, the electron transport rate was decreased in transgenic plants by 27.4%, 21.7%, 21.2%, and 9.2% in young source leaves, source leaves, old source leaves and senescent leaves of H-28 transgenic line as compared to the wild type. Electron transport rate in line H-49 line was comparable to line H-28 line, besides of the electron transport rate (ETR) in senescent leaves (60.1% as compared to the wild type).

**Figure 3-22) - chlorophyll fluorescence parameters in source leaves of wild type and tobacco HPT:RNAi transgenic plants**

Effect of tocopherol deficiency on chlorophyll fluorescence parameters in source leaves of transgenic and wild type tobacco plants at different stages was determined by fluorescence imaging with a walz imaging PAM. (A); Assimilation rate, (B); nonphotochemical quenching (NPQ), (C); non regulated energy dissipation Y(NO), (D); electron transport rate (ETR) were analysed in source leaves (young source leaf, source leaf, old source leaf and senescent leaf). Data are means of four plants ± SD of these four plants.
As shown in Figure 3-22C, the amount of the non-regulated energy dissipation (Y (NO)) was increased in young source, source and old source leaves of the transgenic lines H-28, H-49 and H-44 as compared to the wild type throughout development. The amount of the Y (NO) was not different in senescent leaves of wild type and transgenic lines. The assimilation rate of line H-28 transgenic was decreased by 81%, 81%, 83% and 87% in young source leaves, source leaves, old source leaves and senescent leaves in comparison to the wild type. Whereas the assimilation rate in source leaves, old source leaves and senescent leaves was decreased by 79%, 84% and 55% in line H-49 and 95%, 95% and 63% in line H-44, respectively. However, the assimilation rate was not altered in young source leaves of both H-49 and H-44 transgenic lines as compared to the wild type (94% and 105% respectively).

The amount of the NPQ does not show any significant differences in young source and old source leaves of all transgenic and wild type plants, whereas NPQ was increased by 162%, 234% and 219% in senescent leaves of H-28, H-49 and H-44 as compared to the wild type (Figure 3-22B).

3.2.2.1.5.10. Severe tocopherol deficiency is paralleled by increased lipid peroxidation in source leaves of transgenic tobacco plants

To study the effect of the tocopherol deficiency on lipid peroxidation, the amount of the lipid peroxidation was measured in transgenic and wild type plants by determining the amount of malondialdehyde (MDA) as a final product of lipid peroxidation. MDA content was determined in leaves of 7-week old transgenic and wild type plants at two different leaf ages: (i) upper source leaf (leaf number 5 from the top, which did not show a phenotype), and (ii) lower source leaf (leaf number 12 from the top, which showed chlorotic phenotype) at the same time point (Figure 3-23). As shown in Figure 3-23A, upper source leaves of the transgenic plants had the same amount of MDA as compared to the wild type indicating that tocopherol deficiency does not increase lipid peroxidation in upper source leaves of transgenic plants. Nevertheless, MDA content in lower source leaves of transgenic plants, which displayed sugar accumulation, was increased by 147%, 140% and 124% in transgenic line H28, H-49 and H-44, respectively, whereas transgenic line H-39 had the same MDA content as the wild type (Figure 3-23AB).
Figure- 3-23) Lipid peroxidation in leaves from HPT: RNAi and wild type tobacco plants.
Lipid peroxidation was assayed by determining the amount of Malondialdehyde (MDA) in leaves of 7-week-old transgenic and wild type tobacco plants. The data are given as nmol per mg fresh weight and are means of five wild type plants and five T3 offsprings of each independent transgenic line ± standard deviation

3.2.2.1.5.11. The effect of tocopherol deficiency on ascorbate and glutathione content in transgenic HPT:RNAi tobacco plants
To study whether tocopherol deficiency would influence the antioxidant network in transgenic tobacco plants, the concentration of the soluble antioxidants, ascorbate, and glutathione, was determined in lower and upper source leaves of greenhouse grown HPT:RNAi transgenic and wild type tobacco plants. Under these growth conditions, non-phenotypic leaves did not show significant differences in antioxidant contents as compared to the wild type (data not shown). In phenotypic leaves, which showed increased lipid peroxidation, the total ascorbate content was also not significantly different from wild type plants (Figure 3-24A). As shown in Figure 3-24B, the reduced ascorbate content in transgenic lines, was slightly decreased as compared to the wild type. The ratio of the dehydroascorbate to the total ascorbate was varying between 17% to 26% and 19% and 35% in wild type, transgenic lines H-28, H-49, H-44 and H-39 lines, respectively. Taken together, these data indicate that, although total and reduced ascorbate content in leaves of transgenic HPT:RNAi tobacco plants were slightly decreased compared to those in wild type, the ascorbate pool in transgenic tobacco plants was not significantly affected by even severe tocopherol deficiency.
Results

Figure 3-24) Ascorbate and glutathione contents in source leaves of transgenic HPT:RNAi and wild-type tobacco plants

Total, reduced ascorbate and the ratio of dehydroascorbate to total ascorbate (A, B and C) of wild type and transgenic HPT:RNAi tobacco plants were assayed spectrophotometrically from lower source leaves of 7-week-old plants. The data are given as nmol per mg fresh weight. The data shown are means from five replicate samples ± SD from one representative experiment. Total, oxidized and the ratio of non-oxidized to total glutathione (D, E and F) of wild type and transgenic plants were assayed from lower source leaves of 7-week-old plants by HPLC. The data are given as pmol per mg fresh weight. Data are means of five wild type plants and three T3 offspring of each independent transgenic line ± standard deviation.
The non-oxidized, oxidized and total glutathione content was measured in lower source leaves of wild type and transgenic plants, lower source leaves of transgenic plants which showed increased lipid peroxidation. As illustrated in Figure 3-24D, transgenic plants had the same non-oxidized glutathione and total glutathione content as wild type plants. However, the amount of the oxidized glutathione increased 4.1, 3.4, and 3.8-fold in H-28, H-49 and H-44 transgenic lines as compared to the wild type. Due to the low amount of oxidized glutathione related to total glutathione, the ratio between oxidized and total glutathione is more or less unchanged (this ratio was between 92% and 99% for transgenic lines and wild type, respectively. Collectively, the results of the glutathione measurement indicated that tocopherol deficiency increased the oxidation of the non-oxidized glutathione.

3.2.2.1.5.12. Tocopherol deficiency leads to membrane damage as evident by increased ion leakage

To further characterize the HPT:RNAi transgenic plants and to examine the effect of reduced tocopherol content on membrane damage, ion leakage from different transgenic plants was assayed. For each measurement, six leaf discs (10-mm diameter) were cut from source leaves of wild type and transgenic plants. The leaves were floated on 8 ml of double distilled water for 20 h at 4 °C. Subsequently, ion leakage was measured by the electro conductivity of the solution. To get an impression of the total ion content, the samples were incubated at 95°C for 30 min and after cooling to room temperature, the conductivity of the bathing solution was again determined. For each measurement, ion leakage was expressed as percentage of ion leakage (the first value was divided by the second value and then multiplied by 100). As indicated in table 3-12, there was no significant difference in the total ion of wild type and transgenic lines. Nevertheless, the ion, which leaked from the leaf disc, was increased in transgenic HPT:RNAi tobacco plants compared to the wild type (the value was between about 26 in wild type and 37 in H-28 transgenic lines. As shown in table 3-12, transgenic HPT-39 line, which contained about 70 to 80% of wild type total tocopherol, only showed a slight increase in electrolyte leakage (7%) compared to the wild type, which is not significant. Nevertheless, a more than 90 % reduction in tocopherol content led to remarkable membrane damage in transgenic plants compared to the wild type. As indicated in table 3-12, membrane damage was increased in transgenic HPT:RNAi lines, which showed the strongest tocopherol deficiency, as compared to the wild type. These results
Results

indicated that the severe depletion (more than 90%) of tocopherol led to a parallel increase in membrane damage of transgenic plants.

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Electrolyte leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ion leakage</td>
</tr>
<tr>
<td>WT</td>
<td>26.2 ± 4.45</td>
</tr>
<tr>
<td>H-39</td>
<td>30.1 ± 5.63</td>
</tr>
<tr>
<td>H-44</td>
<td>32.1 ± 5.63</td>
</tr>
<tr>
<td>H-49</td>
<td>36.4 ± 6.83</td>
</tr>
<tr>
<td>H-28</td>
<td>37.1 ± 6.15</td>
</tr>
</tbody>
</table>

% of the membrane electrolyte leakage of wild type and transgenic tobacco plants were measured in leaf discs of 5-week-old plants grown under greenhouse conditions. Data are means of 5 wild type plant and five T3 offspring of each independent transgenic line ± standard deviation.

3.2.2.2. Creation of transgenic tobacco plants with constitutively silenced γ-tocopherol methyl transferase (γTMT)

3.2.2.2.1. Construction of γTMT: RNAi construct

In order to study the function of α-tocopherol in planta, transgenic tobacco plants with decreased γTMT activity were constructed using an RNA interference (RNAi) strategy. To obtain sequence information of the γTMT, the EST database was searched for γTMT orthologous sequences by using the Arabidopsis γTMT as a query sequence using BLASTn (Altschul et al., 1990). One potato EST containing part of the γTMT cDNA sequence was found (accession no. BQ116842) and used to design primers for PCR amplification. To create the RNAi construct, a fragment containing the 625 bp of the St γTMT ORF (representing nts 34 to 659 of EST, BQ116842) was amplified by RT-PCR.
using primers introducing BamHI and SalI recognition sites, respectively, into the sequence. The resulting fragment was inserted in sense orientation downstream of the GA20 oxidase intron in the pUC-RNAi vector. The same fragment was subsequently placed in antisense orientation into the XhoI/BglII sites of pUC-RNAi already carrying the γTMT sense fragments. Finally, the entire RNAi cassette containing sense and antisense fragments of γTMT interspersed by the GA20 oxidase intron was excised from pUC-RNAi using the flanking PstI sites and inserted into the SbfI site of pBinAR between the cauliflower mosaic virus 35S promoter and ocs terminator to yield pBin-γTMT-RNAi construct (Figure 3-25A).

3.2.2.2.2. Plant transformation and screening of transgenic plants

Tobacco plants were transformed with Agrobacteria harbouring the pBin-γTMT-RNAi construct. Due to the close relationship between potato and tobacco, it was assumed that the tobacco γTMT is sufficiently homologous to the potato cDNA to be targeted by the RNAi construct. After regeneration in tissue culture, 86 kanamycin resistant plants were transferred to the greenhouse for further analysis. Tocopherol content was extracted from the leaves of 4-week old transgenics and wild type plant. Tocopherol content and composition was measured in leaves from 4-weeks old plants by HPLC. Alpha- and γ- tocopherol was separated on a C18 column by the retention time (15.9 min and 17.3 min for γ- and α- tocopherol, respectively). Alpha- and γ- tocopherol were added together to calculate total tocopherol content. The total tocopherol content was not significantly changed in transgenic plants as compared to the wild type (Figure 3-26C). However, the amount of α-tocopherol in transgenic plants varied from 5% (line number 55) to 150% (line number 6) of wild type content (Figure 3-26A). Based on their α- tocopherol content, transgenic lines were classified into eight different groups. 3 lines have the lowest α- tocopherol content (about 95% reduction as compared to the wild type), 3 lines only contained 5-10%, 7 lines had 10-20%, 21 lines had 20-40%, 10 lines had 40-60%, 18 lines contained 40-80% α- tocopherol as compared to the wild-type. Nine lines had the same α- tocopherol content as the wild type and 15 lines had 100-150% α- tocopherol as compared to the wild type (Figure 3-26A). Inhibition of γ-TMT in tobacco transgenic plants led to a varying degree of γ-tocopherol accumulation. Based on γ-tocopherol accumulation transgenic plants were grouped into eight different
classes. As shown in Figure 3-26B, eleven lines showed 20-25-fold increase of γ-tocopherol content of wild type, nine lines showed 15-20-fold, six lines showed 10-50-fold, four lines showed 4-10-fold, three lines showed 2-4-fold, eight lines showed 1.5-2-fold increase in γ-tocopherol. 41 lines had almost the same γ-tocopherol content as wild type (28 lines show 1-1.5-fold increase in γ-tocopherol content of wild type and 13 lines contain 0.5-1 fold of γ-tocopherol content of wild type). The ratio of the α- to γ-tocopherol was calculated by dividing γ-tocopherol by α-tocopherol content of each individual transgenic line and wild type plants.

Figure 3-25). Schematic drawing of the pBin: γTMT: RNAi construct and the Screening of the transgenic plants by tocopherol analysis in the T0 generation of γTMT-RNAi tobacco plants

A) The construct was created by inserting the intron, sense and anti-sense γTMT fragment (625bp, nts 31 to 659 of partial EST accession no. BQ116842) from the PUC-γTMT: RNAi construct into the pBin AR vector, between 35S promoter and OCS terminator. B) Tocopherol content of T0 transgenic lines and wild type plants was measured by HPLC method. Tocopherol derivatives were eluted from the C18 column at different retention times (Δ- at the 12.5, γ- at 15.2 and α- at 17.5 min). The pink chromatogram is standard (mixture of 20 ng of α-, γ- and δ-tocopherol), black, is wild type and the blue, represents chromatogram line γTMT-55.
Figure 3-26) Tocopherol content, composition and ratio of γ- to α- tocopherol in leaves of transgenic γ-TMT: RNAi tobacco plants

Tocopherol was extracted from fully expanded leaves of 4-week-old wild type and transgenic plants. Tocopherol content and composition were measured by HPLC. The transgenic plant lines are ordered from left to right with the number of the transgenic lines. A; α-tocopherol, B; γ-tocopherol, C; total tocopherol (sum of α-, γ- and δ-tocopherol) and D; α to γ-tocopherol ratio in wild type and transgenic plants. Data for wild type are means of five wild type plants ± standard deviation and data for transgensics are result of single measurement from T0 transgenic lines.
As illustrated in Figure 3-26D, the ratio of $\alpha$- to $\gamma$-tocopherol was altered following silencing of $\gamma$TMT. The ratio varied from 8.2 to 35.9 in the strongest transgenic lines and 0.98 to 7.1 in the intermediate transgenic lines and 0.21 to 0.65 in the weakest transgenic lines whereas this ratio was 0.036-0.078 in wild type plants. In contrast to these significantly silenced lines some lines exhibited increased levels of $\alpha$-tocopherol compared to the wild-type (Figure3-26A).

Three lines ($\gamma$TMT-RNAi-55, 82, and 22) with very low $\gamma$-tocopherol and high $\alpha$-tocopherol content in leaves were selected for further analysis. These results were comparable to the residual $\alpha$-tocopherol content in the Arabidopsis vte4 null mutant.

### 3.2.2.2.3 Gamma- tocopherol accumulation is inherited in selected $\gamma$TMT: RNAi tobacco transgenic lines

The heritability of the $\gamma$TMT silencing was tested in the three selected transgenic lines. The seeds of T0 transgenic lines TMT-55, 82 and 22 were placed on the kanamycin selective media. As indicated in table 3-13A, all selected transgenic lines showed a 3:1 Mendelian segregation pattern of the kanamycin resistance, which indicates that, the $\gamma$TMT: RNAi construct is inherited as single recessive nuclear gene. To obtain homozygous transgenic lines seeds of the T2 progeny were placed on MS medium containing kanamycin. As indicated in table3-13B, TMT-55, 82 and 22 transgenic lines still show 3:1 segregation, which indicates that, these lines are not homozygous.

To check the heritability of the $\gamma$-tocopherol accumulation, tocopherol content and composition were measured in leaves of four-weeks-old transgenic and wild type plants by HPLC. As indicated in Figure 3-27 inhibition of $\gamma$-TMT in tobacco plants was inherited in the T2 progeny. Alpha- tocopherol was decreased in transgenic plants as compared to the wild type. Transgenic lines $\gamma$-TMT-22, 82 and 55 contain 10.1, 8.9 and 6.8 ng $\alpha$-tocopherol per cm$^2$, respectively, while wild type plant has 177 ng $\alpha$-tocopherol per cm$^2$ (Figure 3-27A). $\Gamma$-tocopherol was measured in wild type and selected transgenic lines. $\Gamma$-tocopherol content was elevated in leaves of transgenic plants in comparison to the wild type plants. $\Gamma$-tocopherol content was 177.4, 171.9 and 147.7 ng per cm$^2$ in $\gamma$-TMT-22, 82 and 55 transgenic lines respectively, while wild type plants accumulated 2.8 ng $\gamma$-tocopherol per cm$^2$ (Figure 3-27B). Total tocopherol was calculated by summation of $\alpha$-, $\gamma$- and $\delta$-tocopherol. Total tocopherol content was 179.9, 187.3, 180.8 and 159.6 in wild type and $\gamma$-TMT-22, 82 and 55 transgenic lines, respectively (Figure 3-27C). The ratio of $\gamma$- to $\alpha$-tocopherol was calculated in transgenic and wild type plants. This ratio was 0.05
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in wild type plants whereas this ratio was 17.7, 20.3 and 23.7 in γ-TMT-22, 82 and 55 transgenic lines, respectively (Figure 3-27D).

Table 3-13A) Segregation patterns of resistance to kanamycin in T1 generation of the transgenic γTMT:RNAi tobacco seedlings

<table>
<thead>
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<th>Plant line</th>
<th>Number of the seed</th>
<th>Number of the seedling</th>
<th>Ratio</th>
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<td>22</td>
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Table 3-13B) Segregation patterns of resistance to kanamycin in T2 generation of the transgenic γ TMT:RNAi tobacco seedlings

<table>
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</table>

These data indicate that inhibition of γ-TMT in tobacco plants leads to α- tocopherol deficiency which is paralleled by an increase in γ-tocopherol (Figure 3-26B and 3-27B), as previously described for the vte4-1 Arabidopsis mutant (Bergmüller et al., 2003). Strong silencing of γ-TMT did not just correlate with a reduction in α- tocopherol, but also correlated with an increase of the γ- to α- tocopherol ratio in the leaves of the transgenic plants (Figure 3-26D and 3-27D). In addition, these data indicated that total tocopherol content remained roughly the same as in wild type (Figure 3-27C). Transgenic γ-TMT-RNAi-55, 82, and 22 with the most dramatic change in the ratio of α- to γ- tocopherol (to far below 1) were selected for a closer study.
Tocopherol was extracted from fully expanded leaves of 4-week-old wild type and transgenic plants. Tocopherol content and composition of transgenic and wild type plants were analyzed by HPLC. A, α-tocopherol content; B, γ-tocopherol content; C, total tocopherol content (sum of α-, γ- and δ-tocopherol) D; The ratio of α- to γ-tocopherol in wild type and transgenic plants. Data are means of five wild type plants and five T2 offsprings of each independent transgenic line ± standard deviation.

To study the effect of α- tocopherol deficiency and γ-tocopherol accumulation on plant growth, some growth parameters were acquired for transgenic and wild type plants grown in the greenhouse under normal growth conditions (Table 3-14). Under tissue culture conditions (compare results for wild type and transgenic γTMT: RNAi line under control conditions in sorbitol and salt stress in section 3-3) and green house condition (data not shown) the transgenic plants were indistinguishable from the wild type. Seeds of transgenic plants were placed on MS medium containing kanamycin. The seedlings were selected for kanamycin resistance in tissue culture and transferred to the greenhouse.
Results

Table 3-14) Growth parameter of γTMT: RNAi and wild type tobacco plants

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Shoot length</th>
<th>Leaf number</th>
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</thead>
<tbody>
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<td></td>
<td>Height (cm)</td>
<td>% of WT</td>
<td>Number</td>
</tr>
<tr>
<td>WT</td>
<td>51.8 ± 4.76</td>
<td>100</td>
<td>21.6 ± 1.63</td>
</tr>
<tr>
<td>γTMT-22</td>
<td>48.3 ± 4.58</td>
<td>93.2 ± 8.8</td>
<td>20.8 ± 2.16</td>
</tr>
<tr>
<td>γTMT-82</td>
<td>49.1 ± 4.63</td>
<td>94.7 ± 8.9</td>
<td>20.5 ± 1.78</td>
</tr>
<tr>
<td>γTMT-55</td>
<td>47.8 ± 5.11</td>
<td>92.2 ± 9.8</td>
<td>21.4 ± 1.63</td>
</tr>
</tbody>
</table>

Growth parameter of γTMT: RNAi tobacco plants were determined of 43 days after transfer to the greenhouse under normal growth conditions. Results are means of five wild type plants and five T2 offsprings of each independent transgenic line ± standard deviation.

