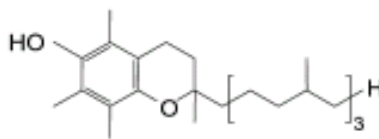


# Investigations on specific functions of $\alpha$ - and $\gamma$ -tocopherol during leaf senescence in higher plants

Dissertation  
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Dean

*Dedicated to my grandparents, Erika & Theodor Ludwig.*

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## IV. Abbreviations

ABA	abscisic acid
APX	ascorbate peroxidase
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
CAB	chlorophyll- <i>a/b</i> -binding protein
<i>cab</i>	gene encoding CAB
CAU	Christian-Albrechts-University
cDNA	complementary DNA
CLSM	confocal laser scanning microscopy
CP1	cysteine proteinase 1
<i>cp1</i>	gene encoding the cysteine proteinase 1
D1	photosystem II protein
DAAD	Deutscher Akademischer Austausch Dienst
DAF-FM	DAF-FM-(4-amino-5-methylamino-27-difluorofluorescein)
DAS	days after sowing
ddH <sub>2</sub> O	double distilled water
DFG	Deutsche Forschungsgemeinschaft
DIN	protein involved in molybdenum cofactor biosynthesis
<i>din</i>	gene encoding a protein involved in molybdenum cofactor biosynthesis
DMPBQ	2,3-dimethyl-5-phytyl-1,4-benzoquinone
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF1 $\alpha$	elongation factor 1 $\alpha$

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<i>ef1a</i>	gene encoding EF1 $\alpha$
FCC	flourescent chlorophyll catabolite
F <sub>v</sub> /F <sub>m</sub>	maximal efficiency of PSII
<i>gdh</i>	gene encoding the glutamate dehydrogenase
GDH	glutamate dehydrogenase
<i>gln1.2</i>	gene encoding the cytosolic glutamin synthase ( <i>Arabidopsis thaliana</i> )
GR	glutathione reductase
GRK	Graduiertenkolleg
GS1	cytosolic glutamine synthase
<i>gs1</i>	gene encoding the cytosolic glutamine synthase (tobacco)
GUS	beta-glucuronidase
<i>gus/GUS</i>	gene encoding the beta-glucuronidase
<i>hgo</i>	gene encoding the homogentisate 1,2-dioxygenase
HGO	homogentisate 1,2-dioxygenase
<i>hpd</i>	gene encoding the <i>p</i> -hydroxyphenylpyruvate dioxygenase
HPLC	High Performance Liquid Chromatography
HPPD	<i>p</i> -hydroxyphenylpyruvate dioxygenase
HPT	homogentisate phytyltransferase VTE2
HRP	horse-radish peroxidase
HST	homogentisate solanyltransferase
INRA	Institut Scientifique de Recherche Agronomique
<i>ipt1</i>	gene encoding isopentenyl transferase
IPT	isopentenyl transferase
LHCII	light-harvesting complex II
LSU	large subunit of Rubisco

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MCS	metal chelating substance
MPBQ	2-methyl-6-phytyl-1,4-benzoquinone
mRNA	messenger RNA
MSBQ	2-methyl-6-solanyl-1,4-benzoquinone
MS	mass spectrometry
NAP	Unité de Nutrition Azotée des Plantes
NCC	non-fluorescent chlorophyll catabolite
NO <sub>x</sub>	nitric oxide
<sup>1</sup> O <sub>2</sub>	singlet oxygen
O <sub>2</sub> <sup>-</sup>	superoxide anion
OH <sup>°</sup>	hydroxyl radical
<i>rbcL</i>	gene encoding the small subunit of Rubisco
<i>rbcS</i>	gene encoding the large subunit of Rubisco
PAM	pulse-amplitude modulation
PAO	pheophorbide- <i>a</i> -oxygenase
PCR	polymerase chain reaction
PPK	phytylphosphatekinase
PR1b	pathogenesis-related protein 1B
<i>pr1b</i>	gene encoding the pathogenesis-related protein 1B
PSII	photosystem II
PUFA	polyunsaturated fatty acid
RCB	Rubisco-containing bodies RCB
RCC	red chlorophyll catabolite
RCCR	RCC reductase
RIL	recombinant inbred line
ROO <sup>°</sup>	lipid peroxy radical
RNA	ribonucleic acid
RNAi	RNA interference

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RNS	reactive nitrogen species
ROS	reactive oxygen species
RT-PCR	reverse transcription PCR
Rubisco	ribulose biphosphate carboxylase/oxygenase
<i>35S</i>	plant specific promoter
SA	salicylic acid
<i>SAG12</i>	senescence-associated gene 12 (cysteine protease of <i>Arabidopsis thaliana</i> )
<i>SAG12-IPT</i>	gene construct containing the <i>SAG12</i> -promoter infront of the bacterial gene <i>ipt1</i>
<i>SAGs</i>	senescence-associated genes
SAM	<i>S</i> -adenosyl methionine
SAV	senescence-associated vacuoles
<i>SDGs</i>	senescence down-regulated genes
SDS	sodium dodecyl sulfate
<i>sdx1</i>	<i>sucrose export deficient1</i> gene
<i>sgr</i>	stay-green gene locus
SGR	stay-green protein
SOD	superoxide dismutase
SPAD	chlorophyll meter
SPPS	Scandinavian Society for Plant Physiology
SSU	small subunit of Rubisco
<i>Taq</i>	<i>Thermus aquaticus</i>
TF	transcription factor
Tris	tris(hydroxymethyl)-aminomethane
PCD	programmed cell death
UV	ultraviolet
<i>vte1</i>	gene encoding the tocopherol cyclase
VTE1	tocopherol cyclase



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<i>vte2</i>	gene encoding the homogentisate phytyltransferase HPT
VTE2	homogentisate phytyltransferase HPT
<i>vte3</i>	gene encoding the MSBQ / MPBQ methyltransferase
VTE3	MSBQ / MPBQ methyltransferase
<i>vte4</i>	gene encoding the $\gamma$ -tocopherol methyltransferase
VTE4	$\gamma$ -tocopherol methyltransferase
<i>vte5</i>	gene encoding the phytol kinase
VTE5	phytol kinase
WAS	weeks after sowing
v/v	volume per volume
w/v	weight per volume

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# 1 Introduction

## 1.1 The senescence syndrome

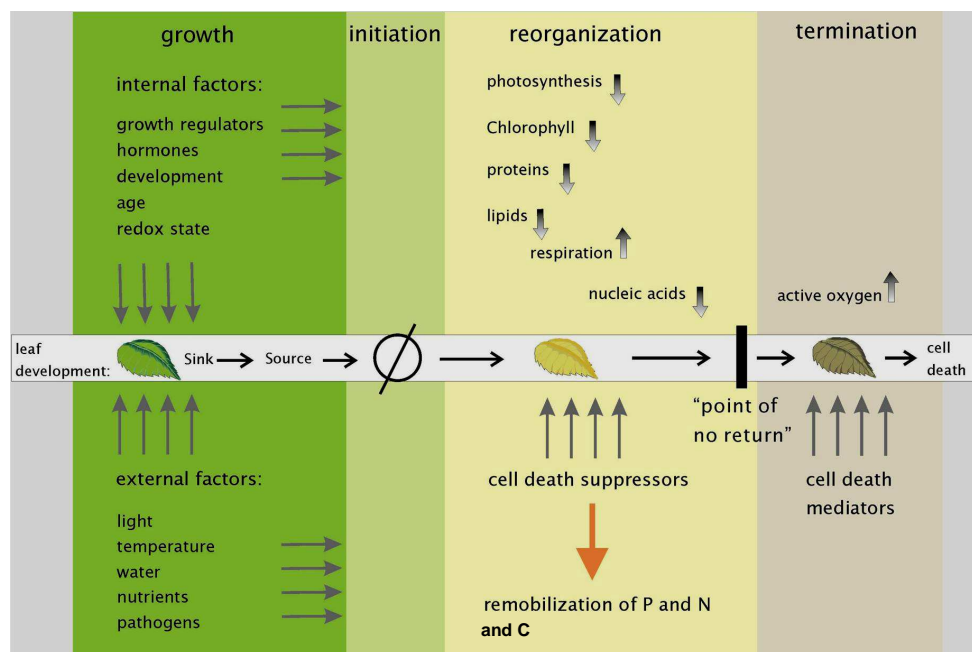
### 1.1.1 Overview

Leaf senescence in autumn leaves is one of the most beautiful phenomena observed in nature (Lim *et al.*, 2003). Green leaves on trees and other perennial plants turn yellow, orange and red before they become brown, die and are discarded from the plant (Buchanan-Wollaston *et al.*, 2003). Annual plants such as crops undergo a similar process turning from green to yellow (Buchanan-Wollaston *et al.*, 2003). This visible change of leaf color reflects the loss of chlorophyll and is accompanied by a variety of physiological, cellular, molecular and metabolic events, that are regulated very precisely (Lim *et al.*, 2003). With respect of agricultural aspects, leaf senescence limits the yield of crop plants and affects the shelf life of leafy vegetables. Therefore, the analysis of the underlying key components and mechanisms that control this last step of development are of great interest (Gan and Amasino, 1997; Quirino *et al.*, 2000; Gepstein, 2004; Lim *et al.*, 2007). Previously senescence was regarded as a negative and inevitable process. However, it is now thought to be an important, integral part of differentiation and development (Gepstein, 2004).

Since the processes, mechanisms and key components of leaf senescence are quite complex, it is useful to divide leaf senescence into several stages (Fig. 1.1): initiation, reorganisation and termination (Noodén *et al.*, 1997; Yoshida, 2003). The induction of leaf senescence that already occurs during normal growth and development is considered as a separate, additional stage (Fig. 1.1).

During normal growth and development of the leaf, several internal and external factors are thought to be responsible for the induction of senescence (Fig.1.1). Internal factors are represented by a variety of phytohormones and other growth regulators. The best evidence for hormonal involvement in the induction of leaf senescence has been shown for the phytohormone cytokinin, which is known to retard the onset of senescence (Buchanan-Wollaston *et al.*, 2003). In contrast, the gaseous phytohormone ethylene is known to induce the senescence syndrome (Buchanan-Wollaston *et al.*,

2003). In addition to these classical phytohormones, other growth regulators such as jasmonic acid (He *et al.*, 2002), salicylic acid (Morris *et al.*, 2000) and brassinosteroids (Clouse, 1996) are involved in the induction of leaf senescence.



**Fig. 1.1 Progression of leaf senescence in higher plants.** The scheme reflects the three-stage theory (Noodén *et al.*, 1997) dividing leaf senescence into initiation, reorganisation & termination. In addition, the processes which take place during normal growth to initiate senescence are indicated. This figure was kindly provided by Prof. Dr. K. Krupinska.

Moreover, sugar metabolism and sugar signaling are amongst the internal factors inducing the initiation of leaf senescence (Wingler *et al.*, 2006; Wingler and Roitsch, 2008; Wingler *et al.*, 2009), though it is still unclear, whether sugar accumulation or sugar starvation causes the onset of leaf senescence (van Doorn, 2008). Moreover, the development (e.g. the reproductive organs), the redox state, the age of the whole organism and the age of the single leaf are crucial internal factors in the initiation of leaf senescence (Fig. 1.1; Noodén and Penney, 2001; Zentgraf *et al.*, 2004). External factors regulating the onset of leaf senescence are the light conditions, ozone and UV-B, the temperature and the maintenance of water and nutrients. Additionally, the infection with pathogens is able to induce the senescence program (Fig. 1.1; Buchanan-Wollaston *et al.*, 2003).

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After leaf senescence is induced by the internal and external factors mentioned above, the initiation phase itself is characterised by first regulatory cascades (Yoshida, 2003). These result in a sink-source transition in the leaf with a switch from metabolism to catabolism (Yoshida, 2003).

The initiation of leaf senescence is followed by the reorganisation phase where the degradation of (macro)molecules and remobilisation of nutrients take place (Fig. 1.1; Quirino *et al.*, 2000; Himelblau and Amasino, 2001). The degradation of chlorophylls, proteins, lipids and nucleic acids is carried out by the enzymes of the chlorophyll catabolism pathway, several endo- and exopeptidases, lipases and nucleases, respectively (Fig. 1.1; Buchanan-Wollaston *et al.*, 2003; Lim *et al.*, 2007). The function of plant organelles is differentially affected. The number of chloroplasts decreases quite early (Krupinska, 2006) and a dramatic decrease of photosynthetic activity is observed (Krupinska and Humbeck, 2004). On the other hand, mitochondria, which provide energy by respiration, remain intact until later stages of senescence (Lim *et al.*, 2007). These degenerative processes are accompanied by a massive remobilisation of metals, phosphorus, carbohydrates and nitrogen to growing areas of the plant, such as young leaves, developing seeds, flowers and fruits (Quirino *et al.*, 2000; Himelblau and Amasino, 2001; Lim *et al.*, 2003). Oshima *et al.* (2007) and Meskauskiene *et al.* (2009) have shown that the remobilisation of nutrients is able to continue as long as so called cell death suppressors (e.g. Bax inhibitor-1 or SOLDAT10) are active (Fig. 1.1).

Once nutrients have been remobilised and recycled in the second reorganisation stage of senescence, a “point of no return” occurs, which renders the senescence syndrome irreversible (Fig. 1.1; Noodèn *et al.*, 1997; Thomas *et al.*, 2003; van Doorn, 2005). The terminal step consists of a programmed cell death (PCD), which is characterised by hallmarks of apoptosis, such as chromatin condensation and DNA laddering (Delorme *et al.*, 2000; Simeonova *et al.*, 2000; Yoshida, 2003). In addition, the vacuole plays a key role during PCD in the terminal stage of leaf senescence, as it accumulates several secondary metabolites and terminates the senescence-related PCD by becoming autolytic (Thomas *et al.*, 2003).

### 1.1.2 Senescence-associated genes (SAGs)

Over the last decade, many research groups have analysed the changes in gene expression that take place during leaf senescence (Fig. 1.1; Gan and Amasino, 1997; Kleber-Janke and Krupinska, 1997; Swidzinski *et al.*, 2002; Buchanan-Wollaston *et al.*, 2003; Gepstein *et al.*, 2003; Zentgraf *et al.*, 2004; Guo *et al.*, 2004; Lin and Wu, 2004). Senescence-associated genes (SAGs) are either exclusively induced or simply up-regulated, so called class I and class II SAGs, respectively (Gan and Amasino, 1997). In contrast to the SAGs, the senescence down-regulated genes (SDGs) show decreased transcription level very early (Gan and Amasino, 1997) and are mainly represented by genes related to photosynthesis such as *cab*, *rbcL* and *rbcS* encoding the chlorophyll-*a/b*-binding protein CAB and the large and small subunit of Rubisco, respectively (Gan and Amasino, 1997). Interestingly, 185 genes for transcription factors (TFs) show altered expression during pre-senescence and early stages of leaf senescence (Balazadeh *et al.*, 2008). Apart from genes of transcriptional regulation, genes involved in the degradation of macromolecules, such as genes encoding proteases, lipases and nucleases, show changes in expression (Tab. 1.1; Gepstein, 2004). Furthermore genes for nutrient recycling appear to be important (Tab. 1.1; Gepstein, 2004): in a large variety of plants, higher mRNA levels of cytosolic glutamine synthase (*gs1*) and mitochondrial glutamate dehydrogenase (*gdh*) genes have been observed during leaf senescence (Masclaux *et al.*, 2000; Masclaux-Daubresse *et al.*, 2005; Masclaux-Daubresse *et al.*, 2006; Pageau *et al.*, 2006; Masclaux-Daubresse *et al.*, 2008). Masclaux-Daubresse and co-workers demonstrated, that GS1 and GDH enzymes, which are involved in the metabolism of amino acids dedicated to phloem loading, play a key role in the remobilisation of nitrogen (Masclaux-Daubresse *et al.*, 2008). Finally, genes related to defense and cell rescue and further signal transduction also show changes in expression levels during leaf senescence (Tab. 1.1; Gepstein, 2004).

**Tab.1.1 Major functional categories of senescence-associated genes** (from Gepstein, 2004, modified)

functional category	associated processes	abundant genes
macromolecule degradation	breakdown of proteins, nucleic acids, lipids & polysaccharides	genes coding for cysteine proteases, nucleases, phospholipases, lipases/acylhydrolases, $\beta$ -glucosidase, glucanases, pectinesterases, polygalacturonase
nutrient recycling & remobilisation	transport of peptides, amino acids, sugars, purines, pyrimidines & ions	genes coding for oligopeptide transporters, ammonium transporters, purine & pyrimidine transporters, glutamine synthase, glutamate dehydrogenase, sugar transporters, ABC transporters
defense & cell rescue mechanisms	abiotic & biotic stress, oxidative stress	genes encoding metallothionein, glutathione <i>S</i> -transferase, protein similar to jasmonate-inducible protein, glutathione peroxidase, cold-regulated protein COR6.6
transcriptional regulation	transcription factors	genes encoding zinc finger proteins, bZIP proteins, HMG-box proteins and transcription factors of the WRKY, NAC, AP2, MYB, HB, TCP & GRAS families
signal transduction	protein phosphorylation and dephosphorylation	genes encoding receptor-like kinases, components of MAP kinase signal cascades, phosphatases, phospholipases, calcium-dependent protein kinases, cytoskeleton-associated proteins

### 1.1.3 Degradation of proteins

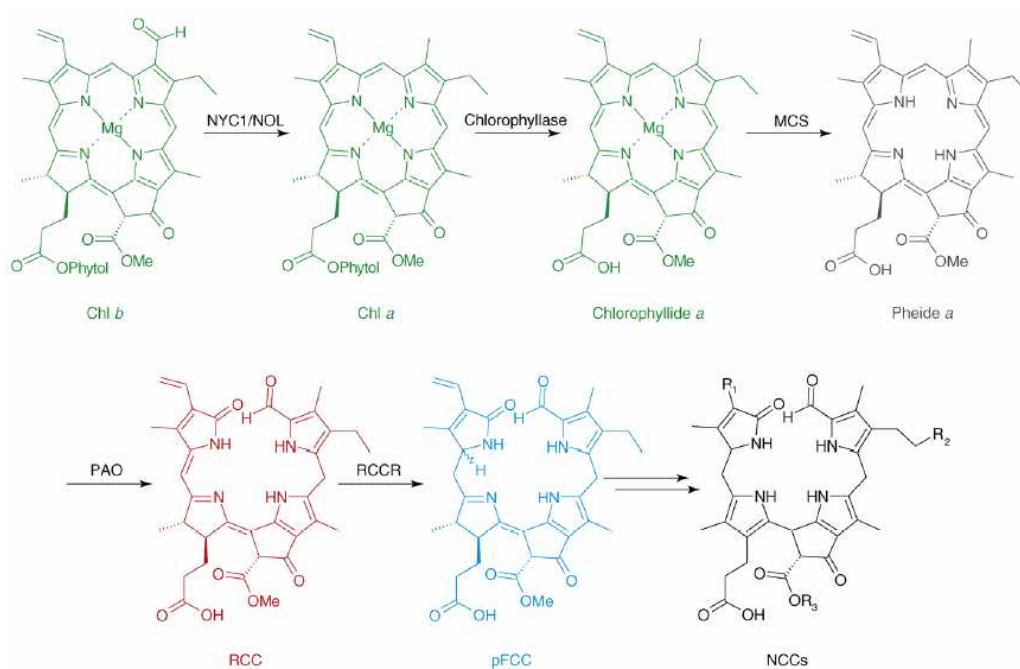
The degradation of proteins has been investigated to a great extent in the past. In particular, the degradation of the plastidic ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is often used to monitor the progression of leaf senescence (Hörtensteiner and Feller, 2002; Feller *et al.*, 2008; Gregersen *et al.*, 2008). Rubisco is the predominant protein in

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photosynthesising parts of plants and the most abundant protein on earth (Feller *et al.*, 2008). The high percentage of leaf nitrogen bound in the Rubisco illustrates the importance of this stromal chloroplast enzyme. Apart from its functions in the assimilation of inorganic carbon Rubisco is essential for the nitrogen budget of a plant (Feller *et al.*, 2008). Despite intensive studies, the proteases, detailed loci and mechanisms responsible for the degradation of Rubisco have not been identified up to date (Gregersen *et al.*, 2008). That degradation takes place within the plastid itself is supported by the observation that chloroplasts contain active proteases. However, it seems that within the plastid this process is mainly related to chlorophyll binding proteins such as LHCII (Martínez *et al.*, 2008). The breakdown of plastidic chlorophyll-binding proteins associated to the thylakoid membrane has been investigated to a lesser extent (Hörtensteiner and Feller, 2002). Stromal Rubisco is released (after an initial pre-cleavage) from the organelle via Rubisco-containing bodies (RCBs), that may fuse with protease-containing, senescence-associated vesicles in the cytoplasm later on which results in further digestion of Rubisco (SAVs; Krupinska, 2006; Gregersen *et al.*, 2008; Martínez *et al.*, 2008). Subsequently, the fusion vesicles could traffic to the central vacuole, where protein fragments and peptides might be degraded to completion and amino acids are stored until they are redistributed and remobilised to other parts of the plant (Martínez *et al.*, 2008).