Shoot growth and leaf number of the wild type and transgenic plants were measured 43 days after transfer to the greenhouse. As indicated in Table 3-14, shoot growth was not changed transgenic γTMT: RNAi lines compared to the wild type. The leaf number of the transgenic plants at the same age as the wild type plants (43 days old) was also not altered in γTMT lines compared to the wild type. There was no obvious difference in flowering time of transgenic lines in comparison to the wild type. These results indicate that γ-tocopherol can compensate the effect of α-tocopherol in the plant growth.

3.2.2.2.4. Inhibition of γTMT leads to seed yield reduction in transgenic tobacco plants

To clarify the effect of α-tocopherol deficiency on seed yield of transgenic plants, the entire seeds from wild type and transgenic plants were harvested. As indicated in table 3-15 seed yield of transgenic plants was decreased by 33.4%, 30.6%, and 26.7% in γTMT-55, 82 and 22 transgenic lines as compared to the wild type. To further study the effect of inhibition of the γTMT on the flower organs, some parameters of flower organs were further characterized.

As illustrated in table 3-15, the weight of the capsule was reduced by 34.6%, 29.6% and 25.4% in γTMT-55, 82 and 22 transgenic lines, respectively, in comparison to the wild type. These results indicate that a severe reduction in α-tocopherol content is paralleled
by a reduction in seed yield, whereas flower initiation, number of the flowers and 100 seed weight were not affected.

| Table 3-15) Flower parameter of TMT: RNAi and wild type tobacco plants |
|--------------------------|-----------------|-----------------|-----------------|
| Plant line               | Number of the flower | Seed per 10 capsid | Seed yield |
|                          | Number          | % of WT          | Weight (gram) | Weight (gram) | % of WT |
| WT                       | 138 ± 10.4      | 100              | 0.58±0.11     | 100           | 5.61±1.16 | 100     |
| rTMT-22                  | 135 ± 12.8      | 96.2±8.9         | 0.43±0.09     | 74.6±10.7     | 4.13±0.85 | 73.3±16.5 |
| rTMT-82                  | 130 ± 11.6      | 92.4±9.7         | 0.41±0.07     | 70.4±10.3     | 3.82±0.74 | 69.4±13.9 |
| rTMT-55                  | 121 ± 14.1      | 86.4±12.6        | 0.38±0.06     | 65.4±9.6      | 3.57±0.63 | 66.6±11.4 |

Floral parameter of γTMT: RNAi tobacco plants were determined when the whole seed riped under normal growth conditions in the greenhouse. Results are means of five wild type plant and five T2 offsprings of each independent transgenic line ± standard deviation.

Except of tocopherol composition and seed yield, γ-TMT transgenic lines were indistinguishable from wild type with respect to growth parameter, chlorophyll content, carbohydrate content, amino acid content and composition, ascorbate and dehydroascorbate (DHA) content and lipid peroxidation in the greenhouse under optimal growth conditions (data not shown).

3.2.2.3. Generation of transgenic tobacco plants with constitutively silenced tocopherol cyclase (TC)

As tocopherol cyclase (TC) catalyses the cyclazation step in Vitamin E biosynthesis, silencing of tocopherol cyclase should result in tocopherol deficiency. Mutation and silencing of tocopherol cyclase in Arabidopsis and potato plants, respectively, led to a reduction of total tocopherol (Dörmann et al. 2003, Hofius et al. 2004). To obtain the transgenic tobacco plants with altered content of tocopherol in analogy to the potato plants previously described, the pBin-SXD1-RNAi vector (Figure 2-28A), which has already been used for potato transformation (Hofius et al. 2004) was used in this work.
Following *Agrobacterium* mediated gene transfer, 80 kanamycin resistant tobacco plantlets were generated. All transformants were transferred to the greenhouse for further analysis.

**Figure 3-28** RNAi-mediated silencing of TC leads to tocopherol deficiency in transgenic tobacco plants.

A, Schematic structure of the binary intron-spliced hairpin RNA (RNAi) expression construct used for transformation of tobacco plants. St.TC fragments (765 bp, nts 503–1,268 of StTC cDNA; accession no. AY536918) in sense and antisense orientation separated by intron 1 of potato GA20 oxidase (200 bp) were placed between the cauliflower mosaic virus 35S promoter and the OCS terminator of the Bin19-derived vector using the indicated restriction sites. B, -tocopherol was separated by HPLC on C18 column. The different tocopherols were eluted from the C18 column at different retention times (δ- at 12.5, γ- at 15.2 and α- at 17.5 min). The pink chromatogram is wild type, black is standard (mixture of 20 ng of α-, γ- and δ- tocopherol) and blue represents a chromatogram of the TC: RNAi-54 transgenic line.
3.2.2.3.1. RNAi mediated silencing of tocoferol cyclase led to total tocoferol deficiency in transgenic tobacco plants

All kanamycin resistant lines were transferred to the greenhouse for further screening. Putative transgenic plants were screened for tocoferol content by HPLC analysis. Tocopherol content of putative TC:RNAi transgenic lines was between 10 to 130% of the wild type tocopherol content (Figure 3-29). Inhibition of tocoferol cyclase in tobacco plants does not lead to same severe tocoferol deficiency as found for HPT: RNAi plants. Among the transgenic lines, eleven lines contained 10-20 % of wild type tocoferol content, twenty-three lines have 20-50%, twenty-five lines have 50-80% of wild type content and twenty-one lines have 80-130% of wild type content (Figure 3-29).

Figure 3-29) Total tocoferol content in leaves of transgenic TC: RNAi tobacco plants

Tocopherol was extracted from fully expanded leaves of 4-week -old wild type and transgenic plants. Total tocoferol contents (α-, γ- and δ-tocopherol) in source leaves of TC: RNAi-silenced transgenic plants were quantified by fluorescence HPLC. The transgenic lines are ordered from left to right with increasing total tocoferol content. Samples were taken from source leaves, 4 weeks after transfer to the greenhouse, and data represent the tocoferol content of five wild type and single individual T0 transgenic plant.
Among the transgenic lines TC: RNAi-73, 54, 45 and 58 three lines contain 10%, 11%, 13% and 15% \( \alpha \)-tocopherol respectively, as compared to the wild type. Except of the transgenic line TC: RNAi 73, which did not produce any seeds, transgenic lines TC: RNAi-73, 54, 45 and 58, which contained the lowest \( \alpha \)-tocopherol content, were selected for further analysis. To further study the effect of tocopherol cyclase deficiency, seeds of selected transgenic lines were placed on kanamycin and resistant seedlings were transferred to the greenhouse.

Physiological and biochemical parameters in wild type and TC-RNAi-45 line, which contained the lowest tocopherol content compared to the wild type, were quantified. Except tocopherol content, transgenic TC:RNAi lines were indistinguishable from wild type with respect to growth parameters, chlorophyll content, carbohydrate content, amino acid content and lipid peroxidation under greenhouse conditions (data not shown).
3.3. **Evaluate the response of the HPT: RNAi and γ-TMT: RNAi tobacco plants to sorbitol and salt stress**

Growth and productivity of plants depends on environmental conditions. Extreme circumstances can limit CO₂ fixation and enhance the generation of reactive oxygen species (ROS), such as superoxide radicals. Plants have developed different mechanisms to control the level of ROS. Several low molecular weight antioxidants (such as tocopherol, ascorbic acid, reduced glutathione) and antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), guaiacol peroxidase (POD) and glutathione reductase (GR) take part in the scavenging of reactive radicals. The levels of antioxidants and the activities of antioxidant enzymes are generally increased in plants under stress conditions. Under stress conditions plants increase antioxidant content to reduce the risk of cell damage by excess ROS (Liebler et al., 1986; Reverberi et al., 2001; Munne-Bosch and Alegre, 2002; Blokhina et al., 2003; Munne-Bosch and Alegre, 2003). High light and cold stress had mostly been applied for functional characterization of tocopherol deficient plants in the past (Porfirova et al., 2002; Bergmuller et al., 2003; Hofius et al., 2004; Havaux et al., 2005; Kanwischer et al., 2005; Maeda M, 2006).

As shown before, α-tocopherol deficient plants (γ-TMT: RNAi) and tocopherol deficient plants with more than 5% of wild type tocopherol level (TC: RNAi and HPT: RNAi) were indistinguishable from wild type plants under normal growth conditions. Therefore, we decided to evaluate the response of the HPT: RNAi and γ-TMT: RNAi tobacco plants to sorbitol (osmotic) and salt, which both result in osmotic and oxidative stress. Superimposed on the final stress outcome, sorbitol causes desiccation and sodium chloride effectuates problems in ion homeostasis. To further characterize the HPT and γTMT silenced plants and to test the function of tocopherols in plants during stress and to study their sensitivity to oxidative stress, transgenic HPT: RNAi, γTMT: RNAi and wild type tobacco seeds were sown on MS medium. The seedlings were grown on MS medium for about 14-20 days in tissue culture. When the fourth leaf appeared and fully expanded, the seedlings were subjected to salt and sorbitol stress imposed by treatment with increasing amounts of NaCl and sorbitol (0, 100, 200, 300,400 and 500 mM) on MS medium (NaCl and sorbitol were added to MS medium), and plant growth, metabolite contents and physiological parameter were scored after four weeks of stress.
3.3.1. Phenotypic alteration in leaves of tobacco plants subjected to salt and sorbitol stress

The phenotype of HPT: RNAi and γ-TMT: RNAi transgenic was not distinguishable from wild type on control plates and on plates supplemented with up to 200mM sorbitol or sodium chloride (see Figures. 3-30A-C and 3-31A-C for the corresponding phenotypes). Sorbitol and sodium chloride of equal or more than 300mM provoked distinct phenotypes for HPT:RNAi and γ-TMT:RNAi tobacco (Figures 3-30A-C and 3-31A-C). Leaves of HPT: RNAi plants were almost entirely chlorotic at 300mM sodium chloride, while the wild type showed chlorosis at 400mM NaCl only (compare Figure 3-30A and 3-30C).

![Figure 3-30](image)

Figure 3-30) Salt-induced oxidative stress in HPT: RNAi and γ-TMT: RNAi transgenic and wild type tobacco plants

Two-weeks old seedlings of WT, γ-TMT:RNAi and HPT:RNAi tobacco plants were subjected to MS medium containing increasing amounts of NaCl. The phenotype four or five representative individuals after four weeks of salt stress is shown for every treatment, with 0, 200, 300 and 400 mM NaCl given in columns from left to right.
As in HPT:RNAi tobacco, silencing of γ-TMT also aggravated the response of the transgenics towards salt stress, this effect was less dramatic for HPT knockdown plants at 300mM NaCl, but similar at 400mM sodium chloride (Figure 3-30A and 3-30B). Surprisingly, at 300 mM and more than 300mM sorbitol concentration, HPT and wild type plants exhibited the same chlorotic phenotype, whereas γ-TMT plants did not show such a phenotype even at higher (400mM) sorbitol concentrations (see Figure 3-31A, B and C).

Figure 3-31) Sorbitol-induced osmotic stress in wild type tobacco and tobacco lines silenced for HPT:RNAi and γ-TMT:RNAi

Two-weeks old seedlings of WT, γ-TMT:RNAi and HPT:RNAi tobacco plants were subjected to MS medium containing different amounts of sorbitol (0, 200, 300 and 400 mM, from left to right). Pictures of four representative individuals were taken after four weeks of sorbitol stress. The phenotype of four representative individuals after four weeks of salt stress is shown for every treatment, with 0, 200, 300 and 400 mM sorbitol given in columns from left to right.
3.3.2. Silencing of γ-TMT results in increased biomass production under osmotic stress, while silencing of both, HPT and γ-TMT, decreases tolerance to salt stress

Biomass production of wild type plants negatively correlated with increasing sorbitol or NaCl concentrations and was diminished by 90% and 65% on 400mM sorbitol and 400 mM sodium chloride, respectively (Tables 3-16 and 3-17, column 1). Vegetative growth of HPT and γ-TMT transgenic plants was not significantly different from wild type on control plates and on plates supplemented with up to 200mM sorbitol or sodium chloride – except for a 25% growth retardation of HPT knockdown plants on 200mM NaCl (Tables 3-16 and 3-17). Silencing of HPT did not alter the response towards osmotic stress significantly (Table 3-16), while the HPT transgenic lines were more susceptible to salt stress than the wild type, exhibiting only 66% of wild type fresh weight on 400mM NaCl (Table 3-17).

<table>
<thead>
<tr>
<th>Sorbitol mM</th>
<th>Wild type mg FW</th>
<th>γ-TMT mg FW</th>
<th>[%] wild type</th>
<th>HPT mg FW</th>
<th>[%] wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.59± 0.34 a</td>
<td>1.45±0.32 a</td>
<td>91,5 ±18,2</td>
<td>1.33±0.25 a</td>
<td>83,3±15,7</td>
</tr>
<tr>
<td>100</td>
<td>1.24± 0.14 a</td>
<td>1.19±0.11 a</td>
<td>95,3 ±8,53</td>
<td>1.10±0.10 a</td>
<td>88,5±8,62</td>
</tr>
<tr>
<td>200</td>
<td>0.76± 0.07 a</td>
<td>0.86±0.09 a</td>
<td>113,1±11,4</td>
<td>0.69±0.08 a</td>
<td>90,4±10,8</td>
</tr>
<tr>
<td>300</td>
<td>0.21± 0.05 a</td>
<td>0.57±0.15 b**</td>
<td>271,3±71,5</td>
<td>0.19±0.05 a</td>
<td>90,3±23,6</td>
</tr>
<tr>
<td>400</td>
<td>0.15 ±0.04 a</td>
<td>0.37±0.06 b***</td>
<td>246,6±40,7</td>
<td>0.12±0.03 a</td>
<td>80,5±20,9</td>
</tr>
</tbody>
</table>

Wild-type (WT), γ-TMT: RNAi and HPT:RNAi tobacco plants were grown on different sorbitol and NaCl concentrations separately. After four weeks, biomass was measured from stressed plants. Data shown are the mean ± standard deviation of four independent measurements. *: P<0.10, **: P<0.05 and ***: P<0.01.

As in HPT: RNAi tobacco, silencing of γ-TMT also aggravated the response of the transgenic plants towards salt stress. This effect was less dramatic than for the HPT knockdown plants at 300mM NaCl, but similar at 400mM sodium chloride (Table 3-16).
Results

Surprisingly, silencing of γ-TMT led to a substantial growth benefit of more than twofold over the wild type in osmotic stress of 300 and 400mM sorbitol (Table 3-15). Despite a decrease in growth of 75% on 400mM sorbitol compared to the control plates without osmolyte, γ-TMT transgenic plants remained virescent at 400mM sorbitol, while wild type and HPT:RNAi plants exhibited advanced chlorosis (Figure 3-31A-C).

<table>
<thead>
<tr>
<th>NaCl mM</th>
<th>Wild type</th>
<th>γ-TMT</th>
<th>[%] wild type</th>
<th>HPT</th>
<th>[%] wild type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg FW</td>
<td>mg FW</td>
<td></td>
<td>mg FW</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>1.41 ± 0.16  a</td>
<td>1.31 ± 0.28   a</td>
<td>92.2±19.7</td>
<td>1.32 ± 0.27 a</td>
<td>93.1±19.6</td>
</tr>
<tr>
<td>100</td>
<td>1.21 ± 0.10  a</td>
<td>1.15 ± 0.12   a</td>
<td>95.2±10.9</td>
<td>1.15± 0.12 a</td>
<td>95.2±10.2</td>
</tr>
<tr>
<td>200</td>
<td>0.90 ± 0.10  a</td>
<td>0.77± 0.08 a b</td>
<td>85.3±9.81</td>
<td>0.67±0.09 b**</td>
<td>74.3±10.9</td>
</tr>
<tr>
<td>300</td>
<td>0.80 ± 0.10  a</td>
<td>0.60±0.09 b**</td>
<td>75.6±11.5</td>
<td>0.50±0.06 b***</td>
<td>62.8±8.51</td>
</tr>
<tr>
<td>400</td>
<td>0.50 ± 0.07  a</td>
<td>0.33± 0.06 b**</td>
<td>66.6±12.4</td>
<td>0.33± .08 b**</td>
<td>66.6±16.3</td>
</tr>
</tbody>
</table>

Wild type (WT), γ-TMT: RNAi and HPT: RNAi tobacco plants were grown on different sorbitol and NaCl concentrations separately. After four weeks, biomass was measured from stressed plants. Data shown are the mean ± standard deviation of four independent measurements. *: P<0.10, **: P<0.05 and ***: P<0.01.

3.3.3. Tocopherol analysis of tobacco transgenic and wild type plants subjected to salt stress

It is well established that the amount of tocopherol increases during stress (Collakova and DellaPenna, 2003, 2003). As we had observed that both HPT:RNAi and γ-TMT:RNAi tobacco plants responded distinctively to osmotic and salt stress, it was instructive to examine, how the disturbances in Vitamin E biosynthesis affected tocopherol accumulation in the studied stress conditions. Tocopherol content and composition in stress acclimated leaves were determined after four weeks of osmotic and salt stress, respectively.
Figure 3-32) Total tocopherol content in wild type and transgenic tobacco leaves subjected to stress

Tobacco seedlings of wild type (black bars); γTMT (white bars) and HPT (grey bars) were subjected to oxidative and osmotic stress by supplementing 0 to 400 mM salt and sorbitol, in 100 mM increments to the MS culture medium. Total tocopherol was extracted from the leaves of 4-weeks-old wild type and transgenic plants and then α- (A and D), γ- (B and E) and total (C and F) tocopherol content was determined by HPLC. The number below the diagrams indicate the following condition: 1; 0 Mm, 2; 100 Mm, 3; 200 Mm, 4; 300 Mm and 5; 400 Mm of sorbitol and sodium chloride. Data represent here are means of four wild type plants and four independent plants for each transgenic line ± standard deviation.
Total tocopherol content in wild type plants was increased 1.46-, 1.63-, 2.96- and 5.91-fold under 100, 200, 300 and 400 mM sorbitol stress, respectively. Although tocopherol content of wild type plants under 100 and 400 mM salt stress was comparable to that under 100 and 400 mM sorbitol, tocopherol content of wild type plant displayed 2.6- and 5.91-fold increase under 200 and 300 mM salt in comparison to the wild type under control conditions (Figure 3-32 C and F). As indicated in Figure 3-32A and D, α-tocopherol content also directly correlated with the concentration of the sorbitol and sodium chloride supplements and α-tocopherol contributed to more than 90% of the tocopherol pool at any time in the wild type. The total tocopherol contents in γ-TMT: RNAi plants were between 60 to 80 ng per cm², which was comparable to wild type in control conditions (see Figure 3-32), except that γ-substituted α-tocopherol in the γ-TMT transgenics. Total tocopherol contents were increased from about 67.65 under control condition to 96.02, 109.94, 192.34 and 382.29 ng per cm² under 100, 200, 300 and 400 mM sorbitol stress and from 86.8 under control condition to 99.2, 191.4, 277.4 and 241.6 ng per cm² under 100, 200, 300 and 400 mM salt stress, respectively. The α-tocopherol deficient γ-TMT: RNAi transgenic tobacco plants accumulated γ-tocopherol instead of α-tocopherol during osmotic and salt stress (Figure 3-32C and F). In the γ-TMT transgenic plants γ-tocopherol contents were about 82.9, 92.1, 172.8 and 370.1 ng per cm² under 100, 200, 300 and 400 mM sorbitol stress and 159.97, 252.74 and 220.55 ng per cm² under 100, 200, 300 and 400 mM salt stress respectively (Figure 3-32B and E). This demonstrates that tocopherol biosynthesis is induced to a similar extent in γ-TMT: RNAi and in wild type tobacco. As a consequence of silencing γ-TMT, a different tocopherol end product accumulates in the transgenic plants at the bottom line.