#### **1.1.4 Degradation of chlorophylls**

Of course, the degradation of chlorophyll-binding proteins requires the simultaneous catabolism of chlorophylls indicating that both degradation pathways are at least partially interconnected (Hörtensteiner and Feller, 2002, Hörtensteiner, 2009). Since the beginning of senescence research the degradation of chlorophyll was a classical biomarker for senescence (Ougham *et al.*, 2008). Interestingly, nitrogen present in chlorophyll is not exported from senescing leaves, but remains within the cells in form of linear tetrapyrrolic catabolites that accumulate in the vacuole (Hörtensteiner and Feller, 2002).



**Fig. 1.2 Pathway for chlorophyll breakdown in higher plants** (from Hörtensteiner, 2009). The chemical structures of chlorophylls and chlorophyll catabolites are shown. R<sub>1</sub>-R<sub>3</sub> indicates the presence of species-specific modification in NCCs from different plants. Abbreviations: chl, chlorophyll; FCC, fluorescent chl catabolite; MCS, metal chelating substance; NCC, non-fluorescent chl catabolite; NYC1/NOL, NON YELLOW COLORING1/NYC-ONE LIKE; PAO, pheide-*a*-oxygenase; pFCC, primary FCC; pheide, pheophorbide; RCC, red fluorescent chl catabolite; RCCR, RCC reductase.

For many years, chlorophyll catabolism during leaf senescence and fruit ripening was considered as a biological enigma (Hörtensteiner, 2009). Only the identification and structure determination of non-fluorescent chlorophyll catabolites (NCCs) as the final products of breakdown allowed the stepwise elucidation of the chlorophyll degradation pathway (Fig. 1.2), which is common in higher plants (Hörtensteiner, 2009). The separation of phytol from chlorophyll *a* by chlorophyllase yields chlorophyllide *a*, which has a small effect on the color (Hörtensteiner, 2009). After a metal chelating substance (MCS) has removed the central Mg<sup>2+</sup>-ion, pheophorbide-*a*-oxygenase (PAO) opens the pheophorbide ring (Hörtensteiner, 2009). The resulting red chlorophyll catabolite (RCC) is converted by RCC reductase (RCCR) to the fluorescent chlorophyll catabolite (FCC), a colorless product that fluoresces under UV-light (Hörtensteiner, 2009). Chlorophyll breakdown ends with the accumulation of one or more non-fluorescent catabolites (NCCs; Hörtensteiner, 2009).



### 1.1.5 Stay-green organisms

In the past, many so called stay-green organisms characterised by the retention of chlorophyll during leaf senescence have been identified (Hörtensteiner, 2009). These stay-green plants can be categorised into two major groups: on the one hand functional stay-greens and on the other cosmetic ones (Howard *et al.*, 2002; Hörtensteiner, 2009). In cosmetic stay-greens the retention of chlorophylls is accompanied by a loss of photosynthetic function, whereas in functional stay-greens the retention of green colour is linked to the retention of photosynthetic activity (Howard, *et al.*, 2002; Hörtensteiner, 2009).

An established representative of cosmetic stay-green plants is the *Lolium/Festuca* mutant generated by the introgression of the *Festuca pratensis* stay-green locus (*sgr*) into *Lolium* species (Howard *et al.* 1997; Howard *et al.*, 2002; Hörtensteiner, 2009). Historically, defects in the *sgr* genes of many plants had been correlated to reduced activity or content of the pheophorbide-*a*-oxygenase (PAO; Fig. 1.2), but recent molecular cloning of PAO and the availability of PAO antibodies showed that SGR acts independently of PAO (Hörtensteiner, 2009). It is more likely that SGR proteins are candidates for protein factors involved in the LHCII disassembly, and the absence of SGR during senescence causes a retention of chlorophyll within the stable apoproteins (Hörtensteiner, 2009).

A well-known example for functional stay-green organisms is the transgenic *SAG12-IPT* tobacco line with an autoregulated, senescence-specific production of cytokinin (Gan and Amasino, 1995). The *SAG12-IPT* tobacco exhibits a the retention of chlorophylls due to reduced chlorophyllase activity and the stimulation of chlorophyll biosynthesis (Hare and Staden, 1997), but also a delay of other senescence-related processes.

Firstly, the decrease of photosynthetic activity is delayed as indicated by measurements of CO<sub>2</sub> uptake, the maximal efficiency of PSII ( $F_v/F_m$ ) and the effective quantum yield of PSII (Gan and Amasino, 1995; Wingler *et al.*,

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1998; Procházková *et al.*, 2008). Accordingly, the decline of photosynthetic proteins such as Rubisco is delayed (Wingler *et al.*, 1998; Jordi *et al.*, 2000).

Secondly, the retention of chlorophylls is accompanied by a retarded remobilisation of nutrients as cytokinin causes an increased sink-strength in mature leaves of higher plants (Roitsch and Ehneß, 2000; Cowan *et al.*, 2005; Sýkorova *et al.*, 2008). The delayed remobilisation of carbohydrates which is represented by a high content of soluble sugars (Wingler *et al.*, 1998) can be explained by the induction of the extracellular invertase and hexose transporters that are crucial for apoplastic phloem unloading (Roitsch and Ehneß, 2000; Balibrea Lara *et al.*, 2004). As a consequence, the import of carbohydrates into the sink-cells is increased whereas the export is decreased (Roitsch and Ehneß, 2000; Balibrea Lara *et al.*, 2004). Also the remobilisation of nitrogen is affected: due to the delayed degradation of photosynthetic proteins senescent leaves of *SAG12-IPT* plants accumulate nitrogen and the translocation to non-senescent leaves is reduced (Jordi *et al.*, 2000; Cowan *et al.*, 2005). Especially under low nitrogen conditions, the impairment of remobilisation processes is responsible for the observation, that *SAG12-IPT* tobacco turns directly into cell death at the end of leaf development (Wingler *et al.*, 2005).

Thirdly, the decrease of the antioxidant system which occurs during leaf ageing is retarded in the functional stay-green organism: the activity of the antioxidative enzymes ascorbate peroxidase (APX), glutathione reductase (GR) and superoxide dismutase (SOD) as well as the content of the water soluble antioxidants ascorbate and glutathione, decreases slower in senescing leaves of the *SAG12-IPT* compared to a normal senescing control line (Dertinger *et al.*, 2003). Interestingly, the amount of the lipid soluble  $\alpha$ -tocopherol, which is also thought to participate in the antioxidative protection, is increased in *SAG12-IPT* tobacco as well as in the control dependent on leaf age suggesting that  $\alpha$ -tocopherol may have another function that is not necessarily related to its antioxidant property (Dertinger *et al.*, 2003).

## 1.2 Tocopherols

### 1.2.1 Common traits

In 1922, Evans and Bishop discovered a nutritional factor essential for the reproduction of rats. The name for that molecule was derived from the Greek *tokos* for childbirth, *pherein* for bringing forth and the suffix *ol* for alcohol tocopherol (Evans *et al.*, 1936; Wagner *et al.*, 2004). Tocopherols belong to the group of vitamin E compounds, which are important for human nutrition and health. They can be divided into four forms, namely  $\alpha$ -tocopherol,  $\beta$ -tocopherol,  $\gamma$ -tocopherol and  $\delta$ -tocopherol. Tocopherols are exclusively synthesised by oxygenic and photosynthetic organisms such as plants, algae and some cyanobacteria (Munné-Bosch, 2005). In higher plants, they are mainly found in seeds, fruits and leaves (Munné-Bosch and Alegre, 2002). On the cellular level tocopherols are found in chloroplasts, more precisely in the chloroplast envelope where they are synthesised (Soll *et al.*, 1985; Arango and Heise, 1998), in plastoglobuli of the chloroplast stroma where they are stored (Lichtenthaler *et al.*, 1981), and in thylakoid membranes (Fryer, 1992; Havaux, 1998).

### 1.2.2 Tocopherols as antioxidants

Due to their localisation tocopherols are thought to be important in maintaining membrane integrity and fluidity (Gomez-Fernandez *et al.*, 1989; Wang and Qinn, 1999; Munné-Bosch and Alegre, 2002). Of course, the function as an antioxidant is closely related to their chemical structure, but the effects in the cell are linked to their localisation: tocopherols are known to reduce reactive oxygen species (ROS) continuously generated in higher plants as byproducts of photosynthesis and to protect polyunsaturated fatty acid chains (PUFAs) from lipid peroxidation (Li *et al.*, 2008). Tocopherols are not only able to scavenge hydroxyl radicals ( $\text{OH}^\bullet$ ), superoxide anions ( $\text{O}_2^-$ ) and lipid peroxyl radicals ( $\text{ROO}^\bullet$ ) by being converted into the tocopheroxyl radical, they also play a key role as antioxidants by physically quenching or chemically scavenging singlet oxygen ( $^1\text{O}_2$ ) (Munné-Bosch and Alegre, 2002). Under conditions of photoinhibition and extensive D1 protein turnover  $\alpha$ -tocopherol may have a

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photoprotective function as singlet oxygen scavenger in PSII (Kruk *et al.*, 2005). Cellular redox systems such as ascorbate, glutathione, carotenoids and tocopherols represent the nonenzymatic part of the antioxidative network, and might also play an important role under environmental stress conditions (Apel and Hirth, 2004). The enzymatic part of the antioxidative network is represented by enzymes like the superoxide dismutase (SOD) converting the super oxide anion to hydrogen peroxide ( $H_2O_2$ ) and the catalases that convert  $H_2O_2$  to water. The same reaction is catalysed by the ascorbate peroxidase (APX) which is an enzyme of the water-water-cycle (Mittler, 2002; Mittler *et al.*, 2004). The water-water-cycle, also called Halliwell-Asada cycle (Asada, 1999), is not only involved in the detoxification of  $H_2O_2$ , it is also required for the conversion of tocopheroxyl radicals back to tocopherol (Li *et al.*, 2008).

### **1.2.3 Tocopherols as signaling molecules**

For mammals and humans, it is well-established that tocopherols might also play a role as signaling molecules or transcriptional regulators (Azzi *et al.*, 2004; Zingg and Azzi, 2004). Accordingly, recent studies on higher plants showed that tocopherols may have similar functions (Li *et al.*, 2008). Tocopherols appear to function as signaling molecules in the regulation of carbohydrate metabolism (Dörmann, 2007; Li *et al.*, 2008). In 1996, Russin *et al.* described the *sdx1* (*sucrose export deficient1*) mutant of maize that was defective in sugar export from source leaves. Some years later, this mutant was characterised in more detail and shown to be a VTE1 tocopherol cyclase mutant, and thus deficient in tocopherols (Provencher *et al.*, 2001; Porfirova *et al.*, 2002; Sattler *et al.*, 2003; Kumar *et al.*, 2005). By a transgenic approach using a RNAi-potato the *vte1* gene encoding the tocopherol cyclase was silenced. This showed that the tocopherol deficiency resulted in a sugar export block (Hofius *et al.*, 2004). This data suggests, that tocopherols are involved in the regulation of carbohydrate metabolism (Li *et al.*, 2008; Dörmann, 2007). Furthermore, studies on the cyanobacterium *Synechocystis* sp. PCC 6803 indicate, that  $\alpha$ -tocopherol probably has a non-antioxidant function in macronutrient homeostasis (Sakuragi *et al.*, 2006).

### **1.2.4 Biochemistry and biosynthesis of tocopherols**

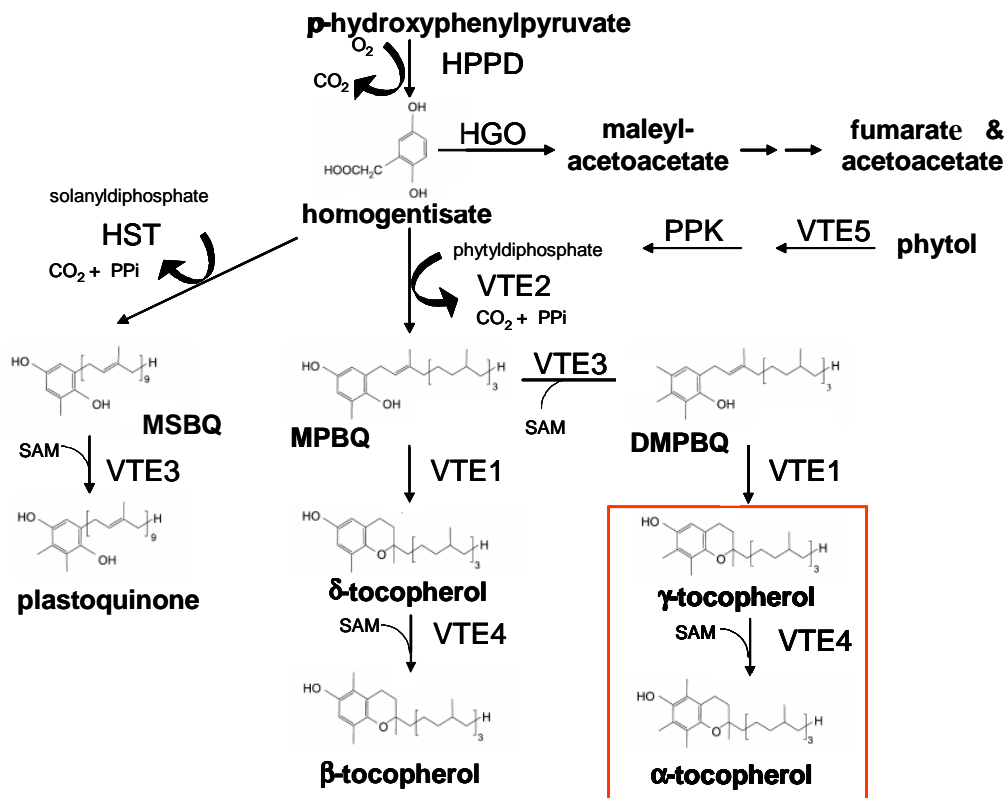
Tocopherols are amphipathic molecules with a hydrophilic head group, which is generated from homogentisate, and a hydrophobic phytyl tail derived from phytyldiphosphate. In conjunction with tocotrienols, which contain an unsaturated phytyl tail derived from geranylgeranyl diphosphate, tocopherols are known as vitamin E in medicine and nutritional sciences. Tocopherols, as well as tocotrienols, exist in four forms differing in the number and position of methyl groups on the aromatic ring, known as  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -forms (Li *et al.*, 2008).

The pathway of tocopherol biosynthesis (Fig. 1.3) was elucidated in the mid of the 1990s using the model organisms *Arabidopsis thaliana* and *Synechocystis* sp. PCC6803 by cloning the genes coding for the enzymes of the pathway (Li *et al.*, 2008). The head groups of tocopherols are derived from the shikimate pathway via the *p*-hydroxyphenylpyruvate dioxygenase (HPPD) that converts *p*-hydroxyphenylpyruvate into homogentisate (Schulz *et al.*, 1993; Forbes and Hamilton, 1994; Kleber-Janke and Krupinska, 1997; Falk *et al.*, 2003). In contrast to all the other enzymes of the pathway, the HPPD is not localised in the plastids, but in the cytosol (Garcia *et al.*, 1997). Homogentisate represents a key molecule, as it serves as a precursor of all forms of tocopherols, tocotrienols and plastoquinone (Whistance and Threlfall, 1970; Soll *et al.*, 1980, DellaPenna, 2005). In addition, homogentisate is part of the tyrosine aromatic amino acid catabolism as homogentisate 1,2-dioxygenase (HGO) is able to convert it into maleylacetoacetate, which is degraded further to fumarate and acetoacetate (Dixon and Edwards, 2006). The involvement of homogentisate in plastoquinone biosynthesis was demonstrated by a tocopherol and plastoquinone deficient mutant of *Arabidopsis thaliana* affected in HPPD activity (Norris *et al.*, 1995, 1998). For the synthesis of plastoquinone, homogentisate is converted into 2-methyl-6-solanyl-1,4-benzoquinone (MSBQ) by the homogentisate solanyltransferase (HST) and MSBQ yields plastoquinone by methylation via MSBQ methyltransferase (VTE3; Sadre *et al.*, 2006). To synthesise tocopherols, homogentisate is prenylated with phytyl diphosphate by homogentisate phytyltransferase (HPT, VTE2) to yield 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) DellaPenna, 2005).

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The recent characterisation of the *vte5* mutant of *Arabidopsis thaliana* revealed that phytol is phosphorylated by the phytol kinase VTE5 (Valentin *et al.*, 2006). The *vte5* mutant of *Arabidopsis thaliana* shows reduced seed and leaf tocopherol, and a knock-out mutant of the *Synechocystis* homologue also led to reduced tocopherol accumulation in cyanobacteria, which underlines the importance of this gene in tocopherol biosynthesis (Valentin *et al.*, 2006). Interestingly, gas chromatography-mass-spectrometry analysis of the *Arabidopsis* and *Synechocystis* mutants showed an accumulation of free phytol and the synthesis of *Arabidopsis* VTE5 protein in *E. coli* in the presence of free phytol led to the formation of phytolmonophosphate (PMP; Valentin *et al.*, 2006). In addition to these findings the activity of a second kinase, the phytyl-phosphate kinase, was identified in *Arabidopsis thaliana* (Ischebeck *et al.*, 2006). Both kinases were localised in the chloroplast envelope, where tocopherol biosynthesis takes place (Ischebeck *et al.*, 2006; Valentin *et al.*, 2006).

The next step in the synthesis of tocopherols in *Arabidopsis thaliana* is either the cyclisation of MPBQ by the tocopherol cyclase (VTE1; Porfirova *et al.*, 2002; Sattler *et al.*, 2003; Kanwischer *et al.*, 2005) or the methylation at the C-3 position by MPBQ methyltransferase (VTE3; Cheng *et al.*, 2003), to yield  $\delta$ -tocopherol or 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ), respectively. VTE3 methyltransferase is essential for both tocopherol and plastoquinone synthesis, since a mutant of *Arabidopsis thaliana* carrying an insertion in *vte3* is also deficient in plastoquinone (Motohashi *et al.*, 2003). Subsequent methylation of  $\delta$ -tocopherol by the  $\gamma$ -tocopherol methyltransferase (VTE4) results in the formation of  $\beta$ -tocopherol (Shintani and DellaPenna, 1998), whereas the cyclisation of DMPBQ by the tocopherol cyclase VTE1 generates  $\gamma$ -tocopherol (Porfirova *et al.*, 2002; Sattler *et al.*, 2003; Kanwischer *et al.*, 2005), the major form found in seeds (Munné-Bosch and Falk, 2004).



**Fig. 1.3 Biosynthesis of tocopherols and plastoquinone.** HPPD, 4-hydroxyphenylpyruvate dioxygenase; HGO, homogentisate 1,2-dioxygenase; HST, homogentisate solanyltransferase; PPK, phytylphosphatekinase; VTE1, tocopherol cyclase; VTE2, homogentisate phytyltransferase; VTE3, MSBQ / MPBQ methyltransferase; VTE4,  $\gamma$ -tocopherol methyltransferase; VTE5, phytolkinase; MSBQ, 2-methyl-6-solanyl-1,4-benzoquinone; MPBQ, 2-methyl-6-phytyl-1,4-benzoquinone; DMPBQ, 2,3-dimethyl-5-phytyl-1,4-benzoquinone; SAM, *S*-adenosyl methionine;  $PP_i$ , pyrophosphate (modified from DellaPenna, 2005).

Finally,  $\gamma$ -tocopherol methyltransferase (VTE4) converts  $\gamma$ -tocopherol to  $\alpha$ -tocopherol (Shintani and DellaPenna, 1998), the major form of tocopherols in green plant tissue such as leaves (Munné-Bosch and Falk, 2004).

### 1.2.5 The regulation of tocopherol biosynthesis

As tocopherols are part of an antioxidative network, it is no surprise, that tocopherol biosynthesis is regulated by ROS and photosynthesis. Photosynthesis can yield ROS and the accumulation of ROS will induce antioxidant-related gene expression (Apel and Hirt, 2004; Li *et al.*, 2008). Interestingly, the promoters of photosynthesis-related genes and genes of tocopherol biosynthesis possess the same ATCTA sequence element,

suggesting common regulatory mechanisms (Welsch *et al.*, 2003). Furthermore, several studies indicate, that tocopherol biosynthesis might be regulated by plastoquinone biosynthesis, as the *hpd* gene as well as the gene encoding the VTE3-methyltransferase play a role in both tocopherol and plastoquinone biosynthesis (Fig. 1.2; Falk *et al.*, 2002; Li *et al.*, 2008). In addition, there is evidence that the phytohormones abscisic acid (ABA) and ethylene and growth regulators such as jasmonic acid and salicylic acid (SA) are able to affect tocopherol biosynthesis. Jasmonic acid is known to accumulate under stress conditions. As a consequence, photosynthesis is inhibited, but genes important for chlorophyll degradation, the synthesis of anthocyanin and antioxidants, including tocopherols, are induced (Falk *et al.*, 2002; Munné-Bosch and Alegre 2002; Sandorf and Holländer-Czytko, 2002; Munné-Bosch and Falk, 2004). In turn, the increased level of tocopherols will cause a negative feedback on the level of jasmonic acid as already mentioned above (Munné-Bosch and Falk, 2004; Li *et al.*, 2008). A relationship between tocopherol biosynthesis and ABA is likely, since tocopherol biosynthesis genes such as *hpd* were shown to contain a ABA response element in the promoter (Dähnhardt *et al.*, 2002; Li *et al.*, 2008). Moreover, it has been reported that SA and the gaseous phytohormone ethylene have an impact on tocopherol biosynthesis, as the endogenous SA concentration correlates with the concentration of  $\alpha$ -tocopherol in drought stressed plants, and mutants of ethylene signaling and perception exhibit a sharp increase in the level of  $\alpha$ -tocopherol during leaf ageing, respectively (Munné-Bosch and Peñuelas, 2004; Cela *et al.*, 2009).

To summarise, it is clear that tocopherols are part of an antioxidant network and their biosynthesis is regulated by various internal and external signals (Li *et al.*, 2008).

#### **1.2.6 The accumulation of tocopherols during leaf senescence**

When isolating cDNA clones for genes exhibiting an enhanced expression in barley leaves during dark-induced and natural senescence under field conditions, Kleber-Janke and Krupinska (1997) found a cDNA with a significant homology to the sequence of the human 4-hydroxyphenyl-



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pyruvate dioxygenase, which was known to play a role in tyrosine metabolism (Ruetschi *et al.*, 1993). Measurement of tocopherols in senescent barley leaves revealed that the senescence-associated expression of the *hpd* gene correlates with an increased tocopherol content of approximately two fold for  $\alpha$ -tocopherol and even approximately ten fold for  $\gamma$ -tocopherol (Chrost *et al.*, 1999). As early as 1989, the  $\alpha$ -tocopherol content was shown to be increased during leaf senescence of *Melia azedarach* (China tree), *Vinca major* (greater periwinkle) and *Citrus sinensis* (orange). Additionally, the  $\alpha$ -tocopherol accumulation was detected in orange fruits and citrus fruit peel (Rise *et al.*, 1989; Sawamura *et al.*, 1986). The level of  $\alpha$ -tocopherol was not elevated in senescent leaves of *Petroselinum sativum* (parsely), *Apium graveolens* (celery) and *Nicotiana tabacum* (Rise *et al.*, 1989). This last finding about tobacco is in disagreement with data published many years later (Dertinger *et al.*, 2003; Falk *et al.*, 2003; Abbasi *et al.*, 2007). Although the same HPLC-method was used, it was clearly demonstrated that the content of  $\alpha$ -tocopherol increases constantly during leaf age of several lines of *Nicotiana tabacum*. Obviously, the sensitivity of the HPLC-method had been improved over the years. The analysis of tocopherols focuses very much on  $\alpha$ -tocopherol, as only few data exist about  $\gamma$ -tocopherol (or the less important isoforms  $\beta$ - and  $\delta$ -tocopherol). In the case of  $\gamma$ -tocopherol, it was observed that senescent leaves of tobacco overexpressing the barley 4-hydroxyphenylpyruvate dioxygenase show an approximately ten fold higher content of  $\gamma$ -tocopherol in senescent leaves when compared to the content in a young leaves (Falk *et al.*, 2003).

The functions of tocopherols during leaf senescence are still not clear to date. On the one hand some functions point to its the classical antioxidative function (Dertinger *et al.*, 2003; Abbasi *et al.*, 2009), on the other hand it is often also considered as a passive consequence of the chlorophyll breakdown that also occurs during leaf senescence (Rise *et al.*, 1989; Dörmann, 2007). It has been hypothesised that excess amounts of phytol resulting from chlorophyll breakdown during senescence might be deposited in form of tocopherols in the chloroplast (Rise *et al.*, 1989; Dörmann, 2007).

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### **1.2.7 Senescence-related phenotypes of mutants and transgenic lines affected in tocopherol biosynthesis**

In order to investigate the relationship between senescence and tocopherols, it is not only necessary to measure the tocopherol content in senescent leaves of various plant species. It is also important to study the senescence behaviour of transgenic lines and mutants impaired in tocopherol biosynthesis.

Very recently, it has been reported that a transgenic *vte2*-RNAi-line of tobacco (in the background of Samsun) which is silenced for homogentisate phytyltransferase (HPT, VTE2), and thus is tocopherol-deficient, showed an accelerated leaf senescence in the lower leaves as indicated by an elevated level of the GS protein, reduced *rbcs* transcript amounts, an earlier decrease of chlorophyll content and an increased level of oxidative stress (Abbasi *et al.*, 2009). Unexpectedly, the transgenic line showed no block of sugar export known from other tocopherol deficient plants like the already mentioned RNAi-potato (Hofius *et al.*, 2004) and *sdx1* maize mutant (Provencher *et al.*, 2001; Porfirova *et al.*, 2002), both lacking a functional tocopherol cyclase (Li *et al.*, 2008).

In addition, the diploma thesis of Thorsten Walter (2004) indicated, that the *vte4* knock-out mutant of *Arabidopsis thaliana* shows a shortened life cycle and accelerated leaf senescence. This suggests a specific role for  $\gamma$ -tocopherol during leaf senescence.

Although the research on the phenotype of transgenic lines and mutants with a disturbance in tocopherol biosynthesis was quite intensive, no further senescence-related phenotype was identified, neither in a transgenic 35S-*vte4*-RNAi tobacco nor in several tocopherol-deficient knock-out mutants or transgenic lines overexpressing a gene of the tocopherol biosynthesis pathway of *Arabidopsis thaliana*.

### **1.3 Aims of this study**

A major goal of this project is to elucidate the role of tocopherols during leaf senescence of higher plants. Since it is known that tocopherols accumulate during that last phase of leaf development, it is hypothesised that this accumulation is an inevitable passive consequence of chlorophyll breakdown. It was never considered or investigated, whether tocopherol might have any real functions, or whether they are also able to accumulate during senescence when chlorophyll breakdown is prevented. Therefore, tocopherols and chlorophylls will be measured in stay-green organisms such as the *SAG12-IPT* tobacco exhibiting a delay of leaf senescence or the *Festuca/Lolium* mutant with a block in chlorophyll catabolism. In contrast to previous research,  $\gamma$ -tocopherol accumulation should be compared to  $\alpha$ -tocopherol accumulation to reveal putative specific functions of both forms.

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## 2. Material and methods

### 2.1 Basic solutions & chemicals

All chemicals and solutions were purchased from Roth (Karlsruhe, Germany), Sigma (Munich, Germany), Merck (Darmstadt, Germany), Life Technologies (Karlsruhe, Germany) and Invitrogen (Karlsruhe, Germany).

- acetone
- *DAF-FM-(4-amino-5-methylamino-27-difluorofluorescein)* (DAF-FM)
- dithiothreitol (DTT)
- 10x DNA sample buffer: 50% (v/v) glycerine, 0.1% (v/v) bromophenol-blue
- double distilled water (ddH<sub>2</sub>O)
- EDTA
- isopropanol
- kinetin solutions : 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM, 0.1 mM
- 1x Laemmli buffer: 0.025 M Tris/HCl, 0.192 M glycine, 0.1% (v/v) SDS
- Luminogen™ TMA-6 Detection Solution A (Amersham Biosciences, Buckinghamshire, UK)
- Luminogen™ TMA-6 Detection Solution B (Amersham Biosciences, Buckinghamshire, UK)
- magnesium hydroxide carbonate
- β-mercaptoethanol
- 5% (w/v) milk powder solution (ALDI Kaffeeweißer)
- NaCl
- n-heptane
- NO<sub>x</sub> extraction buffer (10 mL): 5 mL sodium phosphate buffer, 4.998 L ddH<sub>2</sub>O, 0.002 mg NaEDTA
- protein extraction buffer: 125 mM Tris/HCl (pH 6.8), 4% (w/v) SDS, 200 μL PMSF, 100 μM DTT
- protein sample buffer: 0.25 M Tris/HCl (pH 6.8), 4% (w/v) SDS, 10 M urea, 2% (v/v) β-mercaptoethanol, 20% (v/v) glycerol

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- Roti® Nanoquant Reagent (Roth, Karlsruhe, Germany)
  - sodium dodecyl sulfate (SDS)
  - 10 x TAE buffer (pH 8.5): 0.4 M Tris/HCl, 0.01 M EDTA, 0.2 M acetic acid
  - 1 x TBST: 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20
  - tris(hydroxymethyl)-aminomethane (Tris)
  - polyoxyethylene(20)-sorbitan-monolaurate (Tween 20)

## 2.2 General equipment

- Balance OL 3100-PCE Max 3100 g (Omnilab, Bremen, Germany)
- Blotting System Fastblot 34 (Biometra, Göttingen, Germany)
- Centrifuge 5415D & 5415R (Eppendorf, Hamburg, Germany)
- Centrifuge 5804R with rotors FA45-30-11 and A-2-MTP (Eppendorf, Hamburg, Germany)
- ECL film (Hyperfilm™ ECL Amersham Biosciences, Buckinghamshire, UK)
- Fine Balance A200S (Sartorius Analytic, Göttingen, Germany)
- Gel Chambers for Protein Electrophoresis (Biometra, Göttingen, Germany)
- Gloves Gentle Skin Classic Large (Meditrade, Kiefersfelde, Germany)
- Heatblock Thermomixer Compact (Eppendorf, Hamburg, Germany)
- HPLC including software Class-VP 7.4 SP1 (Shimadzu, Duisburg, Germany)
- Magnetic Mixer MR3001 (Heidolph, Kelheim, Germany)
- Maxi-Imaging-PAM and corresponding software ImagingWin v 2.14 (Waltz, Effeltrich, Germany)
- Microwave 8017 (Privileg, Fürth, Germany)
- Milipore Water Distillation Seralpur PRO 90 CN (Seral, Munich, Germany)
- Minishaker M2 (IKA, Staufen, Germany)

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- OWL Separation Systems Electrophoresis Chambers Easy Cast B1A (Thermo Scientific, New York, USA)
  - OWL Separation Systems Electrophoresis Chambers Easy Cast B2A (Thermo Scientific, New York, USA)
  - PCR-Cycler Primus 96 plus (MWG Biotech, Ebersberg, Germany)
  - pH-Electrode Seven Easy (Mettler Toledo, Giessen, Germany)
  - Pipettes (10  $\mu$ L, 20  $\mu$ L, 200  $\mu$ L, 1000  $\mu$ L) and tips (Eppendorf, Hamburg, Germany)
  - Premium Kombi Freezer -20 °C and Fridge 4 °C (Liebherr, Bulle, Schweiz)
  - PVDF membrane (BioRad, Munich, Germany)
  - Syngene Digital Graphic Printer UP-D890 (Sony Deutschland, Berlin, Germany)
  - Geno/Grinder 2000 (SPEX CertiPrep, Stanmore, UK)
  - Speed-Vac Master Jet (Heto, Oude Wetering, Netherlands)
  - Steam Autoklave 82525 (Webeco, Selmsdorf, Germany)
  - TFX-20.M UV-Transilluminator (Vilber Lourmat, Eberhardzell, Germany)
  - Ultra Low Freezer CFC Free -85 °C (New Brunswick, Edison, USA)
  - UV-VIS recording spectrophotometer UV-2501 PC (Shimadzu, Duisburg, Germany)
  - Voltage Generator Standard Power Pack P25 (Biometra, Göttingen, Germany)
  - Voltage Generators E863 & E834 (Consort, Turnhout, Belgium)
  - Vortex 2 (IKA, Staufen, Germany)

### 2.3 Enzymes

- *Taq* DNA Polymerase, DNase I and <sup>TM</sup>M-MuLV-Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany)

## 2.4 Markers

- Smart Ladder for DNA (Eurogentec, Köln, Germany)
- Page Ruler™ Prestained Protein Ladder (MBI Fermentas, St. Leon-Rot, Germany)

## 2.5 Commercial kits

Spectrum™ Plant Total RNA Kit (Sigma Aldrich, Munich, Germany)

## 2.6 Oligonucleotides for semi-quantitative RT-PCR

Gene-specific primer pairs were ordered from Sigma (Steinheim, Germany).

- *cab* encoding the chlorophyll-*a/b*-binding protein CAB;  
accession number: AY219853;  
forward primer: 5'-gctggacttcagctgatcc-3';  
reverse primer: 5'-actgccaccagggtaaagtg-3';  
temperature of annealing: 60 °C; cycle number: 28  
Conditions according to Wingler *et al.*, 2005
- *cp1* encoding the cysteine proteinase CP1;  
accession number: AY881011;  
forward primer: 5'-attcatggggcagtaaagggtg-3';  
reverse primer: 5'-cagtctaaccaagtctgcctcaa-3';  
temperature of annealing: 66 °C; cycle number: 27  
(gene sequence published in Beyene *et al.*, 2006)
- *din* encoding DIN involved in molybdenum cofactor biosynthesis;  
accession number: AB026439;  
forward primer: 5'-tgagaattcttcctccc-3';  
reverse primer: 5'-ttcatcatcctcccgaac-3';  
temperature of annealing: 56 °C; cycle number: 30

- 
- *ef1 $\alpha$*  encoding the elongation factor EF1 $\alpha$ ;  
accession number: AF120093;  
forward primer: 5'-tcacatcaacattgtggcattgg-3';  
reverse primer: 5'-ttgatctggcaagagcctcagg-3';  
temperature of annealing: 60 °C; cycle number: 27;  
taken from Wingler *et al.*, 2005
  - *gdh* encoding the glutamate dehydrogenase GDH;  
accession number: TC5199;  
forward primer: 5'-gggtgttctctttgctacgg-3';  
reverse primer: 5'-ctaacggtgacacca cctga-3';  
temperature of annealing: 50 °C; cycle number: 30
  - *gs1* encoding the cytosolic glutamine synthase GS1;  
accession number: X95932;  
forward primer: 5'-aatctctgactccactca-3';  
reverse primer: 5'-aagacct ttccag ctccaat-3';  
temperature of annealing: 54 °C; cycle number: 30
  - *gus* encoding the beta-glucuronidase GUS;  
accession number: NC 000913;  
forward primer: 5'-tcgatgcggtcactcattac-3';  
reverse primer: 5'-atcagcacgttatcgaatcc-3';  
temperature of annealing: 58 °C; cycle number: 30;  
obtained from Dr. Bianca Steffens, University of Kiel
  - *hgo* encoding the homogentisate-1,2-dioxygenase HGO;  
accession number: NC 003076;  
forward primer: 5'-ctgaacacaccttccgtcct-3';  
reverse primer: 5'-gagactcaagccgatccag-3';  
temperature of annealing: 55 °C; cycle number: 30



- 
- *hpd* encoding the 4-hydroxyphenylpyruvate dioxygenase HPPD;  
accession number: TC4709;  
forward primer: 5'-gtacctccttcgtcccgttt-3';  
reverse primer: 5'-tccactacaggacca gctc-3';  
temperature of annealing: 50 °C; cycle number: 30
  
  - *ipt1* encoding the isopentenyltransferase IPT;  
accession number: X14410;  
forward primer: 5'-cggccaactgcacaggaa-3';  
reverse primer: 5'-tttctgttcctgtcgacgcg-3';  
temperature of annealing: 54 °C; cycle number: 32
  
  - *pr1b* encoding the pathogenesis-related protein PR1b;  
accession number: X05453;  
forward primer: 5'-tggatgcccataacacagctg-3';  
reverse primer: 5'-cccccccttaattaagaccac-3';  
temperature of annealing: 60 °C; cycle number: 28;  
taken from Wingler *et al.*, 2005
  
  - *rbcL* encoding the large subunit of Rubisco LSU;  
accession number: M16896;  
forward primer: 5'-gcgctctacgtctggaagat-3';  
reverse primer: 5'-cccccgtaagtagtcatgc-5';  
temperature of annealing: 54; cycle number: 25
  
  - *rbcS* encoding the small subunit of Rubisco SSU;  
accession number: J01308;  
forward primer: 5'-agcacggattgtctatcgtgaaa-3';  
reverse primer: 5'-gtaacattcaaaactgcccc-3';  
temperature of annealing: 66 °C; cycle number: 27

- *vte1* encoding the tocopherol cyclase VTE1;  
accession number: AY536918;  
forward primer: 5'-aggatggcctgctgctt-3';  
reverse primer: 5'-cgcaatgtggtccagga-3';  
temperature of annealing: 54 °C; cycle number: 28

## 2.7 Antibodies for Western blot analysis

- The primary antibody generated in a rabbit and raised against the whole Rubisco protein was kindly provided by Prof. Dr. H. J. Schneider-Pötsch (University of Cologne, Germany). It was diluted 1:1000 in TBST.
- The secondary antibody, raised against rabbit and including the horse-radish peroxidase (HRP), was purchased from GE Healthcare (Chalfont St. Giles, UK).