In contrast, silencing of HPT utterly abrogated the capacity for tocopherol biosynthesis in the transgenic HPT: RNAi lines, so that the applied stress conditions did not result in significant tocopherol abundance. Nevertheless, total tocopherol content was induced from 1.6 (i.e. 2% of unstressed wild type) to 9.8 ng·cm² (i.e. 12% of unstressed wild type) after four weeks of either osmotic or salt stress (Figure 3-32 A-F).

### 3.3.4. Pigment analysis of tobacco transgenic and wild type plants subjected to sorbitol and salt stress

Tocopherols are involved in protecting the photosynthetic apparatus against oxidative damage together with the antioxidants ascorbate, glutathione and the photosystem II-associated xanthophyll cycle, (Porfirova et al., 2002; Havaux et al., 2005; Kanwischer et
Results

Therefore, it was reasonable to study the effect of altered tocopherol content (in HPT: RNAi) and composition (in γ-TMT: RNAi) on chlorophyll and carotenoid content in the transgenic tobacco plants under osmotic and salt stress, respectively. Carotenoid contents were measured by spectrophotometer at 470 nm (which is the wavelength of the absorption maximum \( \lambda_{\text{max}} \) for Lutein, \( \beta \)-Carotene, Violaxanthin and Zeaxanthin). Carotenoid contents were similar in wild type and both transgenic lines under 0, 100 and 200 mM salt conditions.

Figure 3-33) Total chlorophyll and carotenoids content in wild type and transgenic tobacco leaves subjected to osmotic and oxidative stress

Tobacco seedlings of wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) plants were subjected to oxidative stress by supplementing 0 to 400 mM salt and sorbitol, in 100 mM increments to the MS culture medium. Total chlorophyll (A and C) and total carotenoids (B and D) were extracted from wild type and transgenic leaves 4 weeks after stress initiation and were measured by spectrophotometer. The number below the diagrams indicate the following condition: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM and 5; 400 mM of sodium chloride or sorbitol. Data are means of four wild type plants and four independent plants for each transgenic line ± standard deviation.

Carotenoid contents decreased by approximately 48% and 57% under 300 mM salt in γTMT:RNAi and HPT:RNAi transgenic plants, respectively, and by around 32%, 69%
and 74% in wild type, γTMT:RNAi and HPT:RNAi transgenic plants respectively under 400 mM salt condition as compared to the wild type (Figure 3-33B). Under sorbitol stress, carotenoid contents were similar in wild type and both transgenic lines under normal and low stress conditions (100 and 200 mM sorbitol). Although wild type and transgenic HPT: RNAi plants showed strongly decrease in carotenoid contents (by approximately 35-85%) under 300 and 400 mM sorbitol stress compared to the control conditions, transgenic γTMT: RNAi plants showed slight decrease in carotenoid content (by approximately 5-25% under 300 or 400 mM sorbitol stress, respectively) compared to the control condition (Figure 3-33D).

In control and low stress conditions (100 and 200 mM sorbitol or salt), there is no significant difference in chlorophyll content of wild type and both transgenic plants (Figure 3-33A and C). Chromophore contents in wild type and transgenic plants were diminished whenever a chlorotic phenotype appeared, paralleling the observations described in Figure 3-30 and 3-31. Compared to the controls, chlorophyll contents were substantially decreased by about 25% each in HPT: RNAi and γ-TMT: RNAi leaves at sodium chloride concentrations of 300 mM and higher, while the wild type showed a 30% reduction in pigments only on 400 mM NaCl (Figure 3-33).

A decline in chromophore contents was also determined for wild type and HPT: RNAi transgenic plants on 400 mM sorbitol (Figure 3-33), while chlorophyll pools in γ-TMT: RNAi plants remained constant irrespective of the sorbitol concentration (Figure 3-33).

Both chlorophyll a and b content in parallel with total chlorophyll content decreased in wild type and both transgenic plants under 100, 200, 300 and 400 mM salt stress (Figure 3-34A and B). The content of chlorophyll a and chlorophyll b was decreased by different pattern in wild type and both transgenic plants under salt stress compared to the sorbitol stress (Figure 3-34D and E). Chlorophyll a content decreased in parallel with total tocopherol in response to salt stress while chlorophyll b content of in wild type, γTMT: RNAi and HPT:RNAi transgenic plants under 400 mM sorbitol, decreased by 34%, 13% and 38%, respectively, in comparison to the control condition (Figure 3-34E). The ratio of chlorophyll a to b was measured in transgenic and wild type plants under both conditions. As indicated in Figure 3-34C, there is no significant difference between the ratio of chlorophyll a/b in wild type and both transgenic plants under any concentration of NaCl.
Figure 3-34) Chlorophyll a, chlorophyll b and ratio of chlorophyll a/b in wild type and transgenic tobacco leaves subjected to osmotic and oxidative stress.

Tobacco seedlings of wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) were subjected to oxidative stress by supplementing 0 to 400 mM salt and sorbitol, in 100 mM increments to the MS culture medium. Total chlorophyll was extracted from wild type and transgenic leaves 4 weeks after stress initiation and chlorophyll a (A and D) and b (B and E) content were measured spectrophotometrically. The ratio of chlorophyll a to chlorophyll b was measured for each individual sample (C and F). The number bellow the diagrams indicate the following condition: 1; 0Mm, 2; 100Mm, 3; 200Mm, 4; 300Mm and 5; 400Mm of sodium chloride or sorbitol. Data are means of four wild type plants and four independent plants for each transgenic line ± standard deviation.
This ratio was 2.5-2.8, 2.5-2.2 and 2.5-2.1 in wild type, γTMT: RNAi and HPT: RNAi transgenics, respectively (Figure 3-34C). The ratio of chlorophyll a/b in both transgenic plants under 0, 100, 200, 300 mM sorbitol stress was also similar to this ratio of wild type plants (Figure 3-34F). The chlorophyll a/b ratio decreased from about 2.4 to 0.8 in both, wild type and HPT: RNAi transgenics while it decreased from 2.5 to 1.72 in transgenic γTMT: RNAi plants under 400mM sorbitol stress (Figure3-34F). These data suggest that the photosynthetic core and accessory complexes become more damaged under osmotic stress than antenna complexes, which are rich in Chl b.

3.3.5. Altering tocopherol composition and quality by silencing HPT and γ-TMT does not lead to strong effects on the ascorbate (ASC) pool in response to salt and osmotic stress

The ascorbate pool size is reportedly increased in response to oxidative stress (Noctor and Foyer, 1998) and there is strong experimental evidence that ascorbate constitutes the interface of the amphiphilic tocopherols to the hydrophilic antioxidant network represented/ dominated by the ascorbate-glutathione cycle (as summarized in e.g. (Munne-Bosch and Alegre, 2002; Foyer and Noctor, 2005). However, it is unclear, whether the ascorbate pool gets induced in the tocopherol deficient *Arabidopsis vte1* mutant, as contrasting results have been obtained (Havaux et al., 2005; Kanwischer et al., 2005), indicating that the physiological status of the leaves might influence the ascorbate pool more than a deficiency in tocopherols. As the altered tocopherol composition in HPT and γ-TMT transgenics influenced the tolerance of the transgenics towards salt and osmotic stress, we assessed, whether total ascorbate contents and the reduction status of the ascorbate pool were markedly shifted when HPT and γ-TMT knockdown tobacco plants were subjected to salt and osmotic stress.

After four weeks of treatment, total and reduced ascorbate contents increased in the wild type Samsun NN with increasing amount of sorbitol and salt (Figure3-35 A, B, D and E). Please note, that salt treatment effectuates a more pronounced increase than sorbitol at identical concentrations (compare Figures 3-35A and 3-35D), e.g. 11.7 nmol·cm⁻² ascorbate on 400mM NaCl compared to 7.4 nmol·cm⁻² ascorbate on 400mM sorbitol.

Reduced ascorbate content was approximately the same in the wild type and in both transgenic plants at the control condition.
Figure 3-35) Total ascorbate, reduced ascorbate and ratio of dehydroascorbate to total ascorbate in leaves of wild type, γTMT: RNAi and HPT: RNAi tobacco plants subjected to salt-induced oxidative stress.

Tobacco seedlings of wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) were subjected to stress by supplementing 0 to 400 mM salt and sorbitol, in 100 mM increments to the MS culture medium. Total and reduced ascorbate were extracted from leaves of wild type and transgenic plants four weeks after stress initiation and then total ascorbate (A and D) and reduced ascorbate (B and E) content were measured spectrophotometrically. The ratio of DHA/ASC was measured by dividing DHA by the total ascorbate of the same sample (C and F). The number below the diagrams indicate the following condition: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM and 5; 400 mM of sodium chloride and sorbitol. Data are means of four wild type plants and four independent plants for each transgenic line ± standard deviation.
Under salt and sorbitol stress, reduced ascorbate content in wild type and both transgenic plants increased progressively (Figure 1A). Reduced ascorbate content was 8.8 in wild type plants, 5.7 and 7.2 for γTMT and HPT transgenic plants under 400mM salt stress while it was about 6.0, 4.2 and 6.1 in wild type, γTMT and HPT transgenic lines respectively under 400mM sorbitol stress. Furthermore, the dehydroascorbate (DHA) content in the wild type and both transgenic plants progressively increased after growth on salt and sorbitol compared to control conditions (Figure 6). dehydroascorbate content increased from 0.48-0.60, 0.34-0.55 and 0.73-0.87 nmol per cm² under control condition to 1.28-1.79, 0.95-1.47 and 1.62-2.81 nmol per cm² in wild type, γTMT:RNAi and HPT:RNAi transgenic plants, respectively, under 400 mM sorbitol and salt, which is indicative of oxidative stress.

The ratio of dehydroascorbate to total ascorbate was calculated in tobacco wild type and transgenic plants. This ratio was 0.18-0.20, 0.18-0.21 and 0.21-0.23 under control condition and 0.18-0.20, 0.17-0.20 and 0.21-0.25 in wild type, γTMT: RNAi and HPT: RNAi transgenic plants, respectively, under 400 mM sorbitol and salt stress. These data indicate that the content of the DHA in leaves of HPT: RNAi plant is higher than in both, wild type and γTMT: RNAi plants under control and both stress conditions (Figure 3-35 C and F). Silencing of γ-TMT resulted in 30% less accumulation of ascorbate than in the wild type during salt and osmotic stress of equal or more than 200mM sodium chloride or sorbitol, respectively (Figure 3-35A and D). Consistently, γ-TMT transgenic plants produced more biomass and exhibited less chlorosis on sorbitol than the wild type (Table 3-15, Figure 3-31). However, knockdown of γ-TMT resulted in elevated susceptibility towards salt stress (Table 3-16, Figure 3-30). In this scenario, increased ascorbate contents compared to the wild type would have been expected. After all, the decrease of the ascorbate pool is not substantial in γ-TMT as compared to the wild type.

**3.3.6. Lipid peroxidation and membrane damage are increased by silencing of HPT and decreased when γ-TMT is silenced under stress condition**

The observed increase in the pools of the antioxidants ascorbate and especially tocopherol under severe sorbitol ( Figures 3-32D, E, F and 3-35 D, E, F) and salt stress (Figures3-32A, B, C and 3-35 A, B, S) suggests that wild type and transgenics suffer from oxidative stress in both conditions. Malondialdehyde (MDA) occurs as a side product during lipid peroxidation and has proven to be a reliable marker for oxidative stress (Baroli et al., 2003; Muller-Moule et al., 2003).
In addition, excess lipid oxidation results in membrane damage, which is caused either by oxidative stress or is triggered during the hypersensitive response in pathogen defence in vivo. The extent of membrane damage can be quantified by ion leakage from the symplast. Unfortunately, determining ion leakage from salt stressed plants is prone to artifacts, so that only data after sorbitol stress could be measured.

To further study the effect of altered tocopherol content and composition on the membrane damage in tobacco plants under stress conditions, the percentage of ion leakage was measured in transgenic and wild type plants four weeks after subjecting to sorbitol stress. As indicated in table 3-18, wild type and γTMT:RNAi transgenic plants have similar ion leakage (44% and 47%) while it is significantly different from ion leakage of HPT:RNAi plants (55%) under control condition. Ion leakage remained constant at about 43-47% in wild type and γ-TMT: RNAi and 55-61% in HPT leaves up to sorbitol concentrations of 300mM (Table 3-18). Although ion leakage rose to about 80% on 400mM sorbitol in the wild type and HPT plants, it remained constant at about 48% in the γ-TMT knockdowns (Table 3-18).

Table 3-18)- Membrane damage is induced by osmotic stress in wild-type and transgenic tobacco plants

<table>
<thead>
<tr>
<th>Sorbitol mM</th>
<th>WT % of ion leakage</th>
<th>γ-TMT % of ion leakage</th>
<th>HPT % of ion leakage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>44.66± 3.09 a</td>
<td>47.49± 3.69 a</td>
<td>55.48±5.06 b*</td>
</tr>
<tr>
<td>100</td>
<td>39.15± 2.96 a</td>
<td>46.31± 3.12 a</td>
<td>63.46±9.76 b**</td>
</tr>
<tr>
<td>200</td>
<td>39.38±7.70 a</td>
<td>47.39± 4.41 ab</td>
<td>55.25±9.56 b**</td>
</tr>
<tr>
<td>300</td>
<td>47.85± 1.71 a</td>
<td>43.71±6.86 a</td>
<td>61.41±9.08 b</td>
</tr>
<tr>
<td>400</td>
<td>78.91± 0.81 a</td>
<td>48.80±8.45 b***</td>
<td>79.85± 8.39 a</td>
</tr>
</tbody>
</table>

Wild type (WT), γ-TMT: RNAi and HPT: RNAi tobacco plants were cultivated on increasing amounts of sorbitol. Four weeks after the onset of osmotic stress, membrane damage was assayed from stressed plants by determining ion leakage from leaf discs. Data shown are the mean ± standard deviation of four independent measurements.

*: P<0.10, **: P<0.05 and ***: P<0.01
MDA contents were determined in transgenic and wild-type plants four weeks after the onset of salt or sorbitol stress (Figure 3-36). MDA content was not significantly different in wild type and both transgenic plants under control condition. Lipid peroxidation in wild type and both transgenic plants was correlated in a near-linear manner with increasing salt and sorbitol stress. MDA content increased 1.07-, 1.61-, 1.76- and 1.94-fold in wild type, 1.18-, 1.23-, 1.29- and 1.39-fold in γTMT:RNAi and 1.04-, 1.33-, 2.44- and 2.69-fold in HPT:RNAi plants under 100, 200, 300 and 400 mM salt, respectively, in comparison to the MDA in wild type under control condition (Figure 3-36A). Whereas MDA content increased by 104%, 113%, 154% and 170% in wild type, 81%, 86% 103% and 114% in γTMT:RNAi and 130%, 159%, 228% and 251% in HPT:RNAi plants under 100, 200, 300 and 400 mM sorbitol compared to that content in wild type plants under control condition (Figure 3-36B). As indicated in Figure 3-36, tobacco plants silenced for γ-TMT were more susceptible to salt (Figure 3-36B) and less susceptible to sorbitol stress than the wild type (Figure 3-36A).

**Figure 3-36** Lipid peroxidation in leaves from HPT: RNAi and γ-TMT: RNAi and wild type tobacco plants after four weeks of osmotic and salt stress.

Wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) tobacco plants were treated with varying amounts between 0 and 400mM of sodium chloride (top panel) or sorbitol (lower panel). Lipid peroxidation was assayed from leaf samples after four weeks of salt (A) and sorbitol (B) stress by determining the amount of Malondialdehyde (MDA). The numbers below the diagrams indicate the following conditions: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM and 5; 400 mM of NaCl (A) or sorbitol (B). Data are means of four wild type plants and four independent plants for each transgenic line ± standard deviation.
This is in good accordance with the decreased accumulation of ascorbate in \( \gamma \)-TMT: RNAi lines in both, salt and sorbitol stress (Figures 3-35A and 3-35D), which points towards a diminished oxidative stress in challenged \( \gamma \)-TMT: RNAi leaves, even though chlorosis is increased under salt stress compared to wild type (Figure 3-30). As \( \gamma \)-tocopherol has been shown to prohibit lipid oxidation in seeds (Sattler et al., 2004), an accumulation of \( \gamma \)-tocopherol instead of \( \alpha \)-tocopherol in \( \gamma \)-TMT: RNAi leaves (Figures 3-32 A-F) most likely prevents lipid peroxidation in the transgenics, thereby ameliorating the effect of oxidative stress. Taken together, our results indicate that a lack of tocopherols in HPT: RNAi plants decreases membrane integrity in the absence of stress, exacerbating lipid oxidation in mild stress conditions already.

3.3.7. Silencing of HPT and \( \gamma \)-TMT alters sugar and amino acid metabolism under stress conditions

Severely tocopherol deficient plants are reportedly compromised in sugar export and accumulate soluble sugars and starch as a result of plasmodesmatal obstruction by callose plugs. The maize TC mutant \textit{sxd1} (Provencher et al., 2001) and potato silenced for TC (Hofius et al., 2004) exhibit this phenotype in standard growth conditions, while the \textit{Arabidopsis} HPT mutant \textit{vte2} displayed this phenotype in cold stress only (Maeda et al., 2006). Furthermore, carbon and nitrogen metabolism interdepend on each other (see Fritz et al., 2006 for a very recent report and the references cited therein). In an oversimplified view, carbon availability triggers nitrogen metabolism (e.g. Henkes et al., 2001; Matt et al., 2001; Gibon et al., 2004) and vice versa, nitrogen availability modulates carbon flow in the leaf (e.g. Scheible et al., 1997; Geiger et al., 1999). Therefore, we assessed how the sugar and amino acid metabolism are affected in the HPT and \( \gamma \)-TMT silenced tobacco plants during salt and sorbitol stress.

Salt and sorbitol stress provoked clearly distinct responses in sugar metabolism in the wild type, although 300mM of both, salt and sorbitol, provoked an accumulation of starch and soluble sugars (Figure 3-37A-D and figure 3-38A and C). Compared to unsupplemented plates, starch accumulated three- and twentyfold in wild type on 300mM sorbitol and salt, respectively, with salt being about five times more potent than sorbitol (compare Figure 3-38B to 3-38D) – less than 300mM of either stressor did not result in a considerable accumulation of starch. The accumulation of soluble sugars in the wild type was found to correlate directly with salt and sorbitol concentrations.
Figure 3-37) Carbohydrate and amino acid contents in leaves from HPT: RNAi and γ-TMT: RNAi and wild type tobacco plants after four weeks of salt and sorbitol stress

Wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) tobacco plants were treated with varying amounts between 0 and 300mM of sodium chloride (left column) or sorbitol (right column). The numbers below the diagrams indicate the following conditions: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM of either NaCl (left) or sorbitol (right panel). The contents of the soluble sugars glucose (A and D), fructose (B and E) and sucrose (C and F) were assayed from leaf samples after four weeks of stress. The depicted data are means ± standard deviation of four independent measurements.
Glucose and fructose levels accounted for the concomitant rise of total soluble sugar contents with increasing sorbitol stress (Figure 3-37A-B and 3-37D-E), while sucrose gradually accumulated with increasing salt stress (Figure3-37C and 3-37 F). Glucose and fructose contents were finally increased about twenty fold on 300mM sorbitol compared to the controls, while sucrose was found to accumulate more than tenfold on 300mM sodium chloride. A similar accumulation of sucrose was observed on the medium with the highest sorbitol concentration only.

**Figure 3-38**) Starch and total soluble sugar contents in leaves from HPT: RNAi and γ-TMT: RNAi and wild type tobacco plants after four weeks of salt and sorbitol stress

Wild type (black bars); γTMT:RNAi (white bars) and HPT:RNAi (grey bars) tobacco plants were treated with varying amounts between 0 and 300mM of sodium chloride (left column) or sorbitol (right column). The numbers below the diagrams indicate the following conditions: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM of either NaCl (left) or sorbitol (right panel). The contents of the total (glucose, fructose and sucrose) soluble sugar (A and C), and starch (B and D), were assayed from leaf samples after four weeks of stress. The depicted data are means ± standard deviation of four independent measurements.
The γ-TMT transgenics displayed comparable steady state pools of soluble sugars as the wild type, with the remarkable exception that glucose and fructose accumulation were twofold lower under sorbitol stress of 200mM or higher compared to wild type (Figures 3-37A-F).