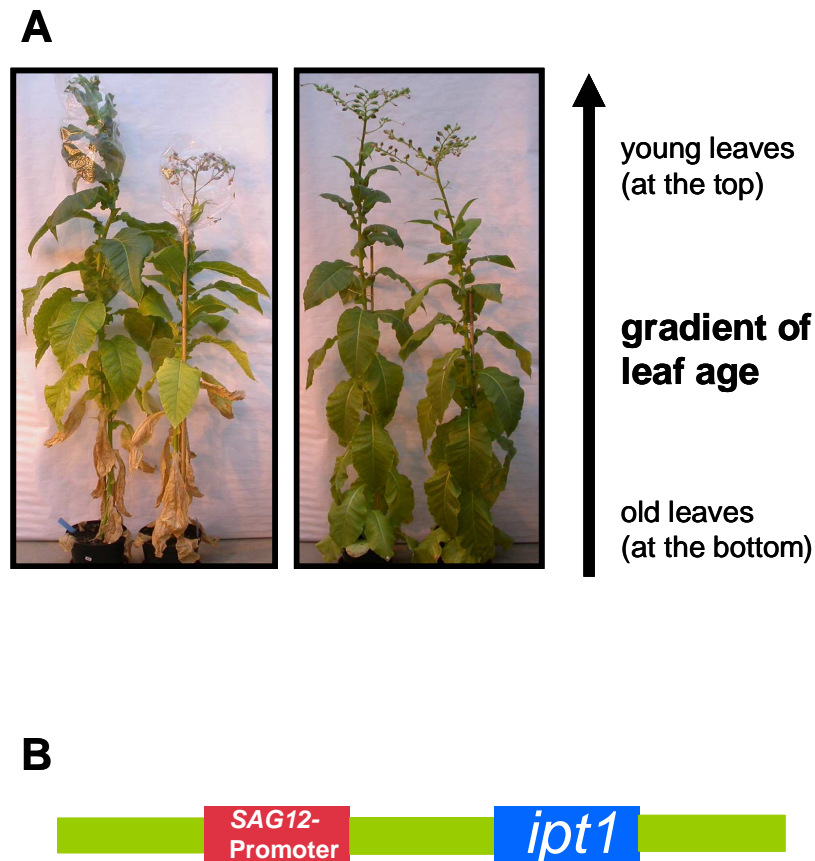
## 2.8 Soils and fertiliser for plant growth

- TKS2 (Floragard) mixed with sand (2:1)
- Stender Vermehrungssubstrat
- Fertiliser COMPO Blaukorn ENTEC

## 2.9 Plant lines

### 2.9.1 *SAG12-IPT* line and *SAG12-GUS* line of *Nicotiana tabacum*

These lines (Fig. 2.1 A) were generated, initially characterised and kindly provided by Prof. Dr. Richard Amasino, University of Wisconsin, Madison, USA (Gan and Amasino, 1995). The *SAG12-IPT* tobacco is transformed with a construct containing the senescence-specific *SAG12*-promoter and the bacterial *ipt1* gene coding for the isopentenyltransferase, a key enzyme of cytokinin biosynthesis (Fig 2.1 B). The control line *SAG12-GUS* contains the *gus* gene coding for beta-glucuronidase instead of *ipt1*.



**Fig. 2.1** *SAG12-IPT* tobacco and *SAG12-GUS* tobacco (control). (A) On the left the control plants (*SAG12-GUS*) and on the right the *SAG12-IPT* line with delayed leaf senescence. The leaf age-gradient is indicated by the arrow on the right: old leaves at the bottom and young leaves at the top of a plant. The transgenic construct in the *SAG12-IPT* line is shown in (B). It is a combination of the senescence-specific *SAG12*-promoter and the *ipt1* gene coding for a key enzyme of cytokinin biosynthesis.

### 2.9.2 *Festuca/Lolium* stay-green mutant and the wild type of *Lolium perenne*

The *Festuca/Lolium* stay-green mutant (Fig. 2.2) was generated, initially characterised and kindly provided by Dr. Helen Ougham, Institute of Grassland and Environmental Research, Aberystwyth, UK (Thomas *et al.*, 1997).

### 2.9.3 Recombinant inbred lines (RILs) of *Arabidopsis thaliana*

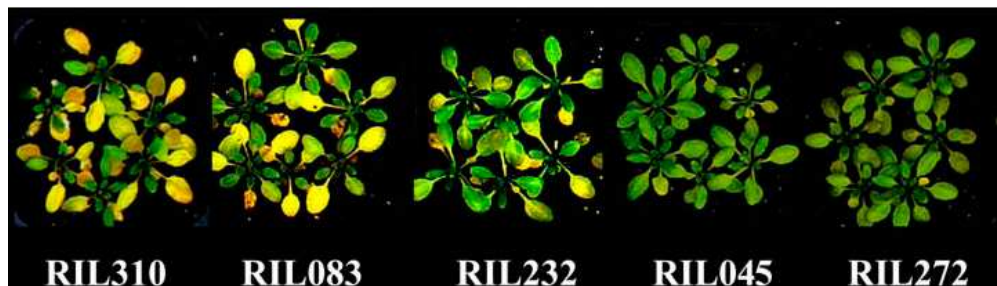
These lines (Fig. 2.3) were generated and described by Loudet *et al.* (2002) and Diaz *et al.* (2005). RIL plant material for the analysis of tocopherols was kindly provided by Dr. Céline Masclaux-Daubresse, INRA, Versailles, France.

### 2.9.4 *Gln1.2* knock-out mutant of *Arabidopsis thaliana*

The *gln1.2* knock-out mutant (FST120E03) belongs to the INRA-collection of T-DNA knock-out mutants and was described in the master thesis of Laure Gaufichon (INRA / Versailles, Unité de Nutrition Azotée des Plantes, 2006, unpublished data). Plant material of the *gln1.2* knock-out mutant for the analysis of tocopherols was kindly provided by Dr. Céline Masclaux-Daubresse, INRA, Versailles, France.



**Fig. 2.2** *Festuca/Lolium* and the wild type of *Lolium perenne*. On the left the wild type of *Lolium perenne*, on the right the *Festuca/Lolium* showing a stay-green phenotype.



**Fig. 2.3** Recombinant inbred lines (RILs). Five RILs (RIL310, RIL083, RIL232, RIL045, RIL272) showing different onsets of leaf senescence (picture from Diaz *et al.*, 2005).

## 2.10 Plant growth conditions

### 2.10.1 Tobacco grown under normal growth conditions

*SAG12-IPT* plants and *SAG12-GUS* plants (control) were grown in a greenhouse under natural light and with additional light for 16 h (7 – 23 h) with a photosynthetic photon flux density of approximately  $300 \mu\text{mol m}^{-2} \text{s}^{-1}$ . Each plant was grown in a large pot (~20 cm Ø) on Floragard TKS2

instant soil containing 50% (v/v) sand, and treated with fertiliser once a week with COMPO Blaukorn ENTEC (10 mM NO<sub>3</sub><sup>-</sup> and 2 mM NH<sub>4</sub><sup>+</sup>) until flowering. Plants were watered every second day.

### 2.10.2 Tobacco grown under nutrient deficiency

*SAG12-GUS*-plants (control) and *SAG12-IPT*-plants were grown in a greenhouse under natural light and with additional light for 16 h (7 - 23 h) at a photosynthetic photon flux density of approximately 300 μmol m<sup>-2</sup> s<sup>-1</sup>. Each tobacco plant was grown in a large pot (Ø of ~20 cm) on nutrient-depleted Stender Vermehrungssubstrat. Plants were not treated with fertiliser and watered every second day.

### 2.10.3 *Festuca/Lolium* and the wild type of *Lolium perenne*

The *Lolium/Festuca* stay-green-mutant and the wild type of *Lolium perenne* were grown in a greenhouse under natural light and with additional light for 16 h (7 – 23 h) by a photosynthetic photon flux density of approximately 300 μmol m<sup>-2</sup> s<sup>-1</sup>. Approximately 20 plants were grown together in one large pot (~30 cm Ø) on Floragard TKS2 instant soil containing 50% (v/v) sand and watered twice a week. Plants were not treated with fertiliser.

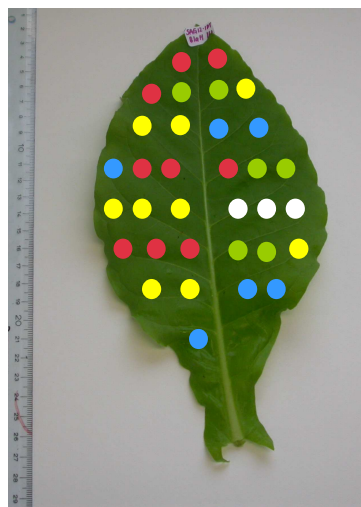
### 2.10.4 RILs and *gln1.2* knock-out mutant of *Arabidopsis thaliana*

RILs, the *gln1.2* knock-out mutant and its corresponding wild type were grown in a growth chamber for eight hours under light with a photosynthetic photon flux density of 150-160 μmol m<sup>-2</sup> s<sup>-1</sup>. The temperature was 21°C in the day and 17°C in the night. Six plants were grown in a small pot (60 mm long, 65 mm wide and 60 mm high) on soil that was not treated with fertiliser (similar to Stender Vermehrungssubstrat) and watered every second day with a solution containing 0.25 mM K<sub>2</sub>SO<sub>4</sub> and 0.25 mM KH<sub>2</sub>PO<sub>4</sub>, 0.25 mM MgSO<sub>4</sub>, 0.25 mM CaCl<sub>2</sub>. Additionally, the watering solution for the RILs also contained 3 mM of KNO<sub>3</sub>. The *gln1.2* knock-out mutant and the corresponding wild type were grown in two groups: the watering solution for one group also contained 2 mM KNO<sub>3</sub> and that for the other group 10 mM KNO<sub>3</sub>.

## 2.11 Plant sampling

### 2.11. Leaves for the kinetics under normal growth conditions

Leaves number 14 and 15 (counted from the bottom) of one tobacco plant per line were collected every week beginning in WAS 9 and ending in WAS 26. Samples of 50-100 mg were always taken from the same, defined areas of the tobacco leaf (2-3 leaf discs of Ø 1,7 cm) to measure the amount of chlorophyll, carotinoid, tocopherol, soluble sugar and to isolate RNA and proteins (Fig. 2.2). Collecting of leaves was always performed at 10 o'clock. Following photographically documentation of the leaf, leaf disc samples were taken, immediately frozen in liquid nitrogen and stored by -80°C until further use. For every week, three independent samples from different, defined areas of the tobacco leaf were analysed. The resulting values are the mean of three measurements and given in relation to leaf surface.



**Fig. 2.4 Sampling of tobacco leaves.** Every week leaf disc samples of tobacco for were taken in the same way: 3 x 3 leaf-discs for tocopherols (red), 3 x 2 leaf-discs for chlorophylls and carotinoids (green), 3 x 3 leaf discs for soluble sugars (yellow) and 2 x 3 leaf discs for RNA (blue) and 1 x 3 leaf discs for proteins (white).

### 2.11.2 Tobacco leaves for the kinetics under nutrient deficiency

Leaves number 15 and 16 of one tobacco plant per line were harvested every week beginning in WAS 12 and ending up in WAS 17. Harvests were performed at 10.00 o'clock. After the photographic documentation the maximum photosynthetic efficiency of the leaf,  $F_v/F_m$ , was measured as a senescence parameter. Samples (3 x 3 leaf discs of Ø 1.7 cm) for tocopherol analysis were always taken from the middle part of the leaf, immediately

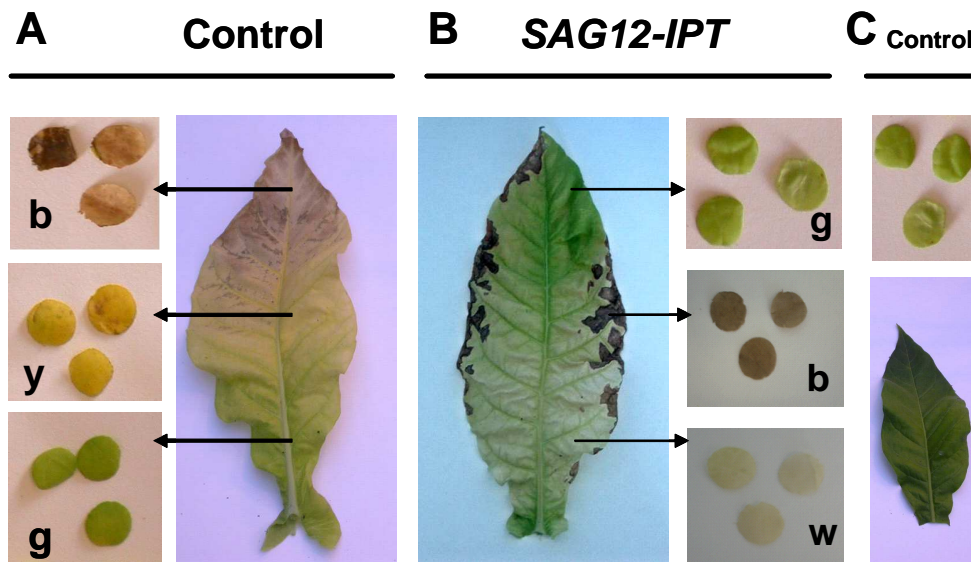
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frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use. For every harvest, three independent samples from different, defined areas of the tobacco leaf were analysed and averaged. Resulting values are given relative to the leaf surface.

### 2.11.3 Tobacco leaves for other experiments

- For the measurement of tocopherol content in the leaf age gradient, tobacco was grown under normal growth conditions (see chapter 2.10.1). Three leaves number 10 (young) and three leaves number 20 (old) from the *SAG12-IPT* line and the *SAG12-GUS* line (control), respectively, were collected in WAS 13. Leaf disc samples ( $\sim 1,7$  cm  $\emptyset$ ) for measuring tocopherols (3 x 3), chlorophyll (3 x 2) and  $\text{NO}_x$  (1 x 3) were taken from the middle part of the leaves, immediately frozen in liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  until further use.
- For the analysis of tocopherol distribution in a non-uniformly senescing tobacco leaf, tobacco was grown under normal growth conditions (see chapter 2.10.1). Leaf disc samples (3 x 3;  $\emptyset$  1.7 cm) were taken from green, yellow and brown tissue of leaf number 15 of the *SAG12-GUS* line in WAS 18 (Fig. 2.5 A). From the *SAG12-IPT* line, leaf disc samples (3 x 3) were taken from green, white and brown tissue in WAS 27 (Fig. 2.5 B). As a control, leaf disc samples were taken from green, non-senescent tissue of the young leaf number 23 of *SAG12-GUS* in WAS 18. Leaf samples were immediately frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further use.
- For the regreening experiment with kinetin, tobacco was grown under normal growth conditions (see chapter 2.10.1). Yellow, senescing leaf discs (2 x 2; 1.7 cm  $\emptyset$ ) were (chlorophylls and carotenoids) taken from leaf number 15 in WAS 18 from the *SAG12-GUS* line (Fig. 2.6). Leaf discs were posed with their lower surface on wet whatman filter paper in a petri dish and, incubated in kinetin

for three days in the dark at room temperature for three days, then frozen in liquid nitrogen and stored at -80 °C until further use.

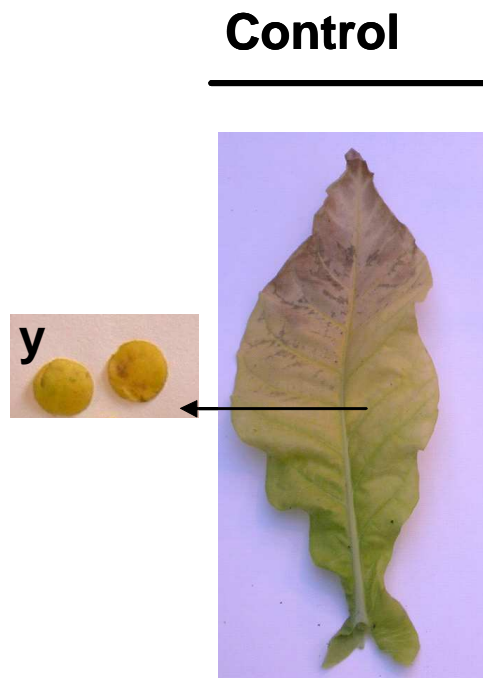


**Fig. 2.5 Sampling for the analysis of tocopherol distribution in a non-uniformly senescing tobacco leaf.** (A) Leaf disc samples (3 x 3) were taken from brown (b), yellow (y) and green (g) tissue of leaf number 15 of the *SAG12-GUS* line in WAS 18. From the *SAG12-IPT* line, leaf disc samples (3 x 3) were taken from green (g), brown (b) and white (w) tissue in WAS 27. (C) As a control, leaf disc samples were taken from green, non-senescent tissue of the young leaf number 23 of *SAG12-GUS* in WAS 18.

#### 2.11.4 *Festuca/Lolium* stay-green-mutant

From the *Festuca/Lolium* stay-green mutant, leaf number one was always collected at 10 o'clock. 1 cm of the leaf tip and 1 cm of the leaf bottom were removed by cutting. After the maximum photosynthetic efficiency,  $F_v/F_m$ , or the SPAD value were measured as senescence parameters, the leaf was frozen in liquid nitrogen and stored by -80°C until further use.





**Fig 2.6 Sampling for the regreening experiment via kinetin.** Yellow (y), senescing leaf discs (2 x 2) for measuring pigments (chlorophylls and) were taken from leaf number 15 in WAS 18 from the *SAG12-GUS* line.

#### 2.11.5 RILs and *gln1.2* knock-out mutant of *Arabidopsis thaliana*

For the measurement of tocopherols in the RILs, only the first six emerging leaves of the rosette were used. For the measurement of tocopherols in the *gln1.2* knock-out mutant and the corresponding wild type, material of the whole rosette was used.

#### 2.12 Quantification of $\alpha$ - and $\gamma$ -tocopherol via HPLC

The contents of  $\alpha$ - and  $\gamma$ -tocopherol were analysed as described by Dähnhardt *et al.* (2002). Briefly, three leaf discs ( $\varnothing$  1.7 cm), which had been frozen in liquid nitrogen, were ground with the Geno/Grinder2000 and extracted with 200 - 1000  $\mu$ L n-heptane at -20 °C for at least 24 h. After centrifugation at 15.000x g, the clear supernatant was analysed by normal phase HPLC. A sample volume of 20  $\mu$ L of the sample was analysed using a LiChrosphere Si 60 (5  $\mu$ m) column (4 x 250 mm) with n-heptane/2-propanol (99 + 1) as eluant and a flow rate of 1.0 mL/min. Tocochromanols were detected and quantified by fluorimetry (model RF10AXL, Shimadzu) set at  $\lambda_{\text{excitation}} = 290$  nm and  $\lambda_{\text{emission}} = 328$  nm. To calibrate the system and to verify the identity of individual peaks, tocopherol standards from Merck (Darmstadt, Germany) were used. Data analysis was performed using the Class-VP 7.4 SP1 software (Shimadzu, Duisburg, Germany).

### **2.13 Extraction and analysis of chlorophylls and carotenoids**

Two leaf discs, which had been kept at  $-80^{\circ}\text{C}$ , ( $\varnothing$  1.7 cm) were ground with the Geno/Grinder2000 and repeatedly extracted with 0.5 – 2 mL ice-cold acetone after addition of a few crystals of magnesiumhydroxycarbonate to avoid pigment oxidation. Chlorophyll and carotenoid content were estimated spectrophotometrically, using the specific absorption coefficients for chlorophyll *a*, chlorophyll *b* and carotenoids reported by Lichtenthaler (1987).

### **2.14 Determination of PSII-efficiency by Imaging-PAM**

Before the sampling, the maximum photosynthetic efficiency of photosystem II ( $F_v/F_m$ ) was analysed using the Maxi-Imaging-PAM (IMAG-MAX/K-Dolphin, Heinz Walz GmbH, Effeltrich, Germany). Measurements were performed as described in Schreiber *et al.* (1986). Briefly, leaves were incubated in the dark for 30 min to oxidise the reaction center of PSII and put under the Imaging-PAM employing pulse-modulated measuring light for fluorescence excitation.

### **2.15 Measurement of SPAD**

The relative chlorophyll content was measured with the SPAD 502 Chlorophyll Meter of Minolta (Japan). On a measuring surface of 2 mm  $\times$  3 mm, the relative content of chlorophyll is determined by measuring the absorption in the red and infrared wavelength range.

### **2.16 Quantification of soluble sugars**

For the determination of soluble sugars (fructose, glucose and sucrose), three frozen leaf discs stored in liquid nitrogen ( $\varnothing$  1.7 cm) were ground with the Geno/Grinder2000 and resuspended in 900  $\mu\text{L}$  of distilled water. After centrifugation at 15.000x *g* for 10 min at  $4^{\circ}\text{C}$ , the supernatant was heated to  $100^{\circ}\text{C}$  for 3 min to denature the enzymes. Soluble sugars in the resulting solution were quantified by HPLC coupled to a pulsed amperometry detector (Dionex 4500i; Dionex Softron, Germering, Germany). Three independent samples from different, defined areas of the tobacco leaf were

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analysed at least twice and averaged. Resulting values are given as the mean relative to leaf surface. These HPLC-analyses was performed in the laboratory of Prof. Dr. T. Roitsch (University of Würzburg, Germany).

### **2.17 Semi-quantitative RT-PCR**

For semi-quantitative RT-PCR, total RNA was extracted from three frozen leaf discs (Ø 1.7 cm) using the Spectrum™ Plant Total RNA Kit according to the manufacturer's protocol (SIGMA®). The resulting pellet was resuspended in 50 µL ddH<sub>2</sub>O. 5 µg of total RNA were treated with 1 unit DNase (1 h, 37°C) to remove genomic DNA contaminants. DNase I treated RNA as a template and primers for the constitutively expressed gene *efla* served as a negative control and a sample with 30 ng of genomic DNA as a positive control. RNA was transcribed to cDNA with 0.5 µg oligo(dT) primers and the RevertAid™ M-MuLV-Reverse Transcriptase (MBI Fermentas) according to the manufacturer's protocol. Subsequently, 30 ng of cDNA was used for PCR. The PCR program started with a 5 min denaturation at 95°C. The cycle profile consisted of 1 min denaturation at 95°C, 1 min annealing at 50-68°C depending on the specific primer pair and 1 min extension at 72°C. Cycle number varied depending on the gene-specific primer pair. An additional extension was carried out for 5 min at 72°C following the cycling. The gene-specific primer pairs, the annealing temperature and the cycle number are given in chapter 2.6..

### **2.18 Western blot analysis**

Soluble proteins were extracted from in liquid nitrogen frozen and ground leaf samples (Ø 1.7 cm) of about 100 mg by homogenisation with 300 µL of warm (60°C) protein extraction buffer. After mixing, incubation for 10 min at 100°C and centrifugation for 15 min at 14.000x g, the supernatants were used for protein concentration determination using the Roti® Nanoquant reagent (Roth, Karlsruhe, Germany). Proteins were denatured in protein sample buffer by incubation at 95°C for 20 minutes. Protein extracts were loaded on a 14% (w/v) SDS containing polyacrylamide gel, 20 µg per lane,

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and gel electrophoresis was performed at 4°C at 28 mA in 1x Laemmli buffer. Proteins were transferred onto a PVDF membrane (BioRad, Munich, Germany) with the help of the Blotting System Fastblot 34 (Biometra, Göttingen, Germany). The transfer was performed in a cold room (4°C) at 0.65 mA / cm<sup>2</sup> of gel. The membrane was incubated in a 5% (w/v) milk powder solution for 15 min at 4°C. After washing with TBST, the membrane was incubated over night with the primary antibody raised against whole Rubisco protein. The primary antibody was generated in a rabbit and kindly provided by Prof. Dr. H. J. Schneider-Pötsch (University of Cologne, Germany) and used at a working concentration of 1: 1000 in TBST. After washing with TBST, the membrane was incubated in a 5% (w/v) milk powder solution for 15 min at 4°C. Subsequently, the membrane was washed with TBST and incubated for 60 min with the secondary horse-radish peroxidase (HRP)-linked antibody generated in a rabbit and ordered from GE Healthcare (Chalfont, St. Giles, UK). The secondary antibody was diluted 1: 2500 in TBST. For detection, equal amounts of detection solution A and detection solution B were mixed and applied to the membrane. Chemoluminescence was detected in a dark room using an ECL film (Hyperfilm™ ECL Amersham Biosciences, Buckinghamshire, UK). Molecular mass standard proteins (Page Ruler™ Prestained Protein Ladder) were purchased from MBI Fermentas (St. Leon-Rot, Germany).

### **2.19 Determination of NO<sub>x</sub> content**

Three leaf discs kept in liquid nitrogen (Ø 1.7 cm) were ground with the Geno/Grinder2000 and extracted with 1 mL NO<sub>x</sub> extraction buffer. After a centrifugation for 15 min at 14.000x g, 490 µL of supernatant were incubated with 10 µL of DAF-FM for 10 min. Subsequently, the reaction mix was transferred to a 96-well plate (150 µL per well) and analysed by fluorimetry (excitation: 480 nm, emission: 515 nm, enhancement: 60, number of flashes: 10, time of integration 40 µs, temperature: 21.5°C).

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### 3 Results

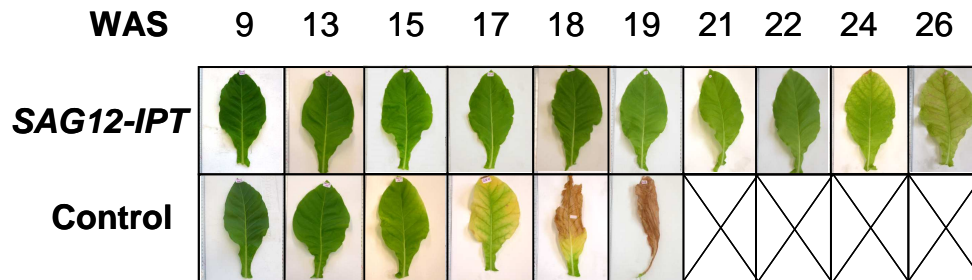
#### 3.1 Temporal, quantitative and functional characterisation of tocopherol accumulation in *SAG12-IPT* tobacco

Gan and Amasino published in 1995 that *SAG12-IPT* tobacco exhibits a delay of leaf senescence caused by the autoregulated production of IPT, a key enzyme of cytokinin biosynthesis (see chapters 1.1.5 and 2.9.1). Cytokinin is known to be a senescence retarding phytohormone and can also reverse senescence (Richmond and Lang, 1957; Gan and Amasino, 1996). In plants synthesising cytokinin during leaf senescence, chlorophyll content remains nearly stable up to the final phase of cell death. Therefore, these plants are suitable to investigate, whether the accumulation of tocopherols is a passive consequence of chlorophyll breakdown. As already mentioned in chapter 1.2.6, it has been hypothesised that excess amounts of phytol resulting from chlorophyll breakdown during senescence might be deposited in form of tocopherols in the chloroplast (Rise *et al.*, 1989; Dörmann, 2007).

##### 3.1.1 Development-dependent changes of tocopherol content in leaves of *SAG12-IPT*-tobacco

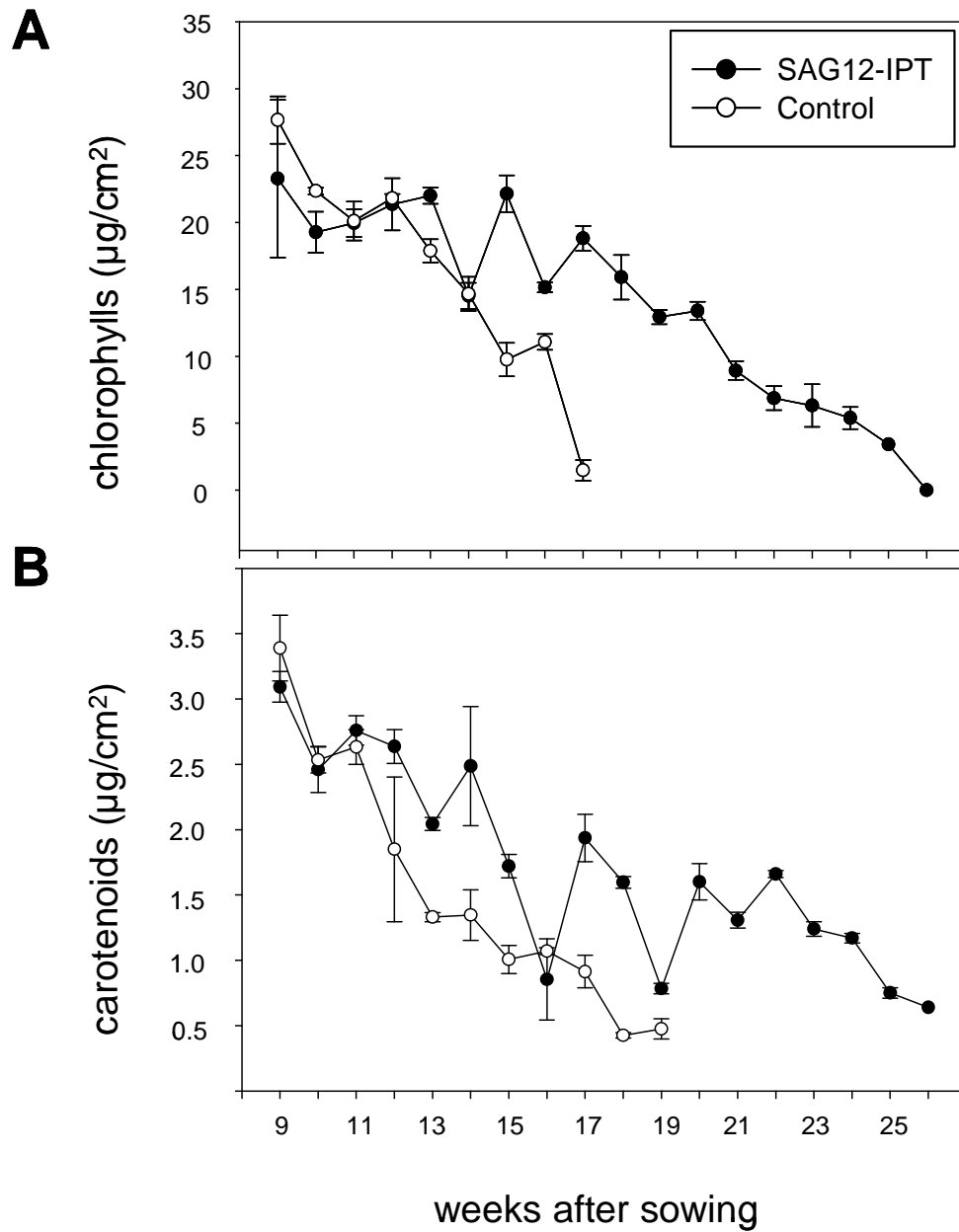
In order to investigate the relationship between tocopherol accumulation and chlorophyll degradation, leaf number 15 of the *SAG12-IPT* line and of the control, respectively, were analysed by HPLC for their  $\alpha$ - and  $\gamma$ -tocopherol and chlorophyll content over a period of 18 weeks, starting in WAS 9 and ending in WAS 26. Through regular treatment of the plants with fertiliser, the life span of leaf number 15 was approximately 10 weeks longer than that of the control plants (Fig. 3.1). The transgenic line stays green until WAS 26 (Fig. 3.1), goes immediately into programmed cell death and dies finally in WAS 27. In the control plants, leaf number 15 undergoes normal leaf senescence which can be divided into three steps: initiation took place in WAS 15 with first visible loss of chlorophylls, especially at the border areas, in WAS 17 the second stage of reorganisation occurred (see chapter 1.1.1). Interestingly, at this point in time the leaf veins used for the transport of nutrients were still green, whereas the mesophyll had already turned

yellow. In WAS 18 the terminal and third stage of leaf senescence programmed cell death occurred (Fig. 3.1).



**Fig 3.1 Development of leaf number 15 in SAG12-IPT-tobacco grown under normal growth conditions.** In a course of 18 weeks, beginning in WAS 9 and ending in WAS 26, every week leaf number 15 was collected from one SAG12-IPT plant and from one control plant, respectively, for further analyses.

To monitor the progression of senescence, the levels of chlorophylls and carotenoids were determined during leaf development via acetone extraction and spectrometry. A constant decrease in the amount of chlorophyll took place in the control plants throughout the whole time course (Fig. 3.2 A). From WAS 9 to WAS 14, about 50 % of chlorophyll was degraded, and in WAS 17 nearly no chlorophyll was left (Fig. 3.2 A). In the SAG12-IPT tobacco, some residual traces of chlorophyll were still present in WAS 26 (Fig. 3.2 A). The carotenoid content of the control plants decreased rapidly from WAS 9 to WAS 13, but a small amount was still detectable in WAS 19 (Fig. 3.2 B). In the SAG12-IPT tobacco carotenoid levels remained fairly constant until WAS 26 (Fig. 3.2 B).



**Fig. 3.2 Development-dependent changes of pigment content in leaf number 15.** In a course of 18 weeks, beginning in WAS 9 and ending in WAS 26, leaf number 15 of the *SAG12-IPT* line (dark dots) and of the control (white dots) was analysed for its content of chlorophylls (A) and carotenoids (B) by acetone extraction and spectrophotometry. The values represent the mean of triplicates and are relative to the leaf surface.

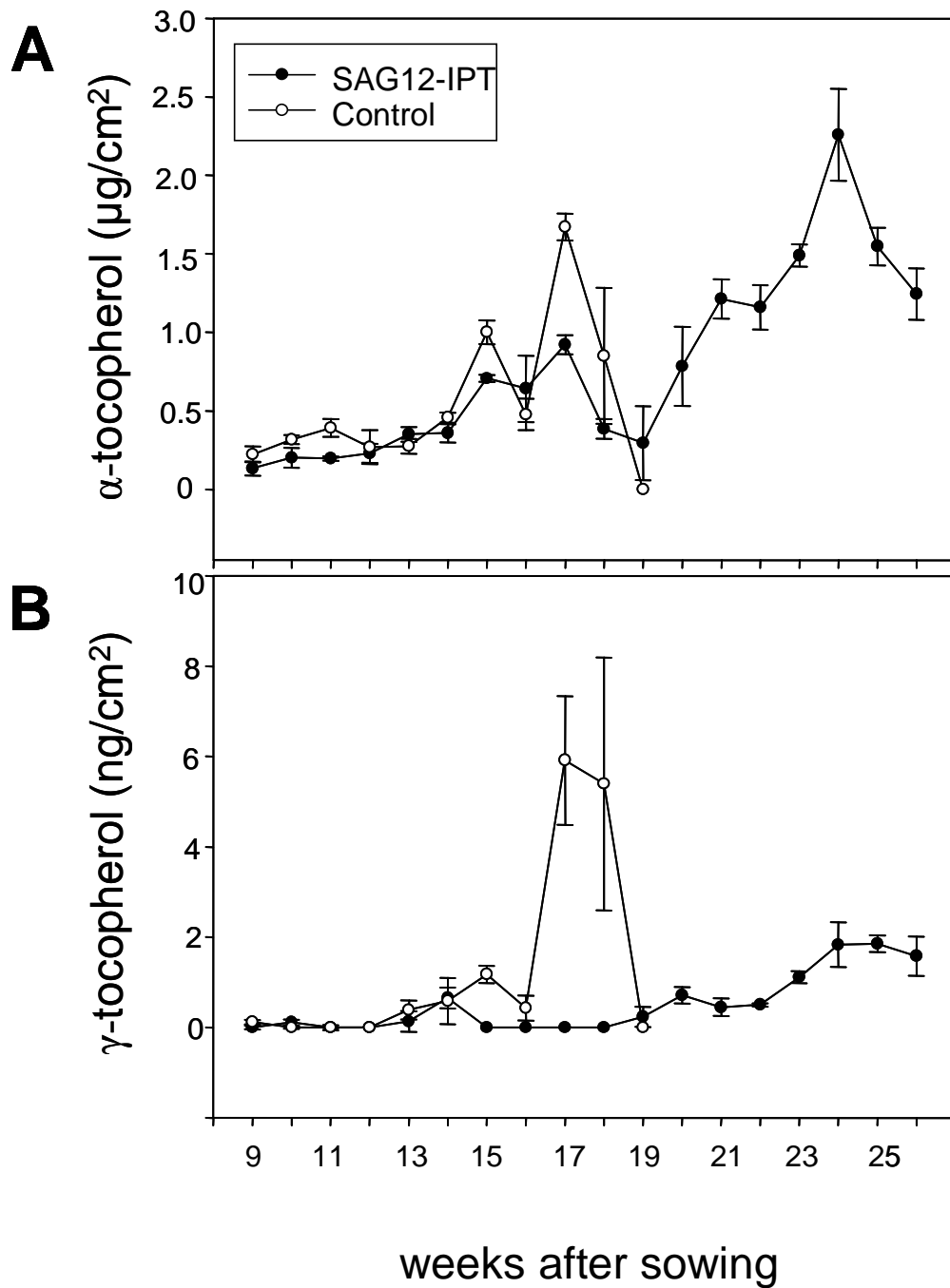
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To investigate the impact of chlorophyll degradation on the accumulation of tocopherols, the  $\alpha$ - and  $\gamma$ -tocopherol content was measured in the leaf over the time course of 18 weeks (Fig. 3.3). In accordance with the results described by Dertinger *et al.* (2003), the content of  $\alpha$ -tocopherol increased during leaf senescence in an approximately leaf age-dependent manner. In the control plants, the accumulation of  $\alpha$ -tocopherol started in WAS 15 and the maximal levels of  $\alpha$ -tocopherol (approximately  $1,7 \mu\text{g}/\text{cm}^2$ ) was found in WAS 17 (Fig 3.3 A). Then the content of  $\alpha$ -tocopherol decreased again, until the leaf finally died in WAS 19 (Fig 3.3 A). The *SAG12-IPT* tobacco showed a quite similar curve shape for  $\alpha$ -tocopherol in the first half of the plant's life span, beginning in WAS 15 with a first maximum of nearly  $1,0 \mu\text{g}/\text{cm}^2$  in WAS 17, followed by a decrease up to WAS 19 (Fig 3.3 A). At this time point the leaf of the *SAG12-IPT* tobacco still seemed to function in the same way as the control leaf that was destined to die. However, from WAS 20 onwards the senescence specific activation of the *SAG12*-promoter initiated the production of IPT resulting in an elongation of the life span. As a consequence, the content of  $\alpha$ -tocopherol increased again reaching its second significant maximum of approximately  $2,3 \mu\text{g}/\text{cm}^2$  in WAS 24 (Fig. 3.3 A). Afterwards the amount of  $\alpha$ -tocopherol declined until cell death terminated the life span of the leaf (Fig. 3.3 A). It is notable, that the maximal value for  $\alpha$ -tocopherol in the control (approximately  $1,7 \mu\text{g}/\text{cm}^2$ ) was just approximately 70% of the maximal value obtained for the *SAG12-IPT* line (nearly  $2,3 \mu\text{g}/\text{cm}^2$ ).