Abrogating HPT activity in HPT: RNAi abolished the correlation of sugar or starch accumulation with the degree of salt or sorbitol stress. Sugar and starch pools in the HPT:RNAi transgenics did not respond to salt stress at all (Figure 3-37A-C and 3-38 A). Total soluble sugar contents in HPT: RNAi lines were already substantially elevated compared to wild type or γ-TMT: RNAi lines in low NaCl (Figure3-37A -C and Figure 3-37 A), while starch contents in the HPT:RNAi transgenics being continuously as high as in wild type or γ-TMT: RNAi transgenics grown on 300mM sorbitol (Figure 3-38B and D ). In osmotic stress conditions up to 200mM sorbitol, starch and soluble sugar contents in HPT:RNAi transgenics remained comparable to untreated wild type on control plates (Figure 3-37D-F and Figure 3-38C-D). In contrast, HPT: RNAi leaves accumulated 30% more sucrose, 75% more glucose and 100% more fructose than wild type or γ-TMT: RNAi plants on 300mM sorbitol, while starch contents rose to similar levels as in the two other genotypes. In contrast to maize sxdl, Arabidopsis vte2 and TC silenced potato (see top), we failed to observe a sugar export block in tocopherol depleted HPT: RNAi transgenic plants in any of the tested conditions, which would involve much stronger starch accumulation accompanied with a more pronounced accumulation of sucrose.

Concerning our amino acid analysis, characteristic changes in the composition of the free amino acid pool coincided with the accumulation of starch or soluble sugars (Figure 3-39 A - F). The content of the compatible solute proline was not statistically different in wild type and both transgenic plants (6.1 - 11.8 nmol per cm²). Under stress condition proline accumulated 7.36 to 11.76- fold and 1.12-15.65- fold in wild type, 0.89 to 11.75- fold and 7.72 to 13.83-fold in γTMT:RNAi and 1.55 to 12.75-fold and 11.83-14.41-fold in HPT:RNAi transgenic plants on 100 - 300mM sorbitol and NaCl, respectively, compared to the unsupplemented wild type control (Figure 3-39 A and D respectively), as did its precursor glutamate in salt stress. Proline accounted for 40% to 50% of the free amino acid pool when 300mM of either salt or sorbitol were supplied (compare values in Figure 3-39 A and D with 3-39 C and F).
Figure 3-39) Amino acid content and composition of leaves from HPT: RNAi and γ-TMT:RNAi and wild type tobacco plants after four weeks of salt and sorbitol stress.

Wild type (black bars); γ-TMT:RNAi (white bars) and HPT:RNAi (grey bars) tobacco plants were treated with varying amounts between 0 and 300 mM of sodium chloride (left column) or sorbitol (right column). Amino acids were extracted from leaves of wild type and transgenic plants 4 weeks after salt and sorbitol treatment and then measured by HPLC. The contents of proline (A and D), the total amino acid minus proline (B and E), and total amino acid content (C and F), were assayed from leaf samples after four weeks of stress. The numbers below the diagrams indicate the following conditions: 1; 0 mM, 2; 100 mM, 3; 200 mM, 4; 300 mM of either NaCl (left) or sorbitol (right panel). The depicted data are means ± standard deviation of four independent measurements.
Table 3-19) Amino acid content and composition of leaves from HPT: RNAi and γ-TMT: RNAi and wild type tobacco plants after four weeks of salt stress

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WT (nmol per cm²)</th>
<th>TMT (nmol per cm²)</th>
<th>HPT (nmol per cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>100</td>
<td>200</td>
</tr>
<tr>
<td>Asp</td>
<td>1.3±</td>
<td>4.4±</td>
<td>2.3±</td>
</tr>
<tr>
<td>Glu</td>
<td>2.3±</td>
<td>8.6±</td>
<td>8.8±</td>
</tr>
<tr>
<td>Asn</td>
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<td>15.6±</td>
<td>16.8±</td>
</tr>
<tr>
<td>Ser</td>
<td>10.9±</td>
<td>5.9±</td>
<td>13.6±</td>
</tr>
<tr>
<td>Gin</td>
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<td>18.7±</td>
<td>20.3±</td>
</tr>
<tr>
<td>Gly</td>
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<td>7.0±</td>
<td>1.6±</td>
</tr>
<tr>
<td>Arg</td>
<td>4.6±</td>
<td>1.0±</td>
<td>2.2±</td>
</tr>
<tr>
<td>Thr</td>
<td>3.6±</td>
<td>3.0±</td>
<td>4.4±</td>
</tr>
<tr>
<td>Ala</td>
<td>3.1±</td>
<td>1.9±</td>
<td>4.0±</td>
</tr>
<tr>
<td>Tyr</td>
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<td>0.3±</td>
<td>0.4±</td>
</tr>
<tr>
<td>Val</td>
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<td>0.8±</td>
<td>1.1±</td>
</tr>
<tr>
<td>Lys</td>
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<td>0.5±</td>
<td>0.6±</td>
</tr>
<tr>
<td>Leu</td>
<td>0.3±</td>
<td>0.4±</td>
<td>0.2±</td>
</tr>
<tr>
<td>Ile</td>
<td>0.5±</td>
<td>0.5±</td>
<td>0.9±</td>
</tr>
<tr>
<td>Phe</td>
<td>0.5±</td>
<td>0.6±</td>
<td>0.6±</td>
</tr>
<tr>
<td>His</td>
<td>0.4±</td>
<td>0.2±</td>
<td>0.9±</td>
</tr>
<tr>
<td>Met</td>
<td>1.0±</td>
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<td>1.2±</td>
</tr>
</tbody>
</table>

Wild type, γ-TMT: RNAi and HPT: RNAi tobacco plants were treated with varying amounts between 0 and 300 mM of sodium chloride. Amino acids were extracted from leaves of wild type and transgenic plants four weeks after sorbitol treatment and measured by HPLC. The data are given as nmol per cm². The depicted data are means ± standard deviation of four independent measurements.

It is noteworthy that 1.47- to 3.24-fold, 1.43- to 2.23-fold and 2.23- to 2.99-fold accumulation of total amino acid in wild type, γTMT:RNAi and HPT:RNAi transgenic lines in comparison to the wild type under control condition could be observed, respectively, when 100-300 mM sodium chloride were supplied, while 0.98 to 1.9-fold, 1.02 to 1.89-fold and 0.56 to 2.08-fold accumulation was measured in wild type, γTMT:RNAi and HPT:RNAi transgenic lines compared to the wild type under control condition when 100-300mM sorbitol were supplied (compare figure 3-39 C and 3-39F).

Apart from this indicative amino acid, we also observed correlations between the
accumulations of asparagine, glutamine, histidine and most branched-chain and aromatic amino acids with hexose contents in increasing sorbitol stress (Table 3-20). Almost the same subset of amino acids accumulated four to ninefold when soluble sugar and starch levels increased dramatically in the wild type on 400mM NaCl, while the transgenic plants did not accumulate these amino acids (Table 3-19). In contrast, the transgenic plants did not show pronounced differences in amino acid contents to the wild type in sorbitol stress in general, with γ-TMT: RNAi plants being indistinguishable from the wild type (Table 3-20).

### Table 3-20 Amino acid content and composition of leaves from HPT: RNAi and γ-TMT: RNAi and wild type tobacco plants after four weeks of sorbitol stress

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>WT (nmol per cm²)</th>
<th>TMT (nmol per cm²)</th>
<th>HPT (nmol per cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
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<td>200</td>
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<tr>
<td>Asp</td>
<td>1.3 ± 0.2</td>
<td>1.0 ± 0.2</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>Glu</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
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</tr>
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<td>0.5 ± 0.1</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>Val</td>
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<tr>
<td>Lys</td>
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<tr>
<td>Leu</td>
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<td>1.0 ± 0.2</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>Ile</td>
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<tr>
<td>Phe</td>
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<tr>
<td>His</td>
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<tr>
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</tr>
</tbody>
</table>

Wild type, γ-TMT: RNAi and HPT: RNAi tobacco plants were treated with varying amounts between 0 and 300 mM of sorbitol. Amino acids were extracted from leaves of wild type and transgenic plants four weeks after sorbitol treatment and measured by HPLC. The data are given as nmol per cm². The depicted data are means ± standard deviation of four independent measurements.
3.4. **Inducible silencing of tocopherol biosynthesis genes in tobacco plants**

Down-regulation of the gene of interest under control of the cauliflower mosaic virus 35S (CaMV 35S) promoter by RNAi is a powerful tool to elucidate gene function in plants. However, the constitutive expression or silencing of target gene often leads to pleiotropic effects and thus masks true gene functions. No phenotypic alteration in transgenic TC:RNAi and γTMT:RNAi tobacco plants might be due to the physiological adaptation to the manipulation of a metabolic function, therefore it is difficult to dissect the consequences of the gene silencing. To overcome these limitations, several inducible gene-switch systems have been developed in plants during the last decade (Aoyama and Chua, 1997; Bohner et al., 1999; Zuo and Chua, 2000; Zuo et al., 2006). The ethanol inducible gene switch (alc) is based on a regulon derived from the filamentous fungus *Aspergillus nidulans* (Caddick et al., 1998; Roslan et al., 2001; Chen et al., 2003).

This system consists of a modified alcA promoter, which controls the gene of interest and the alcR gene, which encodes a protein that can bind to the alcA promoter and activate the transcription from the alcA promoter in the presence of ethanol. Several ways have been developed to effectively induce this system in plants, including root drenching (Caddick et al., 1998; Salter et al., 1998), leaf spraying (Salter et al., 1998) or exposing the plants to ethanol vapor (Sweetman et al., 2002). Since ethanol in concentrations necessary for induction are physiologically affable and exert only minimal physiological side effects, the alc system has been used to study carbon metabolism (Caddick et al., 1998; Junker et al., 2003; Junker et al., 2004).

Although tocopherol is powerful antioxidant, transgenic HPT:RNAi or TC:RNAi tobacco plants with more than 5% of wild type total tocopherol content were indistinguishable from wild type plants. One possible explanation might be the physiological adaptation of the constitutively silenced plants. In order to prevent such possible physiological adaptation in transgenic plants, which were constitutively silenced by TC:RNAi, HPT:RNAi and γTMT:RNAi, these genes (TC, HPT and γTMT) were transiently silenced by using the inducible Alc: RNAi system in tobacco plants.

3.4.1. **Plasmid construction and plant transformation**

In order to create the ethanol inducible RNAi construct for down-regulation of the expression of different genes of tocopherol biosynthesis, the HPT-RNAi and TMT-RNAi cassettes described above were excised from pUC-RNAi using the flanking *PstI*
restriction sites. These fragments were cloned into a pUC-based plasmid between a chimeric alcA promoter and a nos terminator (Caddick et al., 1998). The resulting alcA expression cassettes were subsequently inserted into the HindIII site of p35S: alcR vector, a derivative of pBin19 (Bevan, 1984) carrying the alcR gene from A. nidulans between the CaMV 35S promoter and the nos terminator (Caddick et al., 1998), to yield Alc: TMT: RNAi (3-40A) and Alc: HPT: RNAi (3-40B) constructs.

**Figure 3-40** Schematic representation of alc-γTMT, HPT and TC RNAi constructs

The γTMT-RNAi and HPT: RNAi cassettes were cloned into PUC vector between the chimeric alcA promoter (palcA::Δ35S and OCS terminator (OCS) to create intermediate PUC: Alc: TMT: RNAi and PUC: Alc: HPT: RNAi constructs. The alcA promoter (palcA::Δ35S) consists of the CaMV 35S minimal promoter (-31 to +5) fused at the TATA-box to upstream promoter sequences of alcA (Caddick et al., 1998). These construct were digested by HindIII enzyme and were cloned into the pBin alcR vector downstream of the nos terminator (nos) to yield pBin: Alc: TMT: RNAi (A) and pBin: Alc: HPT: RNAi (B). The inducible TC:RNAi construct(C) had been previously created by Hofius et al. (personal communication).
3.4.2. Screening of putative transgenic plants

Tobacco plants were separately transformed by both mentioned constructs (pAlc: γTMT: RNAi and pAlc: HPT: RNAi) via Agrobacterium-mediated gene transfer. Sixty-two and forty-nine primary transformants were pre-screened on kanamycin for Alc: γTMT: RNAi and Alc: HPT: RNAi, respectively. All resistant plants were transferred to the greenhouse for further analyses. Following the transformation of tobacco plant with pAlc: TC: RNAi construct (Figure 3-40 C) via Agrobacterium-mediated gene transfer, sixty-five primary transformants have been pre-screened on kanamycin by Hofius et al (unpublished data). All resistant Alc: TC: RNAi seedlings were transferred to the greenhouse for further analyses.

3.4.3. Growth characterization of putative transformed plants after induction of silencing with ethanol

Seedlings of Alc: γTMT: RNAi, Alc: HPT: RNAi and Alc: TC: RNAi plants were selected for kanamycin resistance in tissue culture and transferred to the greenhouse. Four weeks after transfer to the greenhouse, transgenic lines and wild type plants were treated with one percent ethanol (1%). Before ethanol induction, all transgenic plants were indistinguishable from wild type plant. However, five to six days after ethanol induction a strong phenotypic alteration was observed in young leaves of Alc-TC: RNAi-7 and 18 transgenic lines (Figure 3-41A and B). Alc-TMT: RNAi plants transgenic lines 5, 22, 26, 41, 45, 48, 61 and 62 also displayed various degrees of phenotypic alteration (Figure 3-41D and E) five to six days after the ethanol induction in young leaves (which is comparable to the phenotype of Alc-TC: RNAi plants after ethanol induction Figure 3-41A and B ). Among the Alc-TMT: RNAi phenotypic plants, pAlc-TMT: RNAi-5 and 26 showed the strongest phenotype (Figure 3-41D and E). Alc-HPT: RNAi plants were also induced by ethanol but from 49 pre-screened lines, all transgenic lines were indistinguishable from wild type plant (not shown). The transgenic lines 18, 7, 25 and 62 for pAlc-TC: RNAi and lines 5, 22, 26 and 61 for pAlc-TMT: RNAi, which showed phenotypic alteration in young leaves as compared to the wild type plant, were selected for further analysis.
Results

3.4.4. Tocopherol analysis from transgenic pAlc-TC: RNAi and pAlc-γTMT: RNAi tobacco plants after ethanol induction

Seeds of transgenic lines 18, 7, 25 and 62 for pAlc-TC: RNAi and lines 5, 22, 26 and 61 for pAlc-TMT: RNAi were placed on kanamycin. The resistant seedlings were transferred to the greenhouse. Four weeks after transfer to the greenhouse, ethanol induction was carried out. Six days after ethanol induction, samples were taken from young leaves, which showed phenotypic alterations as compared to the wild-type control (Figure 3-42 and 3-43). Tocopherol was extracted from the phenotypic leaves and was analyzed by HPLC. Total tocopherol content and composition was measured in pAlc-γTMT: RNAi transgenic plants. Alc-γTMT: RNAi 26, 61, 5, 22 transgenic lines contained 90%, 99%, 109% and 114% α- tocopherol as compared to the wild type (Figure 3-42A). As indicated

Figure 3-41)-Phenotypic alteration of transgenic pAlc-TC: RNAi and pAlc-γTMT: RNAi tobacco plants after ethanol induction.

4 weeks after transfer to the greenhouse putative transgenic plants were induced with 100 ml, 1% ethanol, six days after treatment with ethanol, transgenic plants displayed phenotypic alteration in sink leaves compared to the wild-type plant. A, Alc-TC: RNAi-18; B, Alc-TC: RNAi-7; D, Alc-γTMT: RNAi-5; E, Alc-γTMT: RNAi-22; C and F, wild type tobacco plants six days after ethanol induction.
Results

in Figure 3-42B, Alc-γTMT: RNAi-26, 61, 5, 22 transgenic lines contained 90%, 101%, 111% and 116% of wild type tocopherol content. Tocopherol was also measured in phenotypic leaves of Alc-TC: RNAi transgenic lines and wild types. As indicated in Figure 3-48A, total tocopherol content in wild type and pAlc-TC: RNAi-7, 18, 25 and 62 transgenic lines was 113%, 107%, 90% and 97% of the wild type content, respectively. These results indicated that inducible silencing of TC and γTMT did not change tocopherol content and composition in leaves of transgenic plants (which displayed strong phenotypic alteration) 6 days after ethanol induction.

Figure 3-42)-Tocopherol analysis from transgenic pAlc-γTMT: RNAi tobacco plants after ethanol induction

Tocopherol content and composition were measured from phenotypic leaves pAlc-γTMT: RNAi transgenic lines and wild type leaves by HPLC. A, total tocopherol content of wild type and pAlc-γTMT: RNAi transgenic lines; B, α- tocopherol content of wild type and pAlc-γTMT: RNAi transgenic lines. Samples were taken 6 days after ethanol induction. The data are means of six wild type plants and five individual transgenic plants of each independent transgenic line ± standard deviation.

To further clarify the effect of inducible silencing of tocopherol cyclase on the tocopherol content of transgenic tobacco plants, seeds of pAlc-TC: RNAi-18, which showed the strongest phenotype after induction, were placed on kanamycin and the kanamycin resistant seedlings were transferred to the greenhouse. Transgenic and wild type plant were treated with ethanol and samples were taken from wild type and transgenic young leaves, which later would show the phenotypic effect, at different time points (0, 2, 5, 7, 9, and 11) after ethanol induction.
Transgenic and wild type plant were treated with ethanol and samples were taken from wild type and transgenic young leaves, which later would show the phenotypic effect, at different time points (0, 2, 5, 7, 9, and 11) after ethanol induction.

Figure 3-43) Tocopherol analysis from transgenic pAlc-TC: RNAi tobacco plants after ethanol induction

Tocopherol content was measured from phenotypic leaves of pAlc-TC: RNAi transgenic lines and wild type leaves (with the same age as transgenic leaves) by HPLC. A, total tocopherol content of wild type and pAlc-TC: RNAi transgenic lines, was measured in leaves of samples which were taken six days after ethanol induction. B, total tocopherol content in leaves of wild type and pAlc-TC: RNAi transgenic lines at different days after ethanol induction, samples were taken 0, 2, 5, 7, 9 and 11 days after ethanol induction. The data are means of six wild type plants and five individual transgenic plants of each independent transgenic line ± standard deviation.

As indicated in Figure 3-43 B, before the ethanol treatment, tocopherol content of transgenic plant was very similar to the wild-type plants. During the ethanol treatment, tocopherol content of wild type plant is slightly increased from 21.1 to 24.9 at 11 days after ethanol treatment. Whereas tocopherol content of Alc:TC: RNAi-18 transgenic line was increased from 21.52 to 27.78 at 11 days after ethanol induction. Nevertheless, the tocopherol content of Alc-TC: RNAi-18 transgenic was not statistically different from the wild type tocopherol content. These data indicate that although inducible silencing of TC leads to the development of a strong phenotype, it does not lead to alteration in tocopherol content and composition in phenotypic leaves of transgenic plants at any time point after ethanol induction. It should be mentioned that the phenotype, which was observed in T0, T1 and T2 generation of transgenic Alc:TC: RNAi and Alc:γTMT: RNAi tobacco plants, was not reproducible in third generation of both transgenic plants.
4. Discussion

Under natural conditions, plants are exposed to a variety of biotic and abiotic stresses, including pathogens, adverse temperature, drought, salt and high light. Under these stress conditions, reactive oxygen species (ROS) derived from molecular oxygen can accumulate in leaves resulting in the oxidation of cellular components, including proteins, chlorophyll and lipids. To cope with oxidative stress plants have evolved two general protective mechanisms, enzymatic and non-enzymatic detoxification. Non-enzymatic mechanisms are based on antioxidants including vitamin E, vitamin C, glutathione, carotenoids and flavonoids (Dat, 2000; Alscher, 2002), while enzymatic mechanisms include superoxide dismutase, catalase, ascorbate peroxidase and glutathione reductase (Bowler et al., 1990; Bowler et al., 1991; Dat, 2000).