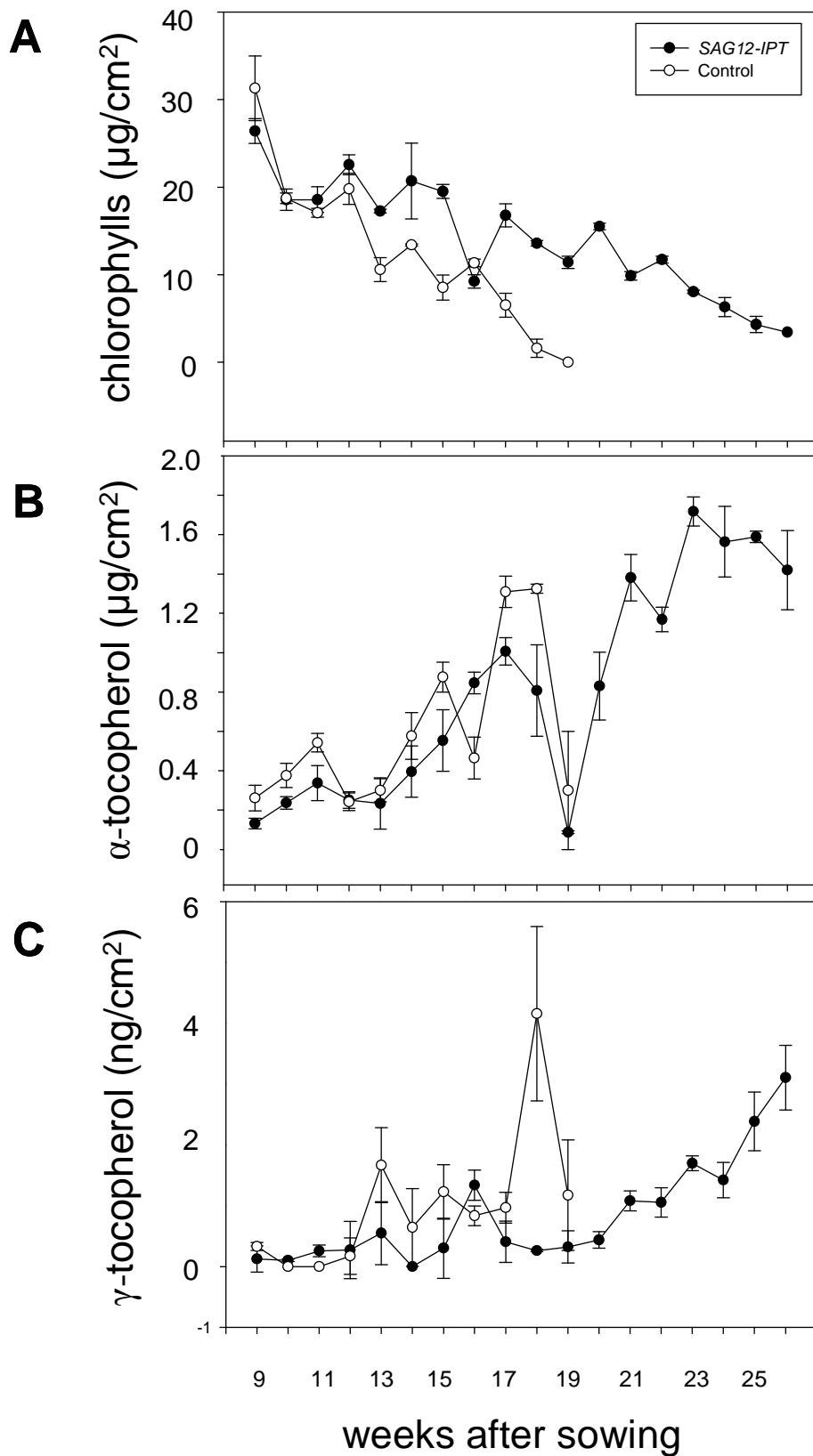
Interestingly,  $\gamma$ -tocopherol accumulated in the amounts of ng and did not seem to be age-dependent but transient at later stages of senescence as evidenced by the significant peak occurring in WAS 17 and WAS 18 of the control (Fig. 3.3 B). However, the *SAG12-IPT* tobacco showed no accumulation of  $\gamma$ -tocopherol in WAS 17 and 18, as observed in the control, and just a low increase in WAS 24 to WAS 26 (Fig. 3.3 B).

The tocopherol and chlorophyll measurements described above were not only performed with leaf number 15, but also with leaf number 14 (Fig. 3.4). The results obtained for leaf number 14 were similar to those for leaf number 15.





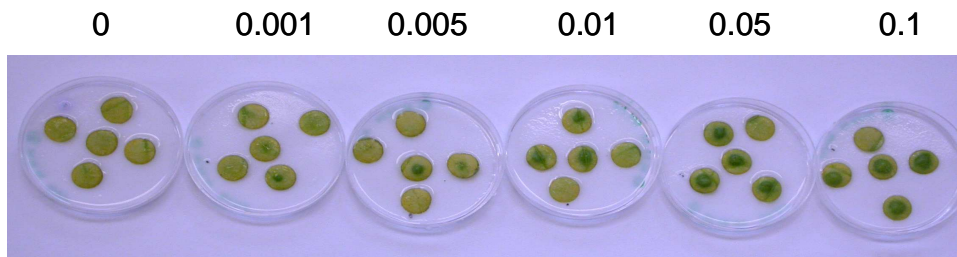
**Fig. 3.3 Development-dependent changes of tocopherol content in leaf number 15.** Over 18 weeks, beginning in WAS 9 and ending in WAS 26, the  $\alpha$ -tocopherol (A) and  $\gamma$ -tocopherol (B) content of leaf number 15 of the *SAG12-IPT*-line (dark dots) and of the control (white dots) were analysed by HPLC. Mean values of triplicate measurements were based on leaf surface.



**Fig. 3.4** Development-dependent changes of tocopherol and chlorophyll content in leaf number 14. During 18 weeks, from WAS 9 - 28, leaf number 14 of the *SAG12-IPT*-line (dark dots) and of the control (white dots) was analysed by spectrophotometry for its chlorophyll content (A) and by HPLC for its  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol content (C). Values are given as the mean of triplicate measurements and were based leaf surface.

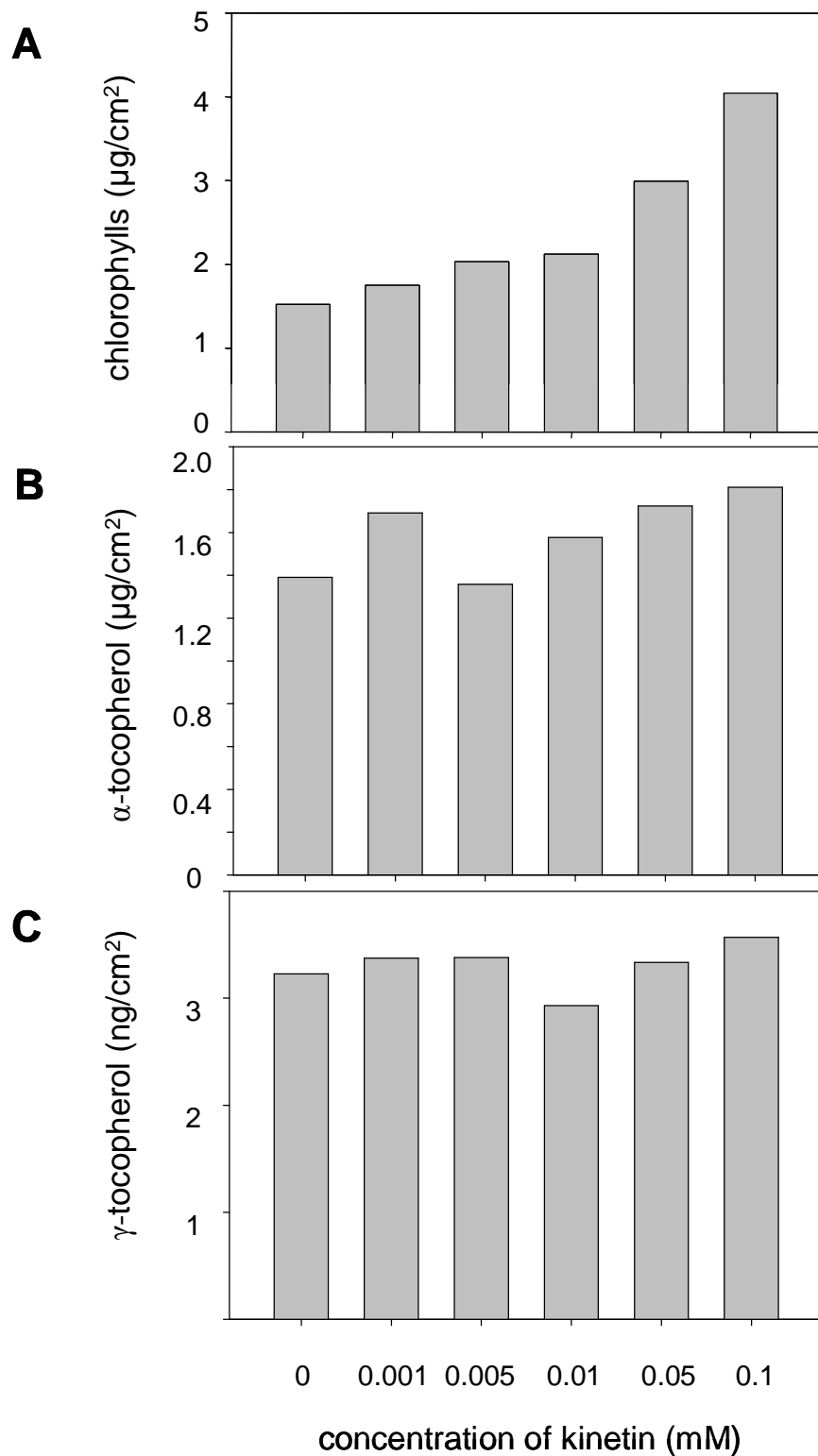
### **3.1.2 Impact of cytokinin on the content of tocopherols and pigments**

To examine, whether tocopherol content is affected by new chlorophyll synthesis, a regreening experiment with the cytokinin kinetin was performed. Senescent, yellow leaf discs from the normally senescing control tobacco were treated with kinetin solutions of different concentrations (Fig. 3.5). After three days of incubation the amount of tocopherols and chlorophylls present in the tobacco leaf discs was analysed. As can be seen in Fig. 3.5 the regreening effect was directly proportional to kinetin concentration. This was confirmed by the chlorophyll measurements (Fig. 3.6 A).



**Fig. 3.5 Regreening of senescent tobacco leaf discs after kinetin treatment.** Yellow leaf discs from the normally senescing control tobacco were posed with their lower surface on wet whatman filter paper in a petri dish and provided on their upper surface with 5  $\mu$ L microliter of kinetin at different concentrations (0 mM, 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM and 0.1 mM). After incubation for three days in the dark and at room temperature, the content of chlorophylls and tocopherols was measured.

The concentration of kinetin seemed to have no direct impact on the content of tocopherols. Neither the  $\alpha$ -tocopherol (Fig. 3.6 B) nor the  $\gamma$ -tocopherol levels (Fig. 3.6 C) were significantly different after the regreening by different concentrations of kinetin. This indicated that tocopherol accumulation is independent of chlorophyll breakdown as it occurred not only when chlorophylls were degraded, but even when chlorophyll was *de novo* synthesised. In addition, these results demonstrated that the accumulation of tocopherols is independent of cytokinin.



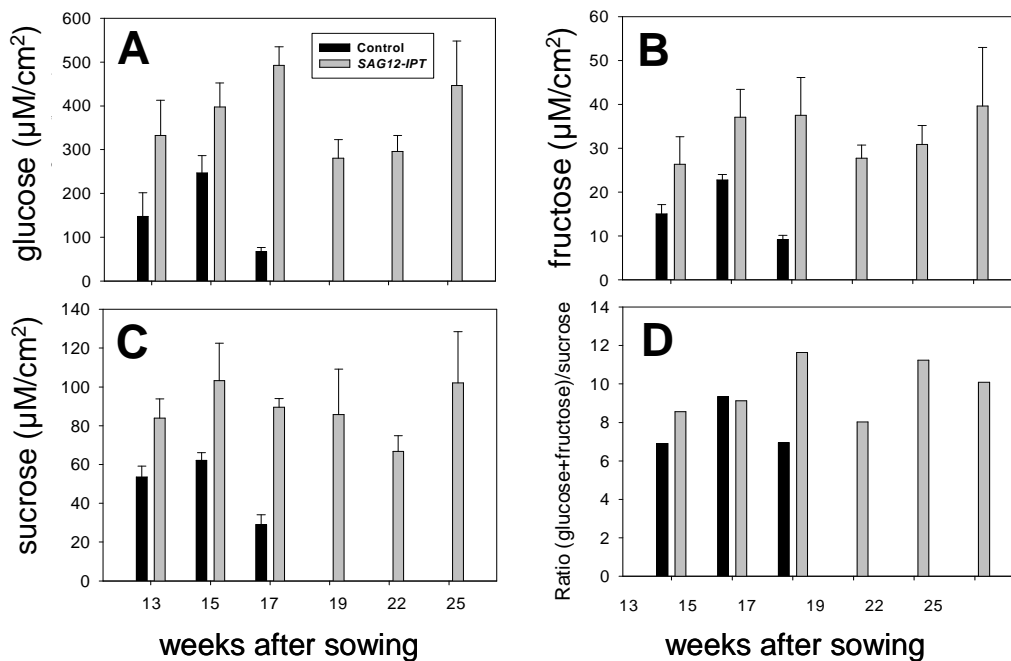
**Fig. 3.6 Content of chlorophylls and tocopherols content in yellow, senescent leaf discs after kinetin treatment.** 5  $\mu\text{L}$  kinetin at different concentrations (0 mM, 0.001 mM, 0.005 mM, 0.01 mM, 0.05 mM and 0.1 mM) were added to the upper surface of yellow, senescent leaf discs taken from the normal senescing control tobacco. After an incubation for 3 days in the dark and at room temperature leaf discs were analysed for their content of chlorophylls (A),  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol (C). The values represent the mean values for two leaf disc extracts.

### **3.1.3 Development-dependent changes of free soluble sugar content in leaves of *SAG12-IPT* tobacco**

A look at the developmental changes in the content of  $\alpha$ -tocopherol (Fig. 3.3 A), might address the question, whether the maximum in WAS 24 is only due to its traditional function as an antioxidant. It has also been proposed, that the function of tocopherols could be related to the regulation of carbohydrate metabolism, especially to the export of sugars from source leaves. In support of this hypothesis the *sxd1*-mutant of maize (Russin *et al.*, 1996) as well as the *vte1*-RNAi-potato (Hofius *et al.*, 2004), both lines deficient in tocopherols, exhibit a significant accumulation of free soluble sugars and starch.

In this work free soluble sugars such as glucose (Fig. 3.7 A), fructose (Fig. 3.7 B) and sucrose (Fig. 3.7 C) were measured during leaf development of the *SAG12-IPT* tobacco to determine, whether the accumulation of  $\alpha$ -tocopherol is accompanied by a disturbance in C-remobilisation. Samples were analysed by a HPLC-system coupled with pulsed amperometry detection in the laboratory of Prof. Dr. T. Roitsch, University in Würzburg. To monitor the changes of C-remobilisation in the *SAG12-IPT* line, the ratio between the amount of total monosaccharides (glucose plus fructose) and the amount of the disaccharide sucrose was calculated (Fig. 3.7 D).

All soluble sugars measured, namely glucose, fructose and sucrose, were significantly increased during the whole leaf development in the *SAG12-IPT* line compared to control plants (Fig. 3.7 A-C). This is in accordance to measurements performed by Wingler *et al.* (1998). Especially at the later stages of development (WAS 19 to WAS 25), which are characterised by an increased level of  $\alpha$ -tocopherol (Fig. 3.3), the *SAG12-IPT* line showed values for free soluble sugars that were all higher than those measured for the control plant from WAS 13 to WAS 17 (Fig. 3.7 A-C). Finally, the ratio between the amount of total monosaccharides (glucose plus fructose) and the amount of the disaccharide sucrose was calculated. This ratio was increased in the *SAG12-IPT* line compared to the control (except in WAS 15) suggesting decreased sucrose transport or the accumulation of hexoses (Fig. 3.7 D).



**Fig. 3.7 Developmental changes in soluble sugar content.** Related to the tobacco kinetics described in chapter 4.1, the content of glucose (A), fructose (B) and sucrose (C) was measured in the *SAG12-IPT* line (white bars) and in the control (dark bars) by HPLC coupled with pulsed amperometry detection. The ratio of (glucose + fructose)/sucrose is shown in (D). The mean of triplicate measurements were based on leaf surface.

Overall, the sugar measurements revealed, that the age-dependent accumulation of  $\alpha$ -tocopherol observed in leaf number 15 of the *SAG12-IPT*-line (Fig. 3.3 A) was accompanied by an accumulation of free soluble sugars (Fig. 3.7).

### **3.1.4 Development-dependent changes of gene expression in leaves of *SAG12-IPT* tobacco grown under normal growth conditions**

To gain further insight into the functions and the regulation of tocopherol biosynthesis during leaf senescence, the expression of a variety of genes was analysed at selected time points of development and compared to the tocopherol levels. Analysis of gene expression was performed by semiquantitative RT-PCR.

The expression of the *ef1a* gene (Fig. 3.8 A) coding for the translation elongation factor EF1 $\alpha$  was used as a control as it is constantly expressed throughout leaf development (Bouquin *et al.*, 2001). When determining the developmental changes of  $\alpha$ -tocopherol content in the *SAG12-IPT* line, it was supposed that the activation of the *SAG12*-promoter and the

corresponding production of IPT does not take place before WAS 19. Indeed, the expression of the bacterial *ipt1* gene, that was transformed into tobacco under control of the *SAG12*-promoter (see chapter 2.9.1; Gan and Amasino, 1995), showed no significant induction up to WAS 22 in the *SAG12-IPT* line (Fig. 3.8 A). In the control tobacco line containing the *gus* gene instead of *ipt1*, the induction of the *SAG12*-promoter already occurred in WAS 13 (Fig. 3.8 A), although the leaf exhibited no visible signs of senescence (Fig. 3.1) and no degradation of chlorophylls (Fig. 3.2) at this time point.

As Welsch *et al.* (2003) found an ATCTA element present in the promoter of photosynthesis-related genes and in genes coding for the enzymes of the biosynthetic pathway for tocopherols, common regulatory mechanisms for tocopherol biosynthesis and photosynthesis were considered. Therefore, the expression level of genes related to tocopherol biosynthesis (3.8 B) and the expression of three photosynthesis marker genes (3.8 C) were analysed.