Vitamin E, as a member of the antioxidative network, is considered as a group of eight structurally related tocopherols and tocotrienols, which differ in the degree of saturation of their hydrophobic prenyl chain and the degree of methylation of their chromonal ring. Sub-cellular distribution and enzymatic steps of vitamin E biosynthesis have been characterized in detail (for recent review see DellaPenna and Pogson, 2006). Recent attempts to identify the enzymatic steps involved in tocopherol biosynthesis lead to a number of different mutants and transgenic plants for this pathway (Porfirova et al., 2002; Savidge et al., 2002; Cheng et al., 2003; Van Eenennaam et al., 2003; Hofius et al., 2004). Vitamin E synthesis is restricted to photosynthetic organisms including plants, algae, and some cyanobacteria. Although tocopherols are specifically synthesized in photosynthetic organisms, most of our current knowledge concerning the antioxidant role of tocopherols originates from human studies.

4.1. The vitamin E composition is tissue-specific in plants

In plants, tocopherol composition differs between different species and between different tissues within one species (summarized in Table 2-2). In agreement with other reports, our attempt to investigate the composition of the tocopherol pools in tobacco wild type plants showed that it consists of more than 90% α- and less than 10% other vitamin E derivatives (either tocopherols or tocotrienols) in vegetative organs such as leaves (Table 3-4). These observations indicate a specific role for α-tocopherol in photosynthetic tissues. Therefore, α-tocopherol has been proposed to participate in the
detoxification of reactive oxygen species together with glutathione and ascorbate (Foyer and Noctor, 2003). Our results suggest that γ-tocopherol is even more potent in preventing ROS-dependent lipid peroxidation during oxidative stress than α-tocopherol (as shown in Figure 3-36).

Based on inhibitor studies it has been shown that tocopherol acts as singlet oxygen scavenger in photosystem II of Chlamydomonas reinhardtii (Trebst et al., 2002; Kruk et al., 2005). The inhibitory effect could be overcome by addition of membrane permeable short chain α- and γ- tocopherol derivatives suggesting that α-tocopherol can be substituted by γ-tocopherol in leaves. This view was further supported by the analysis of the Arabidopsis thaliana vte4-1 mutant deficient in γ-TMT activity (Bergmüller et al., 2003), which lacks the ability to convert α- to γ-tocopherol. Instead of α-tocopherol, vte4-1 mutants accumulate comparable amounts of γ-tocopherol (Bergmüller et al., 2003). Photosynthetic parameters in vte4-1 were found to be indistinguishable from wild type under a variety of stress conditions. In contrast to leaves, we found more than 95 % γ-tocopherol and few percentage of α- and δ- tocopherol in seeds of tobacco plants (Table 3-4). These data exemplify a predominance of γ-tocopherol in reproductive organs of tobacco plants, which is in agreement with other observations that showed γ-tocopherol is dominant in seeds of most dicot plants. In contrast to tocopherols, tocotrienols are the predominant Vitamin E species in monocot seeds (Cahoon et al., 2003; Panfili et al., 2003).

In oil-storing seeds like Arabidopsis, γ-tocopherol has been shown to protect polyunsaturated fatty acids (PUFAs) from oxidation, thereby increasing seed longevity (Sattler et al., 2004). Likewise, γ-tocopherol might also be involved in desiccation tolerance of seeds. With the isolation of a phytol phosphate kinase from Arabidopsis, it has recently been demonstrated that the prenyl moiety for γ-tocopherol biosynthesis in seeds could be derived from phytol recycling (Valentin et al., 2006).

Tocotrienols are abundant in leaves of cereals (Reference!), while they are not detectable in leaves of most dicots like Arabidopsis (Cahoon et al., 2003) or tobacco (Rippert et al., 2004), where tocopherols prevail. In our study, only tocopherols were detected in leaves of Arabidopsis and tobacco plants (Figures 3-7A, 3-13C, 3-25C and Figure 3-28B), while additional peaks were obtained in the HPLC chromatogram from tobacco seed extracts. These peaks could refer to tocotrienols with high probability (not shown). In agreement with other reports (Falk et al., 2003), these data indicate that both tocopherol (mainly γ-tocopherol 95% of detected tocopherol) and tocotrienols accumulate in tobacco seeds.
4.2. **Arabidopsis vte1 mutant is complemented with tocopherol cyclase**

*Arabidopsis* vte1 mutants have been created through ethyl methanesulfonate mutagenesis in *Arabidopsis thaliana* (ecotype Col-2) and were identified by screening the M2 plants for lipid content under stress condition. *Arabidopsis vte1* mutants have been four times backcrossed to wild type Col-2 to reduce the number of background mutations. This mutant, which lacks all forms of tocopherol, was transformed with tocopherol cyclase fused to either GFP or tandem affinity purification tag (TAP-Tag).

Likewise, Sattler et al., (2003) showed that expression of maize tocopherol cyclase is sufficient to complement the tocopherol-deficient phenotype of the *Synechocystis sp. PCC6803 slr1737* deletion mutant.

Kanwischer et al., (2005) showed that transformation of the vte1 mutant with a genomic or cDNA fragment of tocopherol cyclase under the control of the endogenous tocopherol cyclase promoter or strong constitutive 35S promoter could increase tocopherol content in transgenic plants to wild type tocopherol content.

Transformation of the above mentioned vte1 mutant with both, TC-GFP (Figure 3-2) and the TC-TAP-Tag (Figure 3-5), constructs under the control of the strong constitutive 35S promoter dramatically increased the tocopherol content to levels comparable to wild type tocopherol content (see Figures 3-4e, 3-7 and 3-8). An additional result supporting the complementation is that a 66-kDa (54-kDa protein after TEV protease digestion) was detected in protein blots of transgenic plants (see Figures 3-10A and 3-10B) corresponding to the size of TC fused to TAP-Tag.

As an ideal affinity purification tag should not affect the expression, localization, stability, or function of the target protein, the functional complementation of tocopherol cyclase activity by overexpression of tocopherol cyclase either fused to GFP or to a TAP-Tag in the vte1 mutant background demonstrate that these features also hold true for our approach.

4.3. **Looking for interacting partners of Tocopherol cyclase**

Proteins control and mediate many biological activities of the cell. Although some proteins act primarily as monomers, a significant percentage of all proteins are assumed to function in association with interaction partners, or as components of larger protein assemblies. Thus, to obtain a thorough understanding of cellular role, the function of proteins must be understood in context of other interactive proteins. There are different approaches to identify and characterize protein-protein interactions (Reference: Phizicky
and Fields, 1995). One of the most popular and powerful approaches is the yeast two-hybrid system which identifies protein-protein interactions solely on a genetic basis using yeast as a host cell (Fields and Song, 1989). Due to the intrinsic limitations of the yeast two-hybrid system, complementary approaches have been developed over the past years. One such approach is protein-complex affinity purification coupled to mass spectrometry (AP-MS) (Bauer and Küster, 2003). Here, a protein of interest is translationally fused to an affinity tag and subsequently ectopically expressed in a host cell. The protein is then purified from cell extracts and co-purifying proteins are separated on an SDS-gel and can be identified using MS. One of the most widespread used affinity tags for AP-MS is the tandem-affinity-purification tag (TAP-Tag) (Rigaut et al. 1999) which allows affinity purification of fusion proteins in a two-step protocol. This increases purity and reduces the co-purification of unspecifically interacting proteins. Rohila et al. (2004) and Witte et al. (2004) separately showed the utility of a TAP-Tag strategy to study protein-protein interaction in plant systems.

Although several bands were observed on one and two dimensional gel electrophoresis following the colloidal Coomassie brilliant blue staining and silver staining, respectively (Figure 3-11A and 3-11B) and several proteins was detected by peptide analysis of these visualized bands (Table 3-3A and Table 3-3B), no protein which was identified either by Matrix-assisted laser desorption ionization-time of the flight (MALDI-TOF) or by Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS) could be an interacting partner for tocopherol cyclase because:

- No protein identified as a putative interacting protein in this study (Table 3-4) was in agreement with the previous data from a yeast two-hybrid approach (Table 3-1, data from Hofius et al., personal communication).

- No protein found in our analysis was platsidic while tocopherol cyclase is located into the chloroplast.

- No protein detected in mass spectrometry was identified as tocopherol cyclase.

Purification of complex protein via the TAP-Tag strategy required at least five hours due to the combination of two purification steps and an intermediate protease cleavage step, which takes two hours. These 7 hours of purification likely increase the risk of protein degradation, loss of interacting proteins or alteration of informative post-translational modifications.

The absence of inhibitors in complex protein purification may cause degradation problems during the incubation with TEV protease at 16°C, due to the action of co-purified plant proteases. Although Rohila et al., (2004) indicated that the inclusion of the
cysteine protease inhibitor E-64 improved the stability of a TAP-tagged protein during TEV protease cleavage, the general utility of this inhibitor for different proteins remains to be shown.

Witte et al., (2004) transiently transformed *Nicotiana benthamina* with AtSGT1 fused to a TAP-tag or a StreptII-tag. The authors showed that the efficiency of purification of the AtSGT1b protein fused to StreptII-tag is slightly superior to that fused to TAP-tag in *Nicotiana benthamina*. The lower efficiency during TAP-tag purification could be due to degradation during TEV protease cleavage, incomplete release of protein from the IgG column and the combination of two purification steps although increase the purity of the final complex, could reduce overall recovery.

Although a protein of about 54 kDa was detected in protein blots in purified complex protein of transgenic corresponding to the size of tocopherol cyclase and calmodulin binding peptide (approximately 46 kDa for tocopherol cyclase and 8 kDa for calmodulin binding peptide), we failed to detect tocopherol cyclase following the peptide analysis, one explanation for that might be the occurrence of post translational modification in tocopherol cyclase. As in our peptide analysis experiment, we only considered the phosphorilation modification, therefore one could check the possible modification, which might occur in tocopherol cyclase and then consider them during the peptide analysis.

Since protein cross-linking has been recently shown to identify interacting proteins that either were not detected or weakly detected without prior cross-linking in immunoprecipitation experiments (Hall and Struhl, 2002; Rohila et al., 2004), to continue this experiment, one also could investigate the utility of formaldehyde protein cross-linking in intact tissues prior to making the protein extract.

As reviewed by Trepe, (2003) several affinity tags are exist for purification of the complex protein (Terpe, 2003), therefore one could also use another single or multiple affinity tag system which is suitable for plant system.

4.4. **Constitutive silencing of the tocopherol biosynthetic pathway in transgenic tobacco plants**

To study gene function in biosynthetic pathways by reverse genetic approaches is a powerful method. Several reverse genetic approaches have been developed, including Knockout and Knockdown. In Knockout approach, a set of plants with decreased expression of the enzyme of interest must be created either by using T-DNA, gamma radiation or by chemical-induced mutants. Using KO-line only two kinds of mutants can
be generated, hemizygous mutants with 50% reduction of the interested enzyme and homozygous mutants with 100% inhibition. Knockdown strategy is an alternative reverse genetic approach, which allows obtaining a varying degree of inhibition in the expression of a gene of interest. In Knockdown approach the gene of interest will be downregulated by antisense, (van der Krol et al., 1988; Napoli et al., 1990; Smith et al., 1990), a MicroRNAi or RNA-interference (RNAi) technology (Fire et al., 1998; Waterhouse et al., 1998; Chuang and Meyerowitz, 2000). Because of the advantage of the RNAi approaches to obtain a varying degree of inhibition, different genes of tocopherol biosynthesis such as HPT, TC and γ-TMT were silenced to obtain transgenic plants containing altered content and composition of tocopherol.

4.5. **Constitutive silencing of TC results in tocopherol deficiency in tobacco leaves**

Tocopherol cyclase (TC) is one of the enzymes in tocopherol biosynthesis, which adds a second ring at the junction between the aromatic head group and the prenyl tail to create a two-ring structure which is known as chromanol ring (Figure 1-6). Tocopherol cyclase can catalyze the formation of β- and γ-tocopherol from the substrates MPBQ and DMPBQ, respectively, (Soll and Schultz, 1979; Soll et al., 1980; Soll and Schultz, 1981). The tocopherol cyclase (TC) gene has recently been cloned from *Synechocystis sp.* PCC6803 (slr1737), *Arabidopsis thaliana* (VTE1), maize (SXD1) and potato (Sattler et al. 2003; Porfirova et al. 2002; Provencher et al. 2001; Hofius et al., 2004, respectively), and TC deficient *Arabidopsis*, maize and potato plants have been isolated: (i) Porfirova et al., (2002) isolated the *Arabidopsis vte1* mutant while screening EMS mutant pools for lipid content. The *Arabidopsis vte1* mutant was completely devoid of all four forms of tocopherol. Additional *vte1* mutant alleles from *Arabidopsis* were identified from another ethyl methanesulfonate (EMS)-mutagenized population by an HPLC-based screening for reduced Vitamin E contents in leaves (Sattler et al., 2003). Although tocopherols were absent in *vte1-1* seeds, *vte1-2* seeds contained approximately 25% of the tocopherol level in wild type seeds suggesting that *vte1-2* is a weaker allele than *vte1-1*.

(ii) Isolating a maize *sxd1-2* mutant through the Trait Utility System for Corn, Sattler et al., (2003) showed that leaves of both maize *sxd1-2* mutants (which were separately described by Provencher et al., 2001 and Sattler et al., 2003) lack all kind of tocopherols.
(iii) Hofius et al. (2004) showed that silencing of tocopherol cyclase in potato plants by dsRNAi results in a nearly complete depletion of tocopherol in some transgenic potato lines.
(iv) An insertional mutant was created in the slr1737 open reading frame (ORF) of *Synechocystis* sp. PCC6803 through homologous recombination. The SLR1737 protein shows considerable amount of sequence homology to tocopherol cyclase in plants. Like *vte1* mutant in plants, slr1737 mutants lacked all tocopherol derivatives (Sattler et al., 2003).

In the present attempt, tocopherol cyclase gene expression was inhibited in tobacco plants using an RNAi construct under the control of the 35S promoter (Figure 3-28A), which was created with a partial EST fragment of TC of potato. Silencing of TC in tobacco plants resulted in 90% reduction of tocopherol content in leaves of transgenic plants compared to the wild type (Figures 3-28B and 3-29).

Reduction of tocopherol biosynthesis by silencing TC was in agreement with the previous results described above, which showed tocopherol reduction following mutation or silencing of TC.

Although numerous primary transformants (82 lines) were created following silencing of TC in tobacco plants (Figure 3-29), the highest tocopherol deficiency, which was observed in transgenic lines TC-73, TC-54 and TC-45 was less than 10% of wild type tocopherol content. This relatively low efficiency of silencing might be explained, because tocopherol cyclase in potato plants is not completely homologous to TC in tobacco plants. Except of tocopherol content, transgenic TC:RNAi tobacco plants were indistinguishable from the wild type throughout development under optimal growth condition.

4.6. **Constitutive silencing of HPT severely decreased the tocopherol content in leaves of transgenic tobacco plants**

The first committed steps in tocopherol and tocotrienol biosynthesis are the prenylation of homogentisic acid by either phytyldiphosphate or geranylgeranyl-diphosphate yielding 2-methyl-6-phytyl-benzoquinone (MPBQ) or methyl geranylgeranyl benzoquinone (MGGBQ), respectively. HPT catalyses the condensation of HGA with PDP at the entry site of tocopherol biosynthesis while, HGGT links HGA and GGDP at the top of the tocotrienol branch (Cahoon et al., 2003; Figure 2-6).
Savidge et al., (2002) created a HPT null mutant in *Synechocystis* sp. PCC 6803, by inserting the *nptI* gene into the *slr1736* ORF. Tocopherol contents in the mutant were below five ng per mg dry cell mass, which is below the detection limit of conventional HPLC techniques. Sattler et al., (2004) showed that *Arabidopsis vte2* mutants (*vte2-1* and *vte2-2*) lacked tocopherols in seed and leaf tissue. However, Havaux et al., (2005) showed that no tocopherol was measured in leaves of a weak allele of *vte2* in low light, but that these *vte2* leaves contained a small amount of tocopherols (6% of the tocopherol amount accumulated in wild-type leaves) when grown in high light at low temperature.

In the present study, constitutive silencing of the tobacco HPT gene using an *StHPT:RNAi* cassette under the control of the 35S promoter (Figure 3-12) reduced the total tocopherol content in leaves of transgenic primary transformants to a varying degree from zero to 98% compared to the wild type (Figure 3-13). This tocopherol deficiency was in agreement with the results of the mutation of HPT in *Arabidopsis* (Sattler et al., 2004). Although strong tocopherol deficiency was observed in vegetative tissues, tocopherol content in seeds of transgenic plants was only slightly influenced by HPT:RNAi silencing, which was in contrast to the *vte2* *Arabidopsis* mutants.

The *CaMV* 35S promoter is the most widely used promoter for plant (dicots and monocots) transformations. Although it is considered to be active in all tissues (Odell et al., 1985), some reports suggest that it is not expressed in all cell and tissue types, like e.g. during embryonic development (Benfey and Chua, 1989; Terada and Shimamoto, 1990; Ganesan et al., 2002), providing an explanation for the abundance of tocopherols in HPT:RNAi seeds.

The HPT silenced tobacco plants do not accumulate any tocopherol pathway intermediates (see Figures 3-13C and 3-14C). The strong inhibition in tocopherol biosynthetic capacity is paralleled by an obvious growth reduction (Table 3-8), and phenotypic alteration (bleaching and chlorosis phenotype see Figure 3-15) in lower source leaves of transgenic plants compared to the wild type.

In addition, silencing of HPT did not disturb plastoquinone (PQ) biosynthesis (Figure 3-16), which occurred when earlier conversions in the pathway, e.g. *p*-hydroxyphenylpyruvate dioxygenase (HPPD) were compromised, inevitably leading to a deleterious deficiency in the electron carrier PQ, which is indispensable for carotenoid biosynthesis (Norris et al., 1995).
4.7. **Constitutive silencing of γTMT in transgenic tobacco plants alters tocopherol composition in leaves**

Gamma-tocopherol methyltransferase (γ-TMT) is the final enzyme in tocopherol biosynthesis. Gamma-TMT adds a methyl group to the sixth position of the chromanol ring, which converts γ- and β-tocochromanols to α- and δ-tocochromanols, respectively. Gamma-tocopherol methyltransferase was discovered in *Synechocystis* because of its genetic proximity to SLR0090, the *Synechocystis* HPPD homologue (Shintani and DellaPenna 1998). Disruption of the SLR0089 gene in *Synechocystis* by homologous recombination, resulted in the loss of α-tocopherol and accumulation of γ-tocopherol.

Two γ-TMT deficient *vte4* mutants were isolated in *Arabidopsis* (Bergmuller et al., 2003). The *vte4-1* mutant was isolated via a TLC-based screening of a mutant population created by EMS mutagenesis, while *vte4-2* was isolated from a T-DNA insertion mutant population, containing a T-DNA insertion in the third exon of the coding sequence. In leaves of *vte4-1* and *vte4-2* mutants, α-tocopherol and β-tocopherol were absent, while γ-tocopherol and, to a lesser extent, δ-tocopherol accumulated. Total amount of tocopherol in mutant leaves did not differ from wild type.

Mutant with decreased γ-TMT activity have also been isolated from sunflower (*Helianthus annuus*) which led to γ-tocopherol accumulation instead of α-tocopherol. Disruption of the transcriptional activity of both sunflower γ-TMT genes resulted in 10% residual α-tocopherol content in sunflower seeds (Hass et al., 2006).

In the presented work, the γ-TMT gene was silenced in tobacco plants using a dsRNAi construct bearing a potato γ-TMT fragment under control of the 3SS promoter. Following this gene silencing, we achieved an almost quantitative substitution of γ- for α-tocopherol with as few as 5% of foliar α-tocopherol left compared to wild type (Figures 3-26 and 3-27). Although total tocopherol contents in some transgenic plants was increased (Figure 3-26C), the total amount of tocopherol was not very different from wild type in leaves of the strongest transgenic lines and most of the other transgenic plants. Our results are in agreement with the cited previous studies on γ-TMT deficient *Arabidopsis*, sunflower and *Synechocystis* mutants.