As genes which are related to tocopherol biosynthesis the three genes *hpd*, *vte1* and *hgo* were selected (Fig. 3.8 B). While *hpd* and *vte1* gene products are part of the biosynthetic pathway for tocopherols, catalysing the conversion of *p*-hydroxy-phenylpyruvate to homogentisate and the cyclisation, respectively, the *hgo* gene encodes the HGO enzyme that is part of another biosynthetic pathway, which competes with the former pathway for the biosynthesis of tocopherols and plastoquinone (see chapter 1.2.4; DellaPenna, 2005). The HGO enzyme is able to remove homogentisate from the tocopherol biosynthesis pathway converting it to maleylacetoacetate. Therefore, the level of *hgo* gene expression might provide interesting insights into the regulation of tocopherol biosynthesis. In the control plants, all tocopherol-related genes analysed showed an increased expression from WAS 13 to WAS 17 (Fig. 3.8 B) suggesting that the accumulation of tocopherols during leaf senescence is at least partly regulated at the transcriptional level. While the senescence-enhanced expression of *hpd* gene could already be demonstrated in senescing barley leaves (Kleber-Janke and Krupinska, 1997), the senescence-associated expression of *hgo* was found in rosette leaves *Arabidopsis thaliana* (Dixon and Edwards, 2006; Diploma thesis of Christian Sievert, 2008). The increased expression of the *hpd* gene

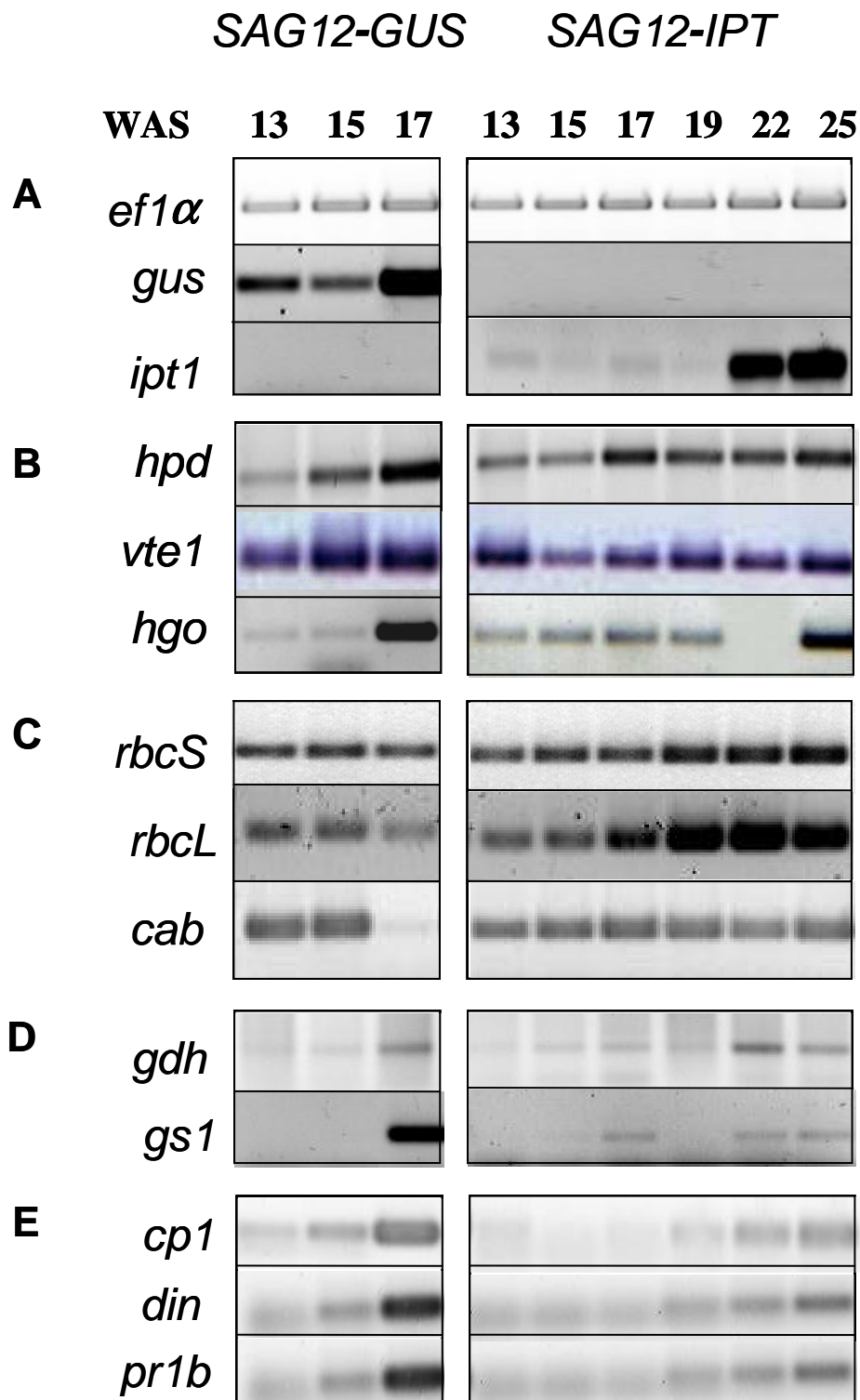
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may be responsible for the higher accumulation of tocopherols as well as the increased *hgo* expression representing the competing pathway that leads via maleylacetoacetat to fumarate and acetoacetate. In the *SAG12-IPT* line, the senescence-associated expression of the three tocopherol-related genes was not as clear as in the control. The *hgo* gene showed a stronger signal in WAS 25 compared to former harvests in WAS 13 – WAS 19 (the missing signal in WAS 22 is most likely due to a PCR artefact), whereas the *vte1* gene gave stonger signals in WAS 13, WAS 19 and WAS 25 (Fig. 3.8 B). By contrast, the *hpd* gene exhibited stronger signals from WAS 17 on, which were most pronounced in WAS 17 and WAS 25.

As genes related to photosynthesis the nuclear *rbcS* gene encoding the small subunit of Rubisco, the plastidic *rbcL* gene encoding the large subunit of Rubisco, and the nuclear *cab* gene that encodes the chlorophyll-*a/b*-binding protein were chosen (Fig. 3.8 C). The *rbcL* gene as well as the *cab* gene were down-regulated in the control from WAS 13 to WAS 17 (Fig. 3.8 C). By contrast, the expression of the nuclear *rbcS* gene appeared to be constant (Fig. 3.8 C). While plastom-containing chloroplasts are degraded quite early in the progression of senescence, the nuclear genome remains active until later stages of senescence thus ensuring the expression of SAGs (Yoschida, 2003). The *SAG12-IPT* line exhibits no down-regulation of these three photosynthesis-related genes. The signals for *cab* and *rbcS* were constant and the signals for *rbcL* even increased throughout the time course.

In order to investigate whether the disturbance of remobilisation in the *SAG12-IPT* line is restricted to processes of carbohydrate metabolism, the expression of two senescence-associated markers for nitrogen remobilisation, namely *ghd* and *gs1* (Masclaux-Daubresse *et al.*, 2005; Pageau *et al.*, 2006), was examined (Fig. 3.8 D). In the control, the expression of *gs1* and *gdh* was induced in WAS 17, which represents a later phases of leaf senescence. In the *SAG12-IPT* line, the expression of both genes was already detectable in WAS 17 and again in WAS 22 and 25. It should be noted that the level of *gs1* expression in *SAG12-IPT* line was very low throughout the whole time course compared to the signal obtained in WAS 17 of the control (Fig. 3.8 D) and that the expression pattern of *gs1* and *gdh* was quite similar to that of *hpd* (Fig. 3.8 B).





**Fig. 3.8 Developmental changes in gene expression.** Gene expression was analysed in the control and in the *SAG12-IPT* line using semiquantitative RT-PCR. The following genes were analysed: (A) the constitutively expressed gene *ef1α* was used as a control and the genes *gus* and *ipt1* indicate the activity of the *SAG12*-promoter in the control (*SAG12-GUS*) and in the *SAG12-IPT* line, respectively (A). The genes *hpd*, *vte1* and *hgo* are genes related to the biosynthetic pathway of tocopherols (B), whereas *rbcL*, *rbcS* and *cab* are genes of photosynthesis (C). The genes *gdh* and *gs1* indicate nitrogen remobilisation (D), while *cp1*, *din* and *pr1b* are used as molecular markers of senescence (E).

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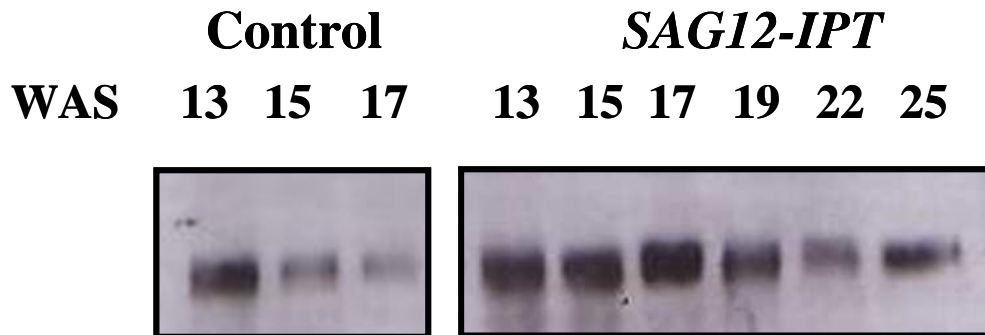
To monitor the onset and the progression of leaf senescence, two different molecular senescence markers were used. Firstly, the *cp1* gene, which encodes the tobacco cysteine proteinase CP1, the endogenous orthologue of the *SAG12* gene in *Arabidopsis thaliana* (Beyene *et al.*, 2006). Secondly, the senescence-associated *din* gene, which possesses 56 % identity to the *Atsen1* gene that is distinctively regulated during natural and artificially induced leaf senescence (Oh *et al.*, 1996, Yang *et al.*, 2003). In the control line a significant induction of both senescence-associated genes occurred in WAS 15, with the strongest signal obtained in WAS 17, when the transient accumulation of  $\gamma$ -tocopherol was observed (Fig. 3.8 E). In the *SAG12-IPT* line, the expression of the senescence-associated genes *din* and *cp1* was clearly detectable in WAS 19 (Fig. 3.8 E). Interestingly, the signals obtained in the *SAG12-IPT* line were not as pronounced as in the control (Fig. 3.8 E). Furthermore, the expression level of the pathogenesis-related gene *pr1b*, which should monitor cell death processes (Wingler *et al.*, 2005), was examined (Fig. 3.8 E). In the control plants, *pr1b* expression was restricted to WAS 17, a time point that exhibits a strong transient accumulation of  $\gamma$ -tocopherol. In the *SAG12-IPT* line, *pr1b* was clearly not induced before WAS 25 (Fig. 3.8 E).

### **3.1.5 Degradation of Rubisco in leaves of *SAG12-IPT* tobacco**

To establish whether increased expression of the *rbcL* gene results in a higher amount of Rubisco, Western blot analysis was performed. Although the antibody was raised against the whole Rubisco protein, the small subunit was not detectable. However, according to previous reports (Wingler *et al.*, 1998; Jordi *et al.*, 2000) the degradation of Rubisco was delayed during the leaf development of the *SAG12-IPT* line (Fig. 3.9).

The level of protein monitored by the Western blot analysis did not follow the expression data obtained for the *rbcL* gene. The expression of *rbcL* increased in the second half of the time course (Fig. 3.8 B), whereas the amount of Rubisco protein decreased during that time (Fig. 3.9). As the degradation of Rubisco is an established indicator for protein remobilisation, these results are in accordance with the observation that

remobilisation processes are impaired by the endogenous production of cytokinin in the *SAG12-IPT* line (see chapter 1.1.5). This suggests that the accumulation of tocopherols, especially  $\alpha$ -tocopherol, counteracts this impairment.



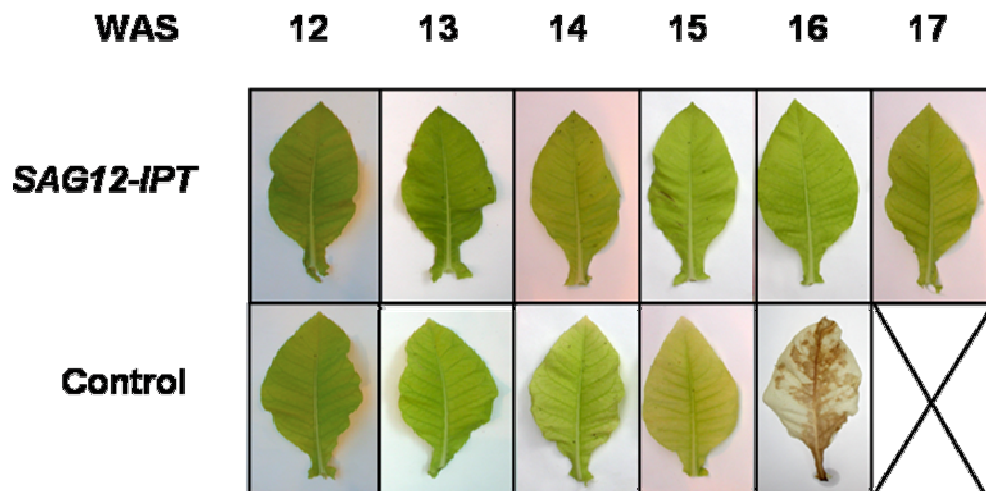
**Fig. 3.9 Immunological detection of the large subunit (LSU) of Rubisco in leaf number 15.** Proteins were extracted from 100 mg leaf material. After denaturation and quantification, 20  $\mu$ g of extract was loaded onto a 10 % SDS polyacrylamide gel. Proteins were blotted to a PVDF membrane and detected with an antibody raised against the whole Rubisco protein.

### **3.1.6 Impact of nutrient deficiency on tocopherol content of leaves of *SAG12-IPT* tobacco**

Many reports state that tocopherols are necessary for a proper remobilisation of carbohydrates, since tocopherol-deficient maize mutants and tocopherol-deficient RNAi-potato lines exhibit a block in sugar export (Provencher *et al.*, 2001; Porfirova *et al.*, 2002; Hofius *et al.*, 2004). In addition, the results presented in this work indicate, that plants which are impaired in remobilisation like the *SAG12-IPT* tobacco show increased levels of tocopherols as the maximal value for  $\alpha$ -tocopherol in the control was just approximately 70% of the maximal value obtained for the *SAG12-IPT* line (Fig. 3.3).

The next question to be addressed was whether tocopherol content is altered when plants have an enhanced need for remobilisation. To this end tobacco plants were grown under nutrient-deficient conditions. It is well-known that nitrogen depletion (or deficiency) causes an increase in the recycling and remobilisation of nitrogen, when the uptake of nutrients from the soil is

limited (Schulze *et al.*, 1994; Diaz *et al.*, 2008). As a consequence, plants are highly dependent on recycling and inner organismic distribution of remobilised macromolecules (Schulze *et al.*, 1994; Diaz *et al.*, 2008). The development of leaf number 15 is shown in Fig. 3.10. The time period of growth was shortened in comparison to normal growth conditions (Fig. 3.1). While the control leaf died in WAS 16, the *SAG12-IPT* leaf was still alive up to WAS 22 (not shown). Interestingly, the life span of leaf number 15 of the control plants was approximately two weeks shorter when grown under nutrient deficiency (compare Fig. 3.1 and Fig. 3.10), whereas leaf number 15 in the *SAG12-IPT* line showed a life span reduced by approximately five weeks when nutrients coming from the soil were limited.



**Fig 3.10 Development of leaf number 15 in *SAG12-IPT* tobacco grown under nutrient deficiency.** Plants were grown on “Stender Vermehrungssubstrat” without fertiliser for 17 weeks. Every week leaf number 15 was collected from a *SAG12-IPT* plant and from one control plant, respectively, beginning in WAS 12 and ending in WAS 17.

Plants of both lines exhibited very small amounts of chlorophylls throughout the whole time, alternatively the ratio of  $F_v/F_m$  was measured to determine the onset and progression of leaf senescence (Fig. 3.11 A). The  $F_v/F_m$  value describes the maximal efficiency of PSII and is a reliable indicator of senescence (Humbeck *et al.*, 1996). In the control plants, the maximum efficiency of PSII started to decrease significantly in WAS 15

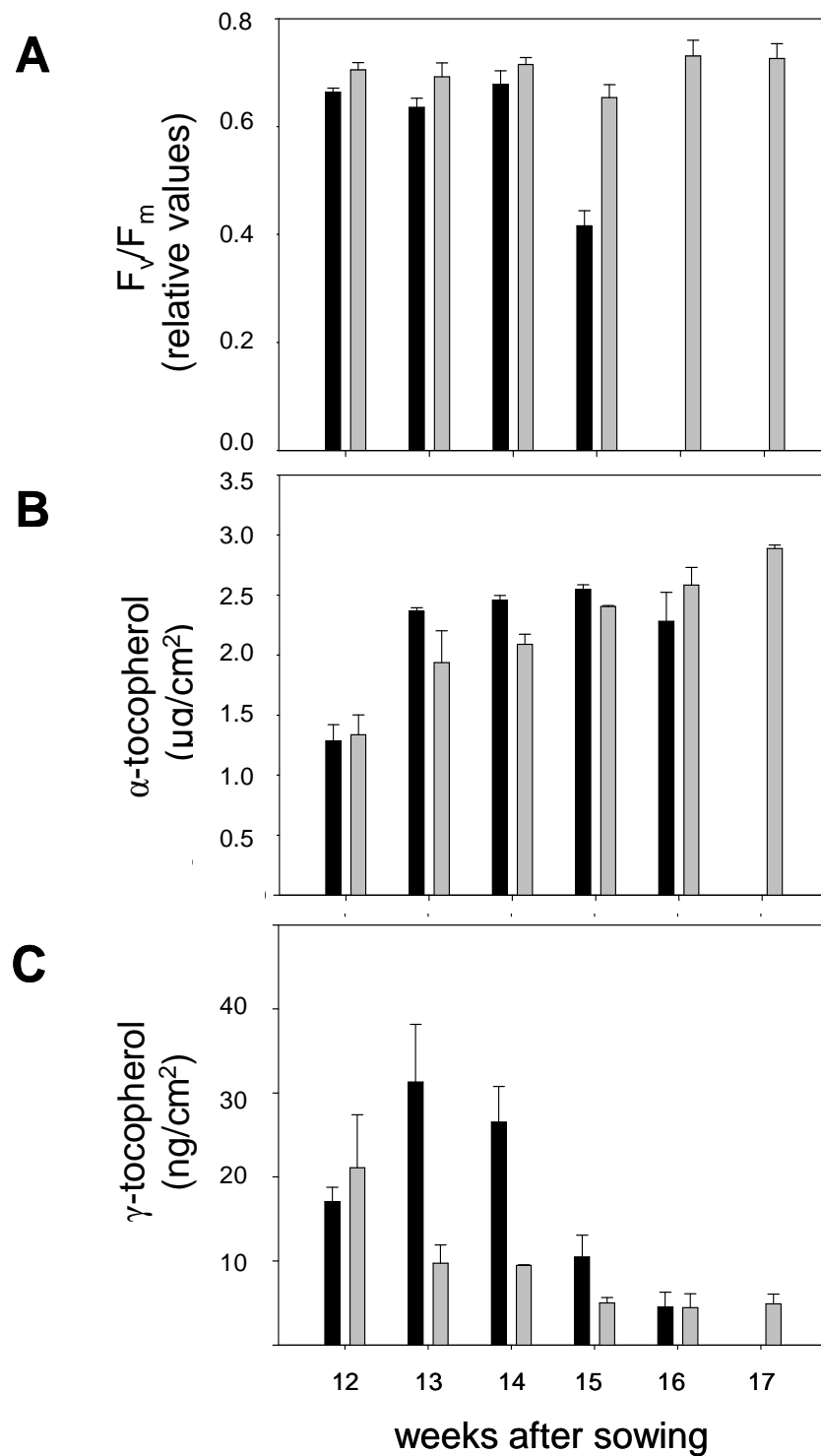
and was below the level of detection in WAS 16 (Fig. 3.11 A). In the *SAG12-IPT* line,  $F_v/F_m$  was constant throughout the whole time span analysed (Fig. 3.11 A).

The values for  $\alpha$ -tocopherol were quite high in young and senescent leaves of both lines (Fig. 3.11 B). Under nutrient-deficient conditions the accumulation of  $\alpha$ -tocopherol was not restricted to senescence. This supports the rationale that  $\alpha$ -tocopherol accumulation is generally related to remobilisation as the need to remobilise is given long before the onset of senescence. Especially the high  $\alpha$ -tocopherol content in WAS 16 of the control is noteworthy as the leaf still contained more than  $2 \mu\text{g}/\text{cm}^2$  (Fig. 3.11 A), although the leaf had already lost all its photosynthetic activity (Fig. 3.11 B).