4.8. **Strong tocopherol deficiency can induce carbohydrate accumulation**

Previous studies on mutant and transgenic plants, which lack tocopherols due to deficiencies in tocopherol cyclase (TC) or homogentisate phytol transferase (HPT) resulted in an alteration of carbohydrate metabolism. Mutation of TC in maize plants...
creates a sucrose export deficient phenotype in leaves (Provencher et al., 2001), resulting in starch and total soluble sugar accumulation. In contrast to maize, the Arabidopsis TC mutant vte1, which is devoid of tocopherols, did not exhibit altered carbohydrate content in leaves compared to the wild type. Later on silencing of TC in potato plants by dsRNAi strategy resulted in transgenic potato plants, lacking all tocopherol derivatives, consistent with Arabidopsis vte1 mutants. As in the maize sxd1 mutant, potato plants also exhibited the accumulation of starch and soluble sugar in lower source leaves of transgenic plants as compared to the wild type. Callose contents were also elevated in SXD1: RNAi potato plants and in maize sxd1 mutants. Recently, Maeda et al., (2006) showed that although Arabidopsis vte2 mutants did not exhibit any carbohydrate accumulation under standard growth conditions, vte2 mutants exhibit carbohydrate and callose accumulation compared to the wild type under low temperature conditions. All together, these data suggest the presence of a carbohydrate export block phenotype in tocopherol deficient plants.

Total soluble sugar measurements in our study showed 1- to 4- fold increase in total soluble sugar (glucose, fructose and sucrose) in lower source leaves of transgenic HPT:RNAi tobacco plants compared to the wild type under normal growth conditions in the greenhouse (Table 3-10 and Figure 3-18), which is in agreement with the previous reports on the accumulation of total soluble sugar in the sxd1 maize mutant and SXD1:RNAi transgenic potato plants. Although we expected the same accumulation as soluble sugar for starch contents, starch remained constant or even decreased in lower source leaves of tobacco HPT: RNAi transgenic plants in comparison to the wild type under optimal growth conditions in the greenhouse. Concomitantly, callose did not accumulate in phenotypic source leaves of HPT: RNAi transgenic plants, which show sugar accumulation. The starch and callose data (Figure 3-17) are in disagreement with the previous reports, which could be explained by two ways: First, the tocopherol deficient maize sxd1 mutants and SXD1: RNAi transgenic potato plants, are deficient in tocopherol cyclase whereas our HPT:RNAi transgenic plants, which did not exhibit starch accumulation, were created by the silencing HPT. This could prevent accumulation of the pathway intermediate MPBQ.

Second, we used tobacco plants for silencing HPT while the other reports are on other plant species like Arabidopsis, maize or potato, respectively (Maeda et al., 2006; Provencher et al., 2001; Hofius et al., 2004). The different effect of tocopherol deficiency on starch accumulation in lower source leaves of SXD1: RNAi transgenic potato, maize
sxdl and HPT:RNAi tobacco transgenic plants studied here might be plant species specific.

Pilon-Smits et al., (1995) showed that total soluble sugar (glucose, fructose, and sucrose) increased 2- to 3.5-fold in wild type plants under drought stress, which was imposed by adding PEG, while drought stress did not have any influence on starch level of the wild type compared to the control condition (Pilon-Smits et al., 1995). Vassy and Sharkey (1989) showed that starch content was 57% of fixed carbon in control plants while mild water stress (-1.0 MPa xylem water potential) led to reduction in starch formation (16% of fixed carbon) (Vassey and Sharkey, 1989). Sucrose content was 39% and 42% of fixed carbon and the ratio of starch to sucrose was about 1.4 and 0.4 under control and mild water stress conditions in leaves of Phaseolus vulgaris cv Linden plants. Lofta et al., (1995) subjected two potato cultivars (Norchip and Up-to-Date) to high temperature (31/29 °C day/night) and they observed that 8 days after treatment, soluble sugar (sucrose and glucose) were increased 1.5-fold (15.8 mg per gram dry weight) in both cultivars compared to the controls (Lafta and Lorenzen, 1995).

Our data also showed considerable increase (1- to 4-fold increase) in total soluble sugar while nearly no obvious differences were observed in starch content of the leaves of transgenic compared to wild type plants (Figure 3-17B). The ratio of starch to sucrose was 1.7 in wild type plants whereas it was 0.3, 0.2, 0.4 and 1.3 in H-28, H-49, H-44 and H-39 transgenic tobacco plants, respectively (Figure 3-17B, Figure 3-18 and Table 3-10). The strongest HPT:RNAi lines show a reduction in the biomass either in control condition (Table 3-8) or under 14-days drought stress (data not shown).

The sugar and starch data obtained in our study for HPT transgenics are in agreement with the accumulation of soluble sugar and decrement of starch in tobacco, potato and phaseolus plants under drought, high temperature and mild water stress conditions cited above. Therefore, it can be hypothesized that HPT transgenic plants suffer somehow from stress in standard growth conditions already, imposing the necessity for soluble sugar accumulation.

Numerous studies have provided strong evidence for the role of the vacuole in the control of cell volume and cell turgor; the regulation of cytoplasmic ions and pH; the storage of amino acids, sugars, and CO₂; and the sequestration of toxic ions and xenobiotics (Sze et al., 1992; Barkla and Pantoja, 1996; Rea et al., 1998).

Additional evidence for the occurrence of stress in the HPT:RNAi transgenics, is provided by a 2-fold increase of proline contents in source leaves of H-28 and H-49 transgenic lines compared to wild type plants (3-19C). Proline has been shown to
function as a protective compatible osmolyte and to accumulate in response to osmotic stress conditions stimulated by salt, cold, drought, and abscisic acid (Hare et al., 1999; Hasegawa et al., 2000), as well as by elevated hexose contents (Bussis et al., 1997). In response to an elevated water stress and maintain the cell turgor, transgenic plants would increase the soluble sugar and proline content compared to the wild type. The accumulated soluble sugar and amino acid will be transported to the vacuole.

4.9. Strong tocopherol deficiency decreased photosynthetic capacity

Photosynthesis is a biological process that harvests solar energy for the formation of chemical bonds. Although light is the energy source for photosynthetic organisms, excessive light (as shown in Figure 4-1) can produce reactive oxygen species which finally lead to oxidative damage of the photosynthetic apparatus (Powles, 1984). Light energy, which is absorbed by chlorophyll, results in singlet-state excitation of Chl-a molecule (\(1\text{chl}^*\)). As shown in Figure 4-1, singlet excited molecule (\(1\text{chl}^*\)) can return to the ground state chlorophyll by several pathways. Excitation energy can be (i) transferred to reaction centers and used to drive photosynthesis, (ii) can be liberated by heat dissipation processes, can be (iii) re-emitted as chlorophyll fluorescence or it can (iv) decay via the triplet state of chlorophyll molecule (\(3\text{chl}^*\)). Four to 25% of excited chlorophyll molecules are converted to the triplet state (\(3\text{chl}^*\)) by intersystem crossing (Harbinson and Foyer, 1991). The \(3\text{chl}^*\) in turn is able to produce singlet oxygen (Asada, 1994; Foyer et al., 1994).

As most plants receive more light than they can utilize for photosynthesis, they have developed different strategies to control unavoidable excess excitation. The formation of ROS can be controlled by several mechanisms such as, dissipation of the energy as heat (Demmig-Adams and Adams, 1996; Niyogi et al., 1998), the increase in electron-consuming pathways like photorespiration (Kozaki and Takeba, 1996), cyclic electron flow around photosystem II (Heber and Walker, 1992), and enzymatic mechanisms involving antioxidants (tocopherol, ascorbate and glutathione) and (Fryer, 1992; Asada, 1994; Foyer et al., 1994; Niyogi et al., 2001).
Chlorophyll is excited from its ground state to the singlet-excited state. This excitation can be used for photosynthesis, can be dissipated as heat or can be re-emitted as chlorophyll fluorescence. The \(^1\text{Chl}\) molecule can also be converted to the ground state molecule via \(^3\text{Chl}\) and reactive oxygen species (Muller et al., 2001).

Several mutants with a reduced non-photochemical energy dissipation capacity (Niyogi, 1999), reduced ascorbate (Conklin et al., 1997), diminished tocopherol (Grasses et al., 2001; Porfirova et al., 2002; Collakova and DellaPenna, 2003; Sattler et al., 2003; Hofius et al., 2004; Maeda et al., 2006) and decreased glutathione (Cobbett et al., 1998) have been identified in higher plants. Although one would expect a reduction in photosynthetic capacity following a mutation in these genes, most of these mutants did not show notable photosynthetic differences in comparison to the wild type plants under optimal growth conditions (Grasses et al., 2001; Porfirova et al., 2002; Havaux et al., 2003; Maeda et al., 2006).

Our photosynthetic data showed that more than 95% reduction in tocopherol content in HPT:RNAi transgenic plants compared to wild type was paralleled by a 25% - 58% reduction of photosynthetic capacity in transgenic HPT:RNAi tobacco plants compared to the wild type under greenhouse conditions (Figure 3-21B). Additionally, a reduction in CO\(_2\) assimilation rate at any photon flux density (PPFD 50-2000 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) indicated that light utilization was significantly affected in source leaves of transgenic plants even under very low photon flux density (PPFD 50 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\)) compared to the wild type (Figure 3-21A). These results indicate a direct or indirect role...
of tocopherol in photosynthetic performance under greenhouse conditions. Our data are in contrast to the previous results obtained for *Arabidopsis vte1* and *vte2* mutants (Porfirova et al., 2002, Collakova and DellaPenna, 2001) and partially tocopherol deficient tobacco plants (Grasses et al., 2001), which did not show any differences in photosynthetic capacity compared to the wild type under optimal growth conditions. However, potato plants silenced for TC showed a reduction in photosynthetic capacity that was attributed to an inhibition of photosynthesis by a carbohydrate mediated feedback effect in lower source leaves of transgenic TC:RNAi potato plants that accumulated large amounts of soluble sugars in addition to starch (Hofius et al., 2004). The observed differences could be due to (i) the plant system under study - tobacco instead of *Arabidopsis* (Porfirova et al., 2002, Collakova and DellaPenna, 2001) or potato (Hofius et al., 2004), (ii) or the different gene which was affected (HPT versus TC in Porfirova et al., 2002 and Hofius et al., 2004) or (iii) the amount of residual tocopherol in our plants (less than 5% in HPT:RNAi tobacco) compared to transgenic chlP tobacco plants (40%-50% of wild type tocopherol - Grasses et al., 2001).

As both senescence-associated (see Figure 3-22) and stress-associated phenotypes (see Figure 4-1) were observed for HPT:RNAi plants, changes in PSII photochemistry in leaves of HPT:RNAi transgenic plants during leaf aging were examined under greenhouse conditions. The fluorescence characteristics during the steady state of photosynthesis were investigated by fluorescence imaging with a walz imaging PAM instrument. In addition, photochemical quenching (qP) decreased in source leaves of transgenic HPT:RNAi tobacco plants compared to wild type. Non-regulated non-photochemical quenching (NO) was increased 31% - 48% in leaves (young source, source and old source leaves) of transgenic H-28 and H-49 tobacco plants compared to wild type (Figure 3-22C).

The observed reduction in photochemical quenching (qP) in HPT:RNAi tobacco plants compared to the wild type was also recorded in partially tocopherol deficient tobacco plants under high light stress conditions (Grasses et al., 2001) and in *Arabidopsis vtc-1* mutant under salt stress conditions (Chenghong et al., 2005). This decrease in qP could indicate an increase of reduced QA donor sites in photosystem II (Genty et al., 1989), indicating an elevated saturation of the photosynthetic electron transport chain in the transgenics.

An increase in the excitation pressure on PSII in the steady-state of photosynthesis (Öquist and Huner, 1993) could result in further damage to PSII if excess excitation pressure was not dissipated safely, since the excitation pressure on PSII has been
shown to be a determining factor for photodamage of PSII (Demmig-Adams and Adams, 1992; Chow, 1994).

4.10. Tocopherol deficiency results in notable retardation in flower initiation

Although the *in vivo* function for tocopherol in animals is known in some detail, the *in vivo* role of tocopherols in plants is still not clear. Nevertheless, characterization of several tocopherol deficient plants suggests different roles of tocopherol in plant physiology, which have to be further studied. One of the suggested functions for tocopherol is its involvement in flower induction. Sirnoval (1950) showed that treatment of vegetative *Fragaria vesca* plants with un-saponifiable oil, which was extracted from leaves of *Fragaria vesca* plants during flowering, resulted in flower initiation under non-inductive photoperiodic conditions. Later on Baszynski (1967) showed that *Calendula officinalis* plants did not show reproductive development in short day whereas feeding of short day-grown *Calendula officinalis* plants with different tocopherol concentrations resulted in flower initiation. Severe tocopherol deficiency in transgenic HPT:RNAi tobacco plants resulted in considerable delay in flower initiation (Table 3-8), which appeared to be in concordance with previous data demonstrating the function of tocopherol in flower initiation (Sirnoval, 1950; Baszynski, 1967).

In addition to flower initiation that was affected in transgenic HPT:RNAi silenced tobacco plants, we also observed a notable decrease in seed yield of HPT:RNAi (Table 3-9) and TMT:RNAi (Table 3-15) transgenic tobacco plants compared to the wild type.

Taken together, one could conclude that tocopherol is involved in flower induction and that $\alpha$-tocopherol can be replaced by $\gamma$-tocopherol, as flower initiation was not affected in TMT:RNAi (Table 3-14). As indicated in Table 3-9 and Table 3-15, transgenic HPT:RNAi and $\gamma$TMT:RNAi tobacco plants, which lack $\alpha$-tocopherol, showed notable seed yield reduction in comparison to wild type plants. Based on these data, one could conclude that the seed yield might specifically be affected by $\alpha$-tocopherol.

On the other hand, no obvious differences between pollen tube growth and seed yield of those capsid of wild type and both transgenic plants, which was cross-pollinated with transgenic and wild type, respectively, indicates that this observation might be a secondary effect of tocopherol deficiency. However, this aspect of tocopherol biology seems to be very complex and needs to be further investigated.
4.11. Salt and sorbitol stress have different targets in cellular metabolism and induce characteristic physiological responses

The rationale behind employing salt and sorbitol stress in our study was to elicit specific stress patterns. Both, severe salt and sorbitol stress finally result in oxidative stress caused by hyperosmolarity (Wang et al., 2003). However, salt and sorbitol stress will primarily target different subcellular compartments. Excess symplastic salt concentrations endanger protein integrity and therefore, excess salt has to be sequestered in the vacuole or extruded from the cells, concomitantly disturbing pH and ion homeostasis across the tonoplast and the plasma membrane (Serrano and Rodriguez-Navarro, 2001). Increased salt tolerance was achieved by generating transgenic plants overexpressing the plasma membrane Na+/H+ antiporter SOS1 (Shi et al., 2003) or the vacuolar Na+/H+ antiporter AtNHX1 (Apse et al., 1999; Zhang and Blumwald, 2001; Zhang et al., 2001), demonstrating that the sequestration capacity for sodium is limiting in high salinity. Our data on carbohydrate and amino acid metabolism provide evidence that the assumed mode of sodium chloride toxicity also holds true for our experimental setup: in transgenics and wild type, we observed a ninefold accumulation of proline (and its precursor glutamate) in 100mM salt stress (Figure 3-39 and Table 3-19), while proline biosynthesis – evidenced by steady state contents of itself and its precursor glutamate - was not induced in 100mM sorbitol stress (Figure 3-39 and Table 3-20), indicating that the constraint to protect macromolecules with the accumulation of compatible solutes is more specific for salinity. Furthermore, a high salinity of 400mM NaCl led to a dramatic accumulation of starch, sucrose as well as aspartate and pyruvate-derived amino acids in the wild type (Figure 3-39, Table 3-19 and Table 3-20), indicating a severely disturbed central cellular metabolism. Likewise, this was not observed in sorbitol stress. The correlation of sucrose and starch accumulation with increasing salt stress also argues for a progressing sugar export block from source leaves in response to poisoning by salinity, as commonly observed under various stress conditions (Voll et al., 2003; Sam et al., 2004; Maeda et al., 2006).

In contrast to sodium chloride, sorbitol is a compatible solute, which does not interfere with enzymatic activities in plasmatic compartments. Sorbitol would more specifically cause desiccation of the cells in addition to hyperosmotic stress. In the second line, reduced water availability would finally increase oxidative stress in the chloroplasts by stimulating the generation of reactive oxygen species (ROS) and, concomitantly, lipid peroxy radicals in illuminated thylakoids. Paralleling the increased tolerance towards sorbitol stress, the γ-TMT transgenics were less susceptible than wild type towards the
Discussion

electron donor methyl viologen that specifically induces oxidative stress in the thylakoids by generating superoxide anions at PSI reaction centers. This indicates that γ-tocopherol in γ-TMT:RNAi leaves helps to mitigate ROS-induced stress in the chloroplasts. Increasing ROS quenching capacity by, e.g. overexpression of superoxide dismutase (SOD) (Gupta et al., 1993) or glutathione-S transferase and glutathione peroxidase (Roxas et al., 1997), was shown to possess the potential to alleviate such oxidative stress.

In support of our model of sorbitol action, we noted an enhanced hexose accumulation in wild type and γ-TMT:RNAi under sorbitol stress compared to salt stress (Figure 3-37 A-F), indicating a rearrangement of carbohydrates in favor of osmotically competent glucose and fructose. The compatible solute proline accumulated in elevated sorbitol stress of more than 300mM only (Figure 3-39). As obtained in our experiments, the specific accumulation of soluble sugars indicates that desiccation predominates over sorbitol toxicity or oxidative stress in sorbitol treatments. An accumulation of hexoses during drought stress has already been described for tobacco (e.g. Karakas et al., 1997) and was observed for barley leaves (Villadsen et al., 2005) and sweet potato suspension cells (Wang et al., 1999) subjected to drought and osmotic stress. Mostly, drought stress was accompanied by a decrease in starch biosynthesis in favor of soluble sugars (Karakas et al., 1997; Villadsen et al., 2005).

4.12. Tocopherol depletion by silencing of HPT decreases stress tolerance in transgenic tobacco

Growth performance and stress tolerance of HPT transgenics with more than 5% residual total tocopherol were indistinguishable from the wild type, indicating that endogenous tocopherol contents in tobacco plants are far from limiting. In comparison to the wild type, strong HPT:RNAi lines exhibited a 30% growth reduction under greenhouse conditions (Table 3-8), but not in axenic culture when sugar was supplied (Table 3-16 and 3-17). In addition, HPT transgenics displayed a 25% increase in membrane damage in the absence of any stress treatment already (Table 3-18).

As expected, eliminating the antioxidant tocopherol by silencing of HPT resulted in increased sensitivity of tobacco leaves towards salt and sorbitol stress. Biomass production (Table 3-16 and Table 3-17) was significantly diminished by 30%, while lipid peroxidation (Figure 3-36) and loss of photosynthetic pigments (Figure 3-33 and 3-34) were significantly increased in the HPT transgenics compared to the wild type on equal
or more than 300mM sodium chloride. Sorbitol treatment prove to be more gentle, only specifically provoking elevated lipid peroxidation in HPT:RNAi tobacco relative to the wild type (Figure 3-36). Likewise, the response of carbon and nitrogen metabolism in HPT transgenics subjected to sorbitol stress did not differ entirely from what was observed in wild type or γ-TMT:RNAi plants, as was the case for salt stressed HPT:RNAi plants. In general, leaves of HPT:RNAi accumulated less soluble sugars (Figures 3-37 and 3-38) and displayed an increase in the Asn/Asp and Gln/Glu ratio compared to the other two genotypes in sorbitol stress (Table 3-20). In contrast, salt stress utterly changed both carbohydrate and amino acid metabolism in HPT transgenics in comparison to wild type or γ-TMT knockdown plants (Figure 3-39 and Table 3-19). Taken together, this demonstrates a rather specific effect of sorbitol mediated stress on lipid oxidation in HPT transgenics, most likely due to an impaired quenching of lipid peroxy radicals and singlet oxygen in the thylakoid membrane, which are the major antioxidant functions of foliar α-tocopherol in wild type thylakoids (Munné-Bosch and Alegre, 2002; Trebst et al., 2002; Kruk et al., 2005).

Aside from a direct impact of elevated lipid peroxidation, the increased susceptibility of HPT:RNAi tobacco towards oxidative stress caused by salt and (to a lesser extent) sorbitol might be facilitated by enhanced ROS signaling in the transgenics early in the acclimation process. Transcriptome analysis of unchallenged Arabidopsis vte2 seedlings revealed that the increased lipid peroxidation in this HPT mutant triggered ROS regulated genes, as during pathogen challenge or oxidative stress like ozone (Sattler et al., 2006). Interestingly, the induced genes in vte2 seedlings were not positively regulated by MeJA (Sattler et al., 2006), the synthesis of which is elevated when lipid peroxidation occurs. In contrast, Arabidopsis TC mutants (vte1) challenged with low temperature and high light exhibited a transient increase in MeJA (Munne-Bosch et al., 2007). However, the diverging results obtained for vte1 and vte2 could be explained by the different age of the examined plants or by the accumulation of the tocopherol precursor DMPBQ in vte1 (Sattler et al., 2003), which is absent in vte2.

Despite the evidence that Arabidopsis vte2 mutants exhibit an enhanced ROS response (Sattler et al., 2006), the soluble antioxidants ascorbate and glutathione were not substantially induced in the Arabidopsis tocopherol deficient vte1 and vte2 mutants (Havaux et al., 2005; Kanwischer et al., 2005). We also found that the ascorbate pool was not significantly increased in tocopherol deficient HPT knockdown tobacco compared to wild type (Figure 6). But as tocopherols connect the lipophilic xanthophyll cycle to the soluble antioxidant network governed by the ascorbate-glutathione cycle.
(Foyer and Noctor, 2003), high light sensitivity of vte1 was greatly enhanced when other antioxidant systems like the zeaxanthin or the glutathione-ascorbate cycle were compromised concomitantly (Kanwischer et al., 2005; Havaux et al., 2005). This demonstrates compensatory capacity in the foliar antioxidant network, which might also account for the comparable response of the ascorbate pool in HPT transgenics and wild type during acclimation to the imposed oxidative and osmotic stress.

4.13. The substitution of γ- for α-Tocopherol in γ-TMT silenced tobacco increases osmotolerance

The Arabidopsis γ-TMT mutant vte4 did not exhibit an altered stress response towards heat, cold and high light compared to the wild type (Bergmüller et al., 2003) and stress susceptibility was not assessed for the Synechocystis and the sunflower mutants. In contrast to the investigation on stress tolerance of the Arabidopsis vte4 mutant (Bergmüller et al., 2003), we imposed sorbitol and sodium chloride to probe stress tolerance of tobacco silenced for γ-TMT. By imposing stress conditions that had not previously been applied to plants with altered tocopherol biosynthesis, we were able to demonstrate a novel in vivo role for γ-tocopherol in osmotolerance.

As described above, salt primarily exerts a toxic effect by disturbing the structural integrity of enzymes, while hyperosmotic and oxidative stress effectuated by salinity are secondary in nature. On the contrary, sorbitol is a compatible solute that primarily causes desiccation, which in turn implicates oxidative stress as a secondary effect. Silencing γ-TMT in tobacco, thereby exchanging γ- for α-tocopherol, resulted in an elevated susceptibility towards salt, but a diminished susceptibility towards osmotic stress compared to the wild type (Table 3-16, Table 3-17, Figure 3-30 and Figure 3-31). Ascorbate pool size and lipid peroxidation were reduced in the γ-TMT transgenics compared to the wild type in both treatments (Figure 3-36), indicating less oxidative stress in the transgenics in both stress conditions. As judged from carotenoid and chlorophyll contents (Figure 3-33 and 3-34), the photosynthetic apparatus in γ-TMT transgenics remained intact in sorbitol, but not in NaCl stress. While the chlorophyll a to b ratio collapsed in wild type leaves on 400mM sorbitol, leaves of γ-TMT:RNAi plants retained the same ratio as on control plates.
4.14. Conclusion

Our results shed new light on the in vivo function of γ-tocopherol, allowing two major conclusions. First, γ-tocopherol is more potent than α-tocopherol in conferring desiccation tolerance in vivo. This is presumably mediated by the higher in vivo lipid antioxidant activity of γ-tocopherol (which is present in the γ-TMT transgenics) compared to α-tocopherol (abundant in wild type leaves). Consequently, lipid peroxidation is diminished, membrane damage is decreased and pigment loss is reduced in the γ-TMT transgenics compared to wild type when oxidative stress arises during desiccation. This assumption is supported by two observations. (i) γ-TMT:RNAi plants exhibited less membrane damage after targeted oxidative stress was imposed on thylakoids (and the PUFAs contained therein) by stimulating ROS production with methyl viologen and (ii) by the fact that γ-tocopherol is the naturally predominant tocopherol derivative in most oil-storing seeds (Figure 1), and that loss of γ-tocopherol was shown to result in elevated polyunsaturated fatty acid (PUFA) oxidation and diminished seed longevity in Arabidopsis (Sattler et al., 2004). Likewise, we also observed a substantially decreased germination efficiency in the tocopherol depleted HPT transgenics (not shown). Thus, the presence of γ-tocopherol is not only pivotal for seed desiccation tolerance, but can also increase desiccation tolerance in leaves.

In oil-storing seeds, storage lipids are located in oil bodies that reside in the cytosol. It was reported that tocochromanols are tightly associated with oil bodies form sunflower and oat seeds (White et al., 2006; Fisk et al., 2006). As excess lipid peroxidation does not occur in e.g. Arabidopsis wild type, but in γ-tocopherol deficient seeds (Sattler et al., 2004), it can be assumed that tocochromanol contents in oil bodies are sufficient to efficiently protect seed PUFAs from oxidation.

For leaves, it has been demonstrated that tocopherols are abundant in plastoglobules and evidence is emerging that tocopherol biosynthesis also occurs in plastoglobules (Austin et al., 2006; Vidi et al., 2006). Plastoglobules are thylakoid protrusions composed of lipophilic constituents like triacylglycerols, quinones, chlorophyll, carotenoids and also MGDG and DGDG (Ghosh et al., 1994; Austin et al., 2006) that increase in number during senescence and in oxidative stress (Steinmüller and Tevini, 1985; Munne-Bosch and Falk, 2004) when chlorophyll turnover is high. Free phytol from chlorophyll breakdown might directly be salvaged for tocopherol biosynthesis (Ischebeck et al., 2006; Dormann, 2007), which is required for antioxidant protection in these conditions.

As a second conclusion, as shown in section 3-3 γTMT:RNAi plants, which have γ-tocopherol instead of α-tocopherol, are more susceptible to salt stress in comparison
to wild type plants. More susceptibility of γTMT:RNAi plants than wild type under salt stress indicates that γ-tocopherol cannot substitute α-tocopherol to ensure a better survival in salt stress. Therefore, one could conclude that under salt stress α-tocopherol may indirectly better protect macromolecules from denaturation than γ-tocopherol, which might proposed a specific mode of action for α-tocopherol in plants via its participation in intercellular signaling.

Although over the last few years, studies on animal cells suggest the function of tocopherol in regulation of gene expression, which are unrelated to their known antioxidant or pro-oxidant functions (Azzi et al., 2002) direct evidence for the involvement of tocopherols in signal transduction or gene expression in plants has not been provided so far.

Since reactive oxygen species, which occur during oxidative stress, can be detoxified by α-tocopherol, it might be speculated that, α-tocopherol participates in intercellular signaling. As α-tocopherol could break the chain reaction of lipid peroxidation by scavenging the lipid radical, it could alters the concentration of the secondary oxidation product such as jasmonic acid (JA) which participates in intracellular signaling. By controlling the hydroperoxide content in chloroplasts, tocopherols may indirectly regulate the amount of endogenous phytohormone levels such as jasmonic acid in leaves and may affect jasmonic acid dependent gene expression (Munne-Bosch, 2005) and therefore would influence cell signaling in the plants.
5. Materials and Methods

5.1. Chemicals and enzymes

All enzymes and chemicals with appropriate purity were purchased from specialized companies including: Amersham Pharmacia (Braunschweig), Applichem (Darmstadt), Boehringer Mannheim (Mannheim), Bio-Rad (München), Fluka (Buchs, Switzerland), New England Biolabs (Beverly, MA, USA), Merck (Darmstadt), Pierce (Rockford, IL, USA), Qiagen (Hilden), Roth (Karlsruhe), Roche (Mannheim), Sera (Heidelberg), Stratagene (Amsterdam, Netherland), Sigma Aldrich (Steinheim), and Whatman (Maidstone, England).

5.2. Plant material and growth conditions

5.2.1. Arabidopsis thaliana

Arabidopsis seeds (Arabidopsis thaliana cv. Columbia-2 and vte1 mutant) were obtained from Peter Dormann (Golm, Germany). The mutant and wild type seeds were grown in a growth chamber under an 8-h light /16-h dark regime. After 3 weeks, plants were transferred to the greenhouse, under a 16-h light and 8 h dark regime.

5.2.2. Nicotiana tabacum

Tobacco plants (Nicotiana tabacum cv. Samsun NN) were obtained from Vereinigte Saatzuchten eG (Ebsdorf, Germany) and grown in tissue culture under a 16h light and 8 h dark regime (irradiance;150 μmol quanta m⁻² s⁻¹) at 50% humidity on Murashige Skoog medium (Sigma, St. Louis, MO, USA) containing 2% (w/v) sucrose.

To test the physiological parameter under normal growth condition tobacco seedling were transferred to the greenhouse; plants in the greenhouse were kept in soil under a 16 h lights (ca. 250 μmol m⁻² s⁻¹; 25 °C) and 8 h dark (20 °C) regime.

To perform salt induced oxidative stress and sorbitol induced osmotic stress, 14-days old seedling were transferred to MS medium containing increasing amount of either salt (0, 100, 200, 300, 400 and 500 mM NaCl) or sorbitol (0, 100, 200, 300, 400 and 500 mM).
sorbitol). Plants were grown under a 16h light and 8 h dark regime (irradiance; 150 μmol quanta m⁻² s⁻¹) at 50% humidity. 4 weeks after transferring to the new media, samples were taken from stressed plant for further analysis.

5.3. Media and culture

All the bacterial media were prepared as described by Sambrook et al., (1989). The plant tissue culture medium was prepared by adding half MS medium including vitamins (Sigma), 2% sucrose, 0.75% agar and the pH was adjusted to 5.75. Antibiotic (kanamycin) was added to the normal MS medium to prepare the selection medium as a selection marker for pre–selection of the transformed plants.

5.4. Bacterial strains, plasmids and Oligo-nucleotides

5.4.1. Oligo-nucleotides and DNA sequencing

PCR oligo-nucleotides were purchased from Metabion (Martinsried, Germany). DNA sequencing was done at the IPK service facility. All the primers that were used in this work are listed in Table 5-1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Restriction Enzyme</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA1</td>
<td>5'-ggatccGCTTCTAGTGGAAGTACCTCCATGGAG-3'</td>
<td>BamHI</td>
<td>5’- TAP-Tag</td>
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<tr>
<td>AA2</td>
<td>5'-tctagaCTTTGGGGCTTGGGCATCGTGTGAC-3'</td>
<td>XbaI</td>
<td>3’- TAP-Tag</td>
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<tr>
<td>AA3</td>
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<td>Asp718</td>
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<tr>
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<tr>
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<td>SalI</td>
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</tr>
<tr>
<td>AA9</td>
<td>5'-ggatccCTTCAAAAAAGGTAACAGCAGTACTCTCG-3'</td>
<td>BamHI</td>
<td>5’- St. γMT:RNAi</td>
</tr>
<tr>
<td>AA10</td>
<td>5'-gtcgacGGATTAGGGGATAAGGTTTCATTTC-3'</td>
<td>SalI</td>
<td>3’- St. γMT:RNAi</td>
</tr>
</tbody>
</table>
5.4.2. Bacterial strains and plasmid list

E. coli strain XL1-blue (Stratagen) and TOP 10F’ (Invitrogen) were used for plasmid amplification. Agrobacterium tumefaciens was used for plant transformation. All the informations about the bacterial strains have been listed in Table 5-2.

Table 5-2) Bacterial strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype or relevant characteristic</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.coli XL1 Blue</td>
<td>$\text{RecA}1$, $\text{endA}1$, $\text{gyrA}96$, $\text{thi-}1$, $\text{hsdR}17$, $\text{supE}44$, $\text{relA}1$, $\text{lac}$, $[\text{F'}$, $\text{proAB}$, $\text{lac}1^q\Delta M15$, $\text{Tn}^{10}$ (tet $^r$)] $^c$</td>
<td>Bullock et al., 1987 Stratagene</td>
</tr>
<tr>
<td>E.coli TOP10F’</td>
<td>$\text{F'}$ mcrA $\Delta (\text{mrr-hsdRMS-mcrBC})$ $\Phi80\text{lacZ} \Delta M15 \Delta \text{lacX74 recA}1 \text{araD}139$ $\Delta (\text{ara-leu})7697 \text{galU galK rpsL}$ (Str$^R$) $\text{endA}1$ $\text{nupG}$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>Agrobacterium tumefaciens</td>
<td>strain C58C1 carrying the virulence plasmid $pGV2260$</td>
<td>Rosahl et al., 1987</td>
</tr>
</tbody>
</table>

Table 5-3) Plasmid list

<table>
<thead>
<tr>
<th>Vector</th>
<th>Application/resistance</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCR-Blunt</td>
<td>Cloning vector, Kan$^R$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pCR2.1</td>
<td>Cloning vector, Kan$^R$</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBluescript SK-</td>
<td>Cloning vector, Amp$^R$</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pBinAR</td>
<td>Binary vector, Kan$^R$</td>
<td>Höfgen and Willmitzer, 1990</td>
</tr>
<tr>
<td>pUC-RNAi</td>
<td>Cloning vector, hpRNA construct with StGA20-oxidase intron, Amp$^R$</td>
<td>Chen, et al. 2003</td>
</tr>
<tr>
<td>p35S-alcR</td>
<td>Binary vector, alc system, Kan$^R$</td>
<td>Caddick et al. 1998</td>
</tr>
<tr>
<td>pUC-alcA</td>
<td>Cloning vector, alc system, Amp$^R$</td>
<td>Caddick et al. 1998</td>
</tr>
<tr>
<td>pUC18, pUC19</td>
<td>Cloning vector, AmprGenotype or relevant characteristic</td>
<td>Yanisch-Perron et al. 1985</td>
</tr>
</tbody>
</table>
The pCR-Blunt vector (Invitrogen) was used for the cloning of PCR products. The vector pMAL (Qiagen) was used for the expression of tagged fusion proteins. The binary vector pBin19 and its derivatives were used for plants transformation. The vector pUC-RNAi (Chen et al., 2003) was used for dsRNA constructs. All plasmids, which were used for plant transformation in this work, were listed in Table 5-3.

5.5. Vector construction

All the cloning processes like PCR-amplification, RT-PCR amplification, plasmid extraction, DNA fragment purification, enzymatic digestion, and ligations were performed as described by Sambrook et al., (1989).

5.6. Bacterial transformation

5.6.1. E. coli transformation

Plasmid DNA was transferred to competent E. coli cells (Inoue et al., 1990) by heat shock method (cooling on ice-water and heating by incubating at 42°C for 1.5 min and again cooling on ice-water). One ml LB-medium was added to the transformed cells and incubated at 37°C for one hour with gentle shaking, and then the selective antibiotics were added to the culture and incubated overnight. Next day, the culture was spread on LB medium containing the selective antibiotic and incubated at 37°C overnight. After 16-h positive colonies were selected following the DNA extraction and digestion (Sambrook and Russell, 2001).

5.6.2. Agrobacterium transformation

Plasmid DNA was transferred to the competent cells agrobacterium by the freeze-thaw method, which has been described by Höfgen et al. (Höfgen and Willmitzer, 1990). YEP medium containing transformed cells were incubated at 28°C with gentle shaking. After 4-6 hour, the selective antibiotics were added to the culture and incubated overnight. Next day, the culture were spread on YEB medium containing the selective antibiotics and incubated at 28°C. After 2-3 days plasmid DNA from grown colonies were extracted and analyzed by enzymatic digestion.
5.7. Agro-infiltration

Three ml of each individual *Agrobacterium* colonies, which carrying pBin:TC:GFP, pBin:TC:TAP:Tag or pBin:P19, which contains coding sequence of P19 protein of *Tomato bushy stunt virus* (TBSV) (Voinnet et al., 2003), were grown overnight, were grown overnight at 28°C in YEB medium containing selective antibiotics separately. The agro-infiltration was done as described by Vionnet et al. (2003). Briefly, the grown cultures were transferred to 50mL induction medium (0.5% beef extract, 0.1% yeast extract, 0.5% Peptone, 0.5% Suc, 2 mM MgSO4, 20 mM acetasyringone, 10 mM MES, pH 5.6) containing selective antibiotics, and grown again overnight. Next day, the cultures were collected and re-suspended in infiltration medium (10 mM MgCl₂, 10 mM MES, 200 mM acetasyringone). The solution was mixed 1:1 with P19 and incubated at room temperature for a minimum of 2 h. Culture was taken with a syringe, and injected into the tobacco source leaves (Brigneti et al., 1998; Qiu et al., 2002; Voinnet et al., 2003) (Brigneti et al., 1998; Voinnet et al., 2003)

5.8. Plant transformation

5.8.1. Arabidopsis transformation

*Arabidopsis* vte1 mutants were transformed by *agrobacterium* –mediated gene transfer (Bechthold et al., 1993; Clough and Bent, 1998). YEB medium, containing kanamycin, ampicillin and rifampicin antibiotics to select the positive transformed colonies, was inoculated with *Agrobacterium tumefaciens* strains carrying the gene of interest on a binary vector. The *Agrobacterium* culture (OD₆₀₀ = 0.8) was resuspended to OD₆₀₀ = 0.8 in 5% Sucrose, before dipping, Silwet L-77 was added to a concentration of 0.05% (500 µl per liter) and mixed well. Aboveground parts of plants were dipped in *Agrobacterium* solution for two to three seconds with gentle agitation. Dipped plants were placed under a dome or cover for 16 to 24 hours to maintain high humidity and after maturity, dry seeds were harvested. Harvested seed were placed on MS containing kanamycin medium to select the transformed seedlings. Putative transformed plants for further analysis were transferred to soil.
5.8.2. Tobacco transformation

Transformation of tobacco plants by *Agrobacterium*-mediated gene transfer using *Agrobacterium tumefaciens* strain C58C1:pGV2260 (Deblaire et al., 1985) were carried out as described previously (Rocha-Sosa et al., 1989).

5.9. Fluorescence microscopy

Microscopic images of TC fused GFP fusion proteins in transient expression and stably transformed plants were obtained with a confocal laser scanning microscope LSM 510 META (Zeiss, Göttingen, Germany) and a regular fluorescence microscope (Axiovert 135, Carl Zeiss, Jena, Germany). In the confocal laser scanning microscope, excitation light of 488 nm produced by krypton/argon laser and emission filters of 510 to 525 and 645 to 700 nm allowed detection of GFP or chlorophyll-derived red fluorescence, respectively, and images were superimposed by means of the Zeiss LSM Version 3.0 software. In Axiovert 135, emission filters of 645 to 700 and 515 to 700 nm allowed detection of GFP or chlorophyll-derived red fluorescence, respectively. All the excitation and emission wavelengths, which used for GFP and Chlorophyll, were listed in Table 5-4.

<table>
<thead>
<tr>
<th>Table 5-4</th>
<th>Filters with different wavelength for excitation and emission of GFP and Chlorophyll fluorescence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axiovert 135</td>
<td>LSM 410 / 510 META</td>
</tr>
<tr>
<td>Excitation</td>
<td>Emission</td>
</tr>
<tr>
<td>GFP</td>
<td>450 – 490 nm</td>
</tr>
<tr>
<td>Chlorophyll</td>
<td>450 – 490 nm</td>
</tr>
</tbody>
</table>

5.10. Photosynthetic activity measurement

Photosynthetic activity and dark respiration measurements were performed on different plants grown at greenhouse according to Hajirezaei et al., (2002). Plants were clamped with a Li-Cor LI-6400 apparatus and the light intensity within the measuring chamber was changed to 2000-mmol photons m⁻² sec⁻¹. The rate of CO₂ assimilation was then measured (Hajirezaei et al., 2002).
Plant chlorophyll fluorescence was determined by a portable fluorometer (PAM-2000, Walz, Effeltrich, Germany) connected with a leaf-clip holder and with a trifurcated fibre-optic (Lu and Zhang, 1998). Data acquisition software (DA-2000, Walz) was used in a notebook computer to dispose data on-line. The measurements were followed essentially according to Lu and Zhang, (1998).

5.11. Molecular techniques

5.11.1. RNA extraction and reverse transcription PCR (RT-PCR)

Total RNA was extracted as described (Logemann et al., 1987). Briefly, frozen leaves were homogenized with extraction buffer and then extracted with phenol /chloroform / isoamylalcohol (25:24:1), RNA was ethanol-precipitated and washed with 3 M sodium acetate (pH=5.2). After that, pellets were washed with 80% ethanol. RNA was dissolved in DEPC water and incubated at 65°C for 10 minutes with shaking. About 2-10 μg total RNA from Arabidopsis leaf tissue was reverse transcribed in a total volume of 50μl with Promega M-MLV reverse transcriptase. The transcription was performed at 37°C for one hour. Two μl of the reverse transcription product was used for PCR amplification (Sambrook and Russell, 2001).

5.11.2. Protein extraction and western blot analysis

Protein was extracted by grinding leaf material in an ice-cold extraction buffer (50 mM Tris-HCl, pH 6.8, 5 mM MgCl2, 5 mM mercaptoethanol, 15% (v/v) glycerol, 1 mM EDTA, and 1 mM EGTA). The homogenate was centrifuged at 15000 rpm at 4°C for 10 minutes, and the supernatant was used for further analysis. Protein concentrations were determined according to the method of Bradford (Bradford, 1976). Soluble protein was separated on 12.5 % SDS polyacrylamid gels and electro-transferred onto a nitrocellulose membrane (Porablot; Macherey-Nagel, Düren, Germany) in the presence of transfer buffer (39 mM glycine, 48 mM Tris-base, 0.01 % (w/v) SDS and 20% methanol). The membrane was incubated for one hour in 5% non-fat milk powder +TBST buffer (TBST: 20 mM Tris, 500 mM NaCl, and 0.1% (v/v) Tween20). Membrane was rinsed three times using 1% non-fat milk powder in TBST. Then the membrane was incubated
in TBST buffer containing 1% non-fat milk powder plus CBP antibody (Calmodulin binding peptide 1: 5000 dilution) for at least 1 h. After three times washing, the membrane was incubated in TBST buffer containing 1% non-fat milk powder plus 1 to 20000 dilution of anti goat anti rabbit antibody. Following 3 times rinsing with TBST buffer plus 1% milk powder, the immunoblots was developed with the Supersignal® West Pico Chemiluminescent Substrate system (Pierce, Rockford, USA). After that, the blots were exposed to X-ray films (Sigma) and quantified with the BAS 2000 Bio-Imaging Analyser (Fujifilm). For detection of TAP-Tag fused proteins only a PAP antibody (Peroxidase anti peroxidase ) instead of first and second antibody was used and the membrane was incubated with PAP antibody and after 3 times washing, as described above, membrane was incubated by substrate and then blot was exposed to X-ray film.

5.11.3. Protein complex purification from transformed plants

Five gram plant material was ground in liquid nitrogen, homogenized with 3 volumes of extraction buffer (100 mM Tris, pH 8.0; 5 mM EDTA; 150 mM NaCl; 10 mM DTT; one tablet plant protease inhibitor cocktail (Sigma P9599, Taufkirchen, Germany), 0.1% Nonidet-40 (NP-40), and the homogenized material was centrifuged at 12 000 g for 10 min at 4°C. The supernatant was incubated with 250 µl IgG beads with gentle rotation. After centrifugation at 150 g for 3 min at 4°C, the IgG beads were recovered and washed three times with 10 ml of washing buffer (50 mM Tris, pH 8.0; 5 mM EDTA; 150 mM NaCl; 2 mM DTT; 0.1% NP-40) and once with 10 ml of cleavage buffer (50 mM Tris, pH 8.0; 0.5 mM EDTA; 150 mM NaCl; 2 mM DTT; 0.1% NP-40). Elution from the IgG beads was performed by incubation with 10 µl (100 units) of TEV protease (Prescission protease; Amersham Biosciences) in 1.5 ml of cleavage buffer, at 4°C with gentle rotation. Supernatants were recovered after centrifugation at 150 g for 3 min at 4°C and stored. The IgG beads were washed with 5 ml of washing buffer and centrifuged again. Supernatants were recovered and the eluates were collected and added to a calmodulin bead suspension (Amersham) previously equilibrated in 1 ml of CBB (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM β-mercaptoethanol; 1 mM Mg-acetate; 1 mM imidazole; 2 mM CaCl$_2$; 0.1% NP-40 ) and resuspended in 900 µl of CBB. Two microliters of 1M CaCl$_2$ solution were added, and the samples were rotated for 1 h at 4°C to allow binding to the beads. The column was washed once in CBB buffer and twice in RB buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM β-mercaptoethanol; 1 mM Mg-acetate; 1 mM imidazole;
0.1% NP-40). Then the bead was incubated with 200 µl of calmodulin elution buffer (50 mM Tris, pH 8.0; 150 mM NaCl; 10 mM β-mercaptoethanol; 1 mM Mgacetate; 1 mM imidazole; 2 mM EGTA; 0.1% NP-40) at room temperature for 5 min. The eluate was collected by centrifugation (13,000-x g, 1 min). The supernatant was loaded to 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and after Commassie blue staining, bands were cut for further analysis by Liquid chromatography electrospray ionisation tandem mass spectrometry (LC-ESI-MS-MS) or Matrix Assisted Laser Desorption Ionisation -Time Of Flight (MALDI-TOF).

5.12. Biochemical methods

5.12.1. Tocopherol extraction and measurement
Tocopherols were extracted by grinding and homogenizing of 50 mg of frozen leaves in 500 µl of 100% methanol. The homogenate was incubated for 30 minutes at 30 °C. Then the samples were centrifuged at 14000 rpm for 5 minutes. The supernatant was transferred to a new tube, and the pellet was re-extracted twice with 250 µl of 100% methanol and the supernatants were pooled. The samples were analyzed by HPLC. The tocopherol content of the organic extract was determined by Waters HPLC and Dionex HPLC system. Resolution of vitamin E species was achieved by using an phenomenex C18 reverse phase column (250 mm length and 4,6 µm particle size ) and a isocratic solvent system consisting of Acetonitril : Methanol (85:15 v/v) with a flow rate of 1 ml per minute. Sample components were detected and quantified by fluorescence with excitation at 290 and emission at 325 nm (Thompson and Hatina, 1979; Vuilleumier et al., 1983). Tocopherol molecular species were identified and quantified relative to standards.

5.12.2. Plastoquinone extraction and measurement
One gram frozen Leaves of tobacco plants were homogenized with mortar and pistil in 0.25 M sucrose. The resulting slurry was centrifuged at high speed for 20 min the pellet was homogenized and centrifuged once more. Lipids from the homogenate were extracted with chloroform /methanol/ water (CMW) 1:1:0.3 (v/v/v.) for 1 h at room temperature and the extract was adjusted to a final CMW ratio of 3:2:1 (v/v/v.) The
organic phase was evaporated by speed vacuum (Maciejewska et al., 2002). The extracted lipids were dissolved in methanol and the samples were measured by HPLC. The plastoquinone content was determined by using a Dionex HPLC. Resolution of plastoquinone was achieved by using an phenomenex C18 reverse phase column (250 mm length and 4.6 μm particle size) and a solvent system consisting of Acetonitril: Methanol (65:35 v/v) with a flow rate of 1 ml /min. Sample components were detected and quantified by fluorescence with excitation at 320 and emission at 430 nm. Plastoquinone and its precursor were identified and quantified relative to standards (Maciejewska et al., 2002).

5.12.3. Sugar measurement

10-20 mg samples were taken from different leaves and different plants, samples were frozen in nitrogen liquid and then soluble sugar (glucose, fructose, and sucrose) were extracted for 60 min in 400 μl 80% ethanol at 70°C. Supernatants were transferred to new vials and the residue was extracted 2 times more with 80% ethanol as above. 1 ml final volume was evaporated by speed vacuum and the pellet was dissolved in 0.2 ml distilled water. Then soluble sugars were determined according to Sonnewald et al. (Sonnewald et al., 1991). Ten to twenty μl of extract were incubated in 300μl of 100mM imidazol buffer (pH 6.9), containing 5 mM MgCl₂, 2 mM NAD⁺, 1 mM ATP and one unit glucose 6-phosphate dehydrogenase, at room temperature for 5 minutes. Mixture was measured by an Elisa Reader (Tecan Spectra, Germany). One unit hexokinase, phosphoglucose isomerase, and β-fructosidase were added subsequently for glucose, fructose, and sucrose measurements.

5.12.4. Starch measurement

The insoluble debris of the ethanol extraction were homogenized in 400 μl 0.2 N KOH. The homogenate was incubated at 95°C for 1 hour and neutralized with 70 μl 1 N acetic acid. After that, 50-μl supernatant was incubated at 55°C with 50 μl of amyloglucosidase solution (2 mg per ml in 50mM sodium acetate, pH 5.2) for 2 hours. Starch content was calculated by measuring the glucose as described in 2.12.3 and quantified as an equivalent of glucose in the mixtures (Hajirezaei et al., 2000).
5.12.5. Callose determination

Extraction and measurement of callose from leaf material was done as described by Kohle et al., (1985). Callose quantification was based on comparison with the fluorescence of known amounts of the commercial β-1, 3-glucan pachyman (ICN Biomedicals, Irvine, CA), and measurements were performed using a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Ismaning, Germany). Callose contents were expressed as β-1, 3-glucan pachyman equivalents (Kohle et al., 1985).

5.12.6. Chlorophyll and carotenoids measurement

10-20 mg leaf samples were taken from the wild type and transgenic plants, samples were froze on nitrogen liquid and then chlorophyll and carotenoids were extracted with 500 µl 95% ethanol at 70°C for 1 hour. An aliquot of the supernatant was introduced to the photometer. Chlorophyll and carotenoids were quantified by measuring the absorbance at 664, 648 and 470 nm as described by Lichtenthaler, (Lichtenthaler, 1987).

5.12.7. Amino acid measurement

Samples were prepared in 80 % ethanol as described in van Wandelen and Cohen (1997). Ten to twenty mg leaf materials were incubated for 60 minutes at 80°C, after centrifugation at 14000 rpm, the supernatant evaporated to dryness, the samples were re-suspended in purest water and used for HPLC analysis (van Wandelen and Cohen, 1997). Prior to the measurement, primary and secondary amino acids were derivatized using 6-aminoquinolyl-N-hydroxysuccinimidylcarbamate (AQC reagent) as a fluorescing substance. Using a reversed phase HPLC (Dionex summit) concentrations of 17 amino acids were determined. The HPLC consists of a gradient pump, a degasing module, an autosampler and a fluorescence detector. Chromatograms were recorded using the software program chromeleon. The gradient was accomplished with a buffer A containing 140 mM sodium acetate, pH 5.8 (Suprapur, Merck) and 7 mM triethanolamine (Sigma, Germany), acetonitrile (Roti C Solv HPLC, Roth) and HPLC water (Baker) were used as eluents B and C respectively. To separate amino acids, a reversed phase C_{18} column (phenomenex, 4.6 x 250 mm) was used which consists of a modified apolar as matrix. The gradient was produced as follows by 1%, 5%, 9%, 18%, 60% and 0% of
eluent B at 0.5 min, 27 min, 28.5 min, 44.5 min, and 47.5 min and 50.5 min retention time. The column was equilibrated 10 minute with buffer A at a flow rate of 1 ml per minute and heated at 37°C during the whole measurement. Sample components were detected and quantified by fluorescence with excitation at 300 and emission at 400 nm (van Wandelen and Cohen, 1997).

5.12.8. Ion leakage measurement
Membrane damage was assayed by measuring ion leakage from leaf discs as described by Rizhsky et al., (2002). For each measurement, six leaf discs (10-mm diameter) were floated on 8ml of double distilled water for 20 h at 4 °C. Following incubation, the conductivity of the bathing solution was measured with a conductivity meter (value A). The leaf discs were then returned to the bathing solution, introduced into sealed tubes and incubated with the bathing solution at 95°C for 30 min. After cooling to room temperature the conductivity of the bathing solution was measured again (value B). For each measurement ion leakage was expressed as percentage leakage, i.e. (value A/value B)*100 (Rizhsky et al., 2002).

5.12.9. Determination of lipid peroxidation
The level of lipid peroxidation in leaf tissues was determined in terms of MDA produced based on the method of Heat and Packer (1968) with some modifications. Briefly, 50-100 mg leaf material frozen in liquid nitrogen was ground in 1 ml of 0.1% trichloroacetic acid (TCA) and 0.1 ml Butylated hydroxytoluene (BHT, 5mg/ml) was added to the homogenized sample as an antioxidant. The homogenate was incubated at 95°C for 30 min, the homogenate was centrifuged at 15,000×g for 10 min and 0.55 ml of the supernatant was mixed with 0.55 ml of thiobarbaturic acid (TBA) solution (25% thiobarbituric acid). The mixture was heated at 95°C for 30 min, chilled on ice, and then centrifuged at 10,000×g for 5 min. The absorbance of the supernatant was measured at 532 nm (Heath and Packer, 1968; You-Sheng and Zhi-Min, 2005). The value for non-specific absorption at 600 nm was subtracted. The amount of MDA was calculated from a standard curve of malondialdehyde (MDA) in the range of 5-100 nM.
5.12.10. Determination of ascorbate and dehydroascorbate

The level of ascorbate (Asc) and dehydroascorbate (DHA) of crude extracts from tobacco leaves was determined by spectrophotometrically, based on the method of Law et al., (1983). Briefly, 50-100 mg frozen leaf material was homogenized in liquid nitrogen to a fine powder and extracted with 1.1 ml 5% (w/v) 5-sulfosalicylic acid. After centrifugation, the supernatant was diluted 1:1 with 150 mM NaH$_2$PO$_4$ buffer (pH 7.4) and stored on ice. The pH was adjusted to 5.5-6.5 using 10 N NaOH. For determination of reduced ascorbate, 200 μl of extract was successively mixed with 100 μl ddH$_2$O, 200 μl 10% TCA, 200 μl 44 % H$_3$PO$_4$, 200 μl 2, 2’-dipyridyl (in 70% (v/v) ethanol) and 100 μl 3% (w/v) FeCl$_3$. The mixtures were incubated for 60 min at 30 °C and finally the color formation was measured at 525 nm (Law et al., 1983). The ascorbate content was calculated using a standard curve measured with freshly prepared ascorbate standards. For determination of total ascorbate, the neutralized extracts were reduced by immediately adding 50 μl DTT (10 mM) to the samples instead of aqua dest. After 15 min incubation at room temperature 50 μl 0.5% (w/v) N-ethylmaleimide were added followed by the same procedure as described above.

5.12.11. Determination of thiol-containing compounds

Glutathione and cystein of crude extracts from tobacco leaves were determined by an HPLC-based method. Briefly, 25-50 mg frozen leaf material were homogenized in liquid nitrogen to a fine powder and extracted with 1 ml 0.1M hydrochloric acid (HCl). The homogenate was incubated on ice for 15 min. Prior to the measurement, the supernatant was derivatized using monobromobimane as a fluorescing substance (Noctor and Foyer, 1998). Using a reversed phase HPLC (Dionex summit) concentrations of total and oxidized glutathione were determined. The HPLC consists of a gradient pump, a degasing module, an autosampler and a fluorescence detector. Chromatograms were recorded using the software program chromeleon. The gradient was accomplished with a buffer A (100 mM potassium acetate, pH 5.5), and buffer B (pure methanol from Merck).

To separate glutathione, a reversed phase column (phenomenex, 4.6 x 250 mm) was used which consists of a modified apolar as matrix. The gradient was produced as follows by 11% buffer A and 89 % buffer B, at 0-19 min, 100 % buffer B, at 19-26 min and 11% buffer A and 89 % buffer B at 27-37 minute retention time. The column was
equilibrated 10 minute with buffer A at a flow rate of 1 ml per minute and heated at 37°C during the whole measurement (Noctor and Foyer, 1998).

5.13. Ethanol induction

To perform ethanol induction, tobacco plants were transferred to the greenhouse and grown under normal growth condition. Three to four weeks after transferring to greenhouse, wild type and Alc: RNAi transgenic tobacco plants were treated with 100ml of one % (v/v) ethanol solution via root drenching. Samples were taken from treated plants at different days after induction with ethanol for tocopherol measurement (Chen et al., 2003).

5.14. Statistical analysis

Statistical differences between wild type and transgenic plants on different treatments were analyzed following the Duncan multiple range tests (Duncan, 1955). Differences were considered significant at a probability level of P< 0.05.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
</tr>
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<tbody>
<tr>
<td>1chl* and 3chl*</td>
<td>Chlorophyll singlet and triplet state</td>
</tr>
<tr>
<td>α-TTP</td>
<td>Alpha-tocopherol transfer protein</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxy-toluene</td>
</tr>
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<td>CaMV</td>
<td>Cauliflower mosaic virus</td>
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<tr>
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<td>Calmodulin binding peptide antibody</td>
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<td>Complementary DNA</td>
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<td>Chlorophyll</td>
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<td>CLSM</td>
<td>Confocal laser scanning microscope</td>
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<td>Coenzyme Q</td>
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<tr>
<td>GGPP</td>
<td>Geranyl geranyl pyrophosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>Reduced glutathione</td>
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<tr>
<td>GSSG</td>
<td>Oxidized glutathione</td>
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<tr>
<td>γ-TMT</td>
<td>Gamma-tocopherol methyltransferase</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HGA</td>
<td>Homogentisic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>HPP</td>
<td>4-Hydroxy phenyl pyruvate</td>
</tr>
<tr>
<td>HPPD</td>
<td>Hydroxyphenylpyruvate dioxygenase</td>
</tr>
<tr>
<td>HPT</td>
<td>Homogentisate phaytyltransferase</td>
</tr>
<tr>
<td>IC-MS</td>
<td>Ion-exchange chromatography coupled to mass-spectrometry</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin</td>
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</table>
KDa  Kilo dalton
LDL  low-density lipoprotein
MALDI  Matrix-assisted laser desorption/ionization
MDHA  Monodehydroascorbate
MGGBQ  2-methyl-6-geranylgeranyl-1, 4-benzoquinone
MPBQ  2-methyl-6-phytyl-1, 4-benzoquinone
MPBQMT  MPBQ/MSBQ methyltransferase
mRNA  Messenger RNA
MS  Murashige-skoog
NAD  Nicotinamide adenine dinucleotide
NADH  Nicotinamide adenine dinucleotide, reduced
NADP  Nicotinamide adenine dinucleotidephosphate
NADPH  Nicotinamide adenine dinucleotidephosphate, reduced
NOS  Nopalinsynthase
NPQ  Non photochemical quenching
OCS  Octopin synthase
OD  Optical density
ORF  Open reading frame
PAGE  Polya crylamid electrophoresis
PAP  Peroxidase anti peroxidase antibody
PCR  Polymerase chain reaction
pH  Hydrogen ion concentration
PUFA  Poly unsaturated fatty acid
RNAi  RNA interference
ROS  Reactive oxygen species
RT-PCR  Reverse transcription PCR
SDS  Sodium dodecyl sulfate
SNN  Samsun NN (Nicotiana tabacum)
ß-ME  ß-Mercaptoethanol
SXD1  Sucrose export deficient 1
TAP-Tag  Tandem affinity purification Tag
TC  Tocopherol cyclase
TEV  Tobacco etch virus
VTE1 (1, 2, 3 and 4)  Vitamin E deficient (1, 2, 3 and 4)
(V/v)  Volume: volume ratio
(W/v)  Weight: volume ratio
WT  Wild type
Acknowledgement

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**Curriculum Vitae**

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<td>Nationality</td>
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**Education**

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Publication