In the *SAG12-IPT* line, the values for  $\gamma$ -tocopherol increased transiently in WAS 13 and WAS 14 while in the control the maximum value was already observed in WAS 12 (Fig. 3.11 C).

The tocopherol values from the first time course study under normal growth conditions (Fig. 3.3) were compared to those from the time course experiment under nutrient deficiency (Tab. 3.1). In WAS 13, leaf number 15 contained an approximately ten fold higher  $\alpha$ -tocopherol content and an up to 100-fold higher  $\gamma$ -tocopherol content when the *SAG12-IPT* and control plants were grown under nutrient deficiency. As the amounts of  $\gamma$ -tocopherol are minimal and therefore negligible, the total tocopherol content mainly reflects the amounts of  $\alpha$ -tocopherol. The ratio of  $\gamma$ -tocopherol /  $\alpha$ -tocopherol significantly increased to approximately ten fold in the control and about two fold in the *SAG12-IPT* line when plants were grown under nutrient-deficient conditions and not under normal growth conditions (Tab. 3.1).

In summary, this experiment demonstrated that a plant with an increased need to remobilise, for example during senescence or when grown under nutrient deficiency, exhibits a higher content of tocopherols.



**Fig. 3.11** Developmental changes of tocopherol content in leaf number 15 of *SAG12-IPT* tobacco grown under nutrient deficiency. In leaf number 15 of the *SAG12-IPT* line (white bars) and of the control line (dark bars), the maximum PSII efficiency (A) was determined by Imaging-PAM and the contents of  $\alpha$ -tocopherol (A) and  $\gamma$ -tocopherol (B) were measured by HPLC over a period of six weeks, beginning in WAS 12 and ending in WAS 17. Values are given as the mean of triplicate measurement and were based leaf surface.

**Tab. 3.1 Content of tocopherols in leaves of *SAG12-IPT* tobacco grown under normal growth conditions and under nutrient deficiency.** The table compares the tocopherol content in leaf number 15 of *SAG12-IPT* tobacco and control, grown under normal growth conditions, and the tocopherol content of leaf number 15 of *SAG12-IPT* tobacco and the control, grown under nutrient deficiency, in WAS 13. The values for  $\alpha$ -tocopherol and  $\gamma$ -tocopherol (both in  $\mu\text{g}/\text{cm}^2$ ) stated here were determined under the normal growth conditions described in chapter 4.1.1 and those under nutrient deficiency detailed in this chapter. The values for the content of  $\alpha$ -tocopherol and  $\gamma$ -tocopherol are the mean values of triplicate measurements with the standard deviation (SD) indicated in brackets. In addition, total tocopherol (in  $\mu\text{g}/\text{cm}^2$ ) and the  $\gamma$ -tocopherol/ $\alpha$ -tocopherol ratio are given.

Leaf 15 in WAS 13	normal growth conditions		nutrient deficiency	
	Control	<i>SAG12-IPT</i>	Control	<i>SAG12-IPT</i>
$\alpha$ -tocopherol in $\mu\text{g}/\text{cm}^2$	0.27 (0.04)	0.35 (0.04)	2.36 (0.03)	1.92 (0.24)
$\gamma$ -tocopherol in $\mu\text{g}/\text{cm}^2$	0.39 (0.02)	0.13 (0.03)	31.30 (6.85)	9.65 (2.10)
total tocopherol in $\mu\text{g}/\text{cm}^2$	0.21	0.28	2.37	1.93
Ratio $\gamma/\alpha$	$14 \times 10^{-3}$	$2.5 \times 10^{-3}$	$13.2 \times 10^{-3}$	$5.0 \times 10^{-3}$

### **3.1.7 Tocopherol content in the leaf age gradient**

To find support for the observation that the accumulation of  $\alpha$ -tocopherol is age-dependent whereas the accumulation of  $\gamma$ -tocopherol senescence-dependent, tocopherols were measured in different leaves of one and the same plant grown under normal growth conditions. Tobacco is characterised by a leaf age gradient, as old leaves grow at the bottom of the plant and young, new emerging leaves on the top (Fig. 2.1). In WAS 15, the tocopherol content of the young leaves number 20 from the top of one *SAG12-IPT* plant and of one control plant was compared to the tocopherol content of the old leaves number 10 taken from the bottom of a *SAG12-IPT* plant and a control. The young leaves number 20 from the top of the plants appeared very similar (Fig. 3.13 A).

The old leaf number 10 of the *SAG12-IPT* plant, which exhibits an auto-regulated production of senescence retarding IPT, still showed no visible signs of senescence, whereas the old leaf number 10 of the control

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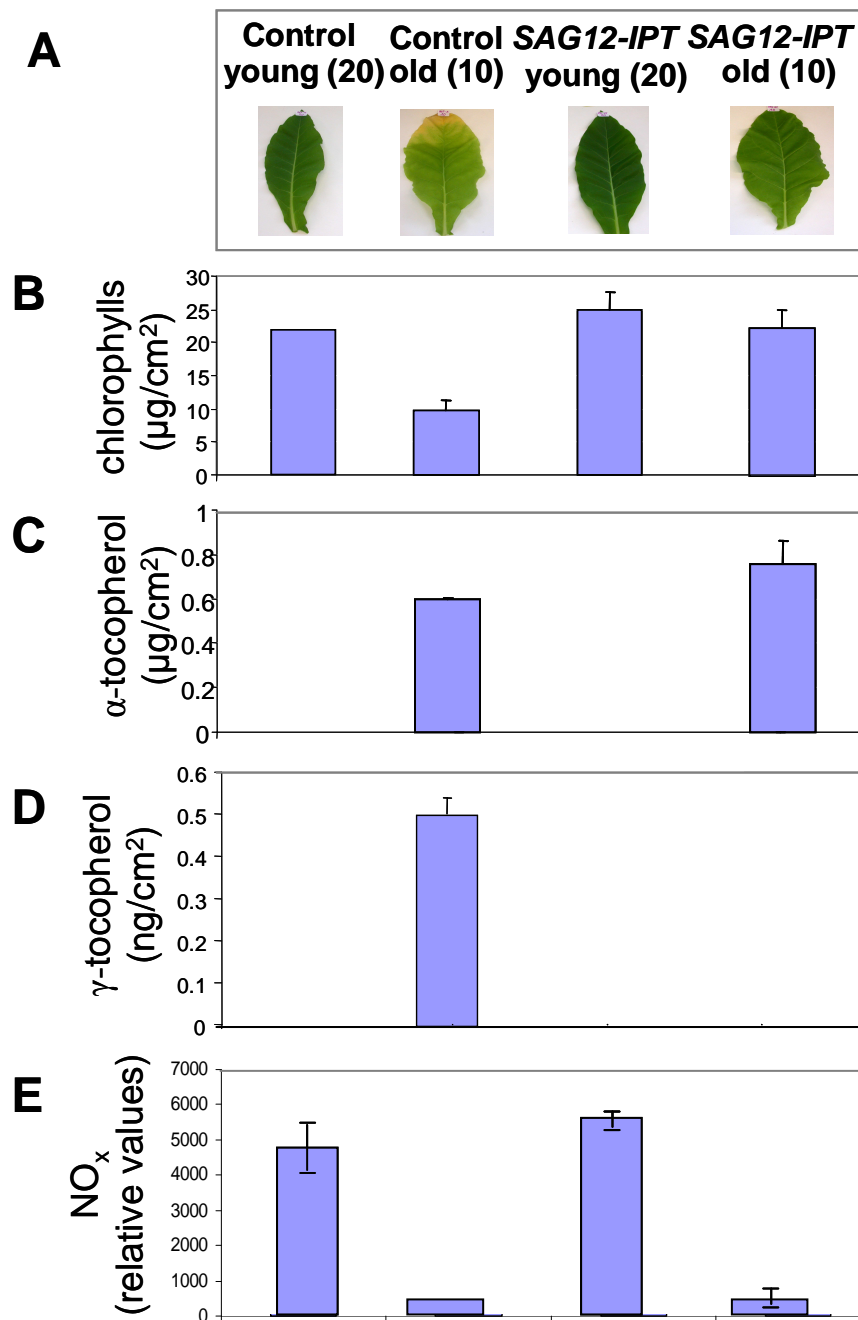
yellowed considerably. This observation was underlined by the values for chlorophylls (Fig. 3.13 B). In both lines, the young leaf number 20 possessed a high chlorophyll content of approximately 20-25  $\mu\text{g}/\text{cm}^2$ . In the old leaf number 10 of the *SAG12-IPT* plant, the chlorophyll content was still comparable to that in the young leaves. However, in the old leaf of the control the chlorophyll content was reduced by over 50 % to approximately 10 $\mu\text{g}/\text{cm}^2$ , demonstrating that the old leaf number 10 of the control plant was senescent, while the corresponding old leaf of the *SAG12-IPT* plant was not.

The measurement of  $\alpha$ -tocopherol (Fig. 3.12 C) and  $\gamma$ -tocopherol (Fig. 3.12 D) revealed that  $\alpha$ -tocopherol accumulated in both old leaves number 10, whether the leaf was senescent (control) or not (*SAG12-IPT* line) (Fig. 3.12 C). By contrast,  $\gamma$ -tocopherol accumulated only in the senescent leaf number 10 from the control, whereas it is not detectable in the non-senescent leaf number 10 from the *SAG12-IPT* (Fig. 3.12 D).

Since it is known from experiments on seeds of *Arabidopsis thaliana* that  $\gamma$ -tocopherol reacts with the signal molecule NO to regulate germination (Desel *et al.*, 2007), it was hypothesised that  $\gamma$ -tocopherol might also scavenge NO in leaves. As NO is discussed as a senescence-retarding molecule (Leshem *et al.*, 1998), the postulated reaction with  $\gamma$ -tocopherol was considered a mechanism to initiate senescence. To test this hypothesis, the relative content of NO was measured by a DAF-FM assay (Fig. 3.12 E). In both lines, the content of NO decreased with leaf age. From this point of view a interaction between  $\gamma$ -tocopherol and NO seemed quite unlikely.

This experiment supported the findings from the time course experiment under normal growth conditions that the accumulation of  $\alpha$ -tocopherol is dependent on leaf age, whereas the accumulation of  $\gamma$ -tocopherol is senescence-specific.

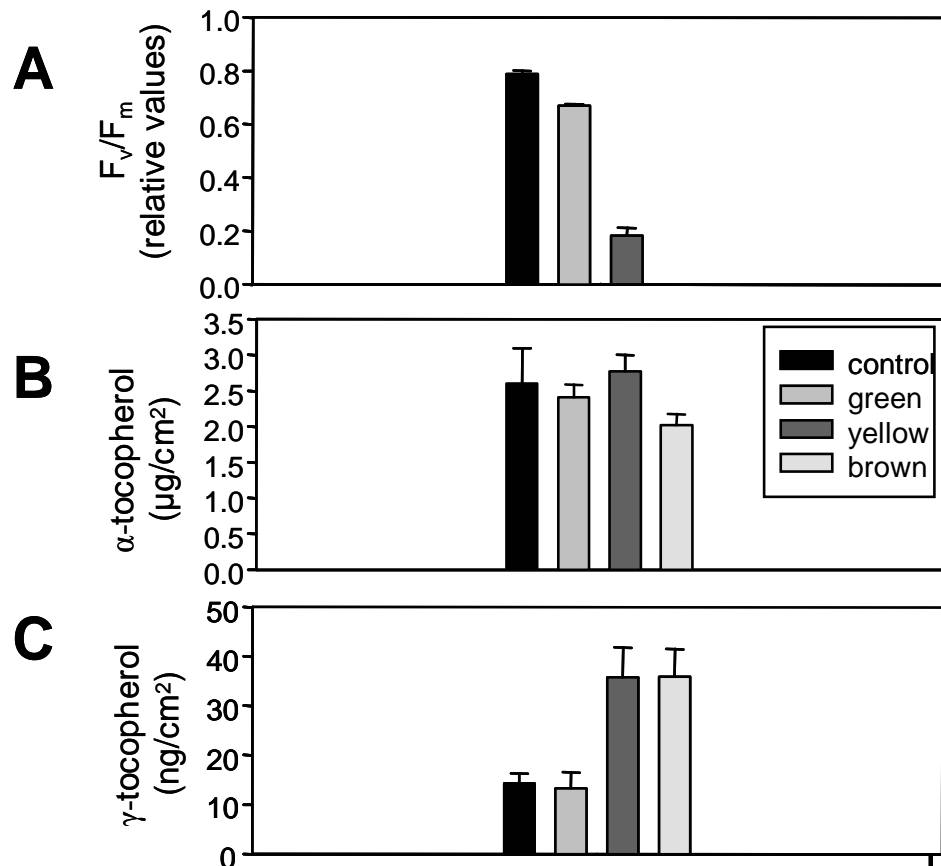




**Fig. 3.12 Analysis of tocopherols in tobacco leaves taken from different stages of a plant.** (A) In WAS 15 a young leaf from the top (leaf number 20) and an old leaf from the bottom (leaf number 10) were analysed in the *SAG12-IPT* line (right) and in the control (left). Chlorophylls were measured as a senescence parameter (B). The  $\alpha$ -tocopherol (C) and  $\gamma$ -tocopherol (D) content was determined by HPLC. The relative  $\text{NO}_x$  values (E) were measured by the DAF-FM-assay. All values are the mean of three measurements and stated relative to leaf surface.

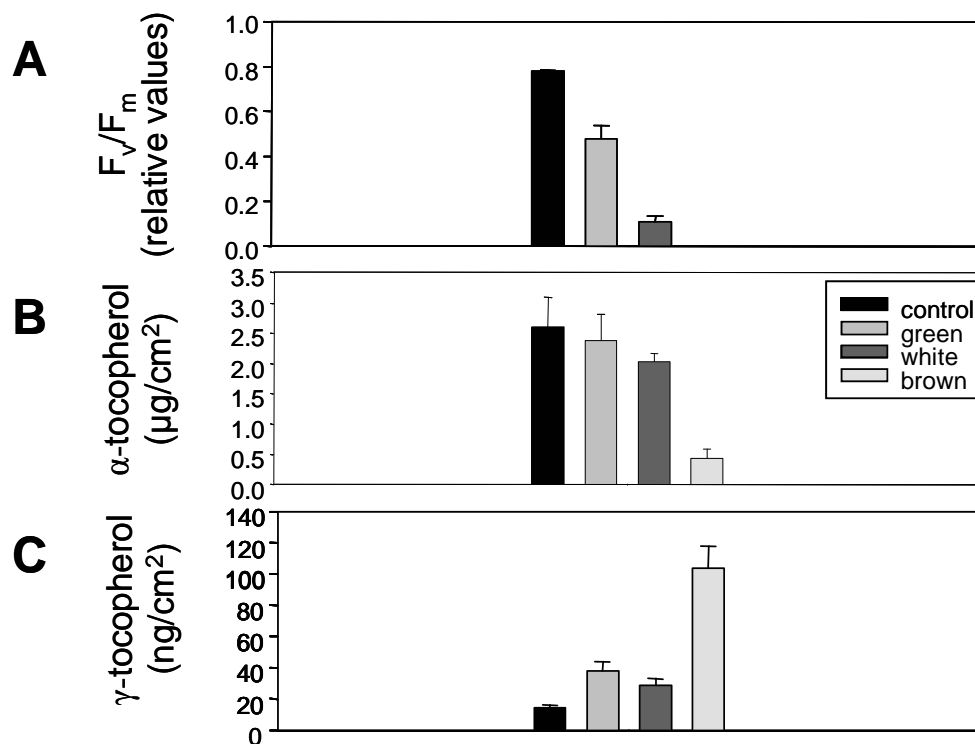
### **3.1.8 Distribution of tocopherols in a non-uniformly senescing tobacco leaf**

A tobacco leaf does not senesce uniformly, hence heterogeneous tissues at different stages of senescence are present in a single tobacco leaf.



**Fig. 3.13 Distribution of tocopherols in a senescent leaf of the *SAG12-GUS* tobacco.** Green, yellow and brown leaf discs were taken from leaf number 15 of the *SAG12-GUS* line in WAS 18. As a control, green and non-senescent leaf discs from the top of a control plant were used. Following  $F_v/F_m$  measurements, which indicate the efficiency of PSII (A), leaf discs were analysed for their  $\alpha$ - (B) and  $\gamma$ -tocopherol (C) content.

In a senescent leaf of the control plants, there were still some green parts undergoing the initiation of senescence, where chlorophyll and photosynthetic competence was still maintained. At the same time yellow parts were present in the leaf, especially in the interveinal areas, that were apparently about to remobilise nutrients from the source organ. Additionally, some leaf areas were already brown indicating PCD which occurs during the terminal phase of leaf senescence. A senescent leaf of the *SAG12-IPT* line, also showed green and brown areas. However, instead of yellow tissue the senescent leaf of the *SAG12-IPT* line exhibited white parts. This observation may be due to the disturbance of remobilisation as in this organism chlorophylls are not degraded and remobilised to the vacuole, but retarded in the leaf until photooxidative stress causes the destruction of chlorophylls and the bleaching of the senescent tissue.



**Fig. 3.14 Distribution of tocopherols in a senescent leaf of the *SAG12-IPT* tobacco.** Green, white and brown leaf discs were taken from leaf number 15 of the *SAG12-IPT* line in WAS 27. As a control, green and non-senescent leaf discs were derived from a young leaf taken from the top of a control plant (A). After the measurement of  $F_v/F_m$  indicating the efficiency of PSII (A), leaf discs were analysed for their  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol (C) content.

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In order to gain information about the biological significance of tocopherol accumulation, the tocopherol content of leaf discs taken from green, yellow and brown tissue of leaf number 15 of the control in WAS 18 was measured. In parallel, the green, white and brown tissue of leaf number 15 of the *SAG12-IPT* line in WAS 27 were analysed for their tocopherols content. Leaf discs derived from a young leaf of the control line, respectively, were taken as a non-senescent reference.

Prior to tocopherol measurements,  $F_v/F_m$  was determined by Imaging-PAM indicating the maximum PSII efficiency (Fig. 3.13 A, 3.14 A). The leaf discs from still green tissue undergoing the initiation of senescence exhibited a value for  $F_v/F_m$  that was approximately reduced by 20% in the control plants and approximately by 40% in the *SAG12-IPT* line when compared to leaf discs taken from the young control leaf (Fig. 3.13 A, 3.14 A). Remobilising and thus yellow tissue from the control line (Fig. 3.14 A) as well as the white, bleached tissue from the *SAG12-IPT* line (Fig. 3.14 A) showed a decrease in the maximal PSII efficiency of about 80% (in comparison to the young control leaf). Finally, brown tissue of both lines had completely lost its photosynthetic ability (Fig. 3.13 A, 3.14 A).

The measurement of tocopherols in the *SAG12-GUS* line (control) revealed a similar amount of  $\alpha$ -tocopherol in all tissues analysed (Fig. 3.13 B). By contrast, the amount of  $\gamma$ -tocopherol was significantly increased in yellow and brown tissue (Fig. 3.13 C). The analysis of tocopherols in different tissues of a leaf taken from a *SAG12-IPT* line showed that the content of  $\alpha$ -tocopherol is comparable in the non-senescent control tissue, green and white tissue, whereas the content in brown tissue is significantly decreased (Fig. 3.14 B). In the *SAG12-IPT* line, the amount of  $\gamma$ -tocopherol was significantly increased exclusively in brown tissue (Fig. 3.14 C).

### 3.2 Developmental changes in the tocopherol content of recombinant inbred lines (RILs)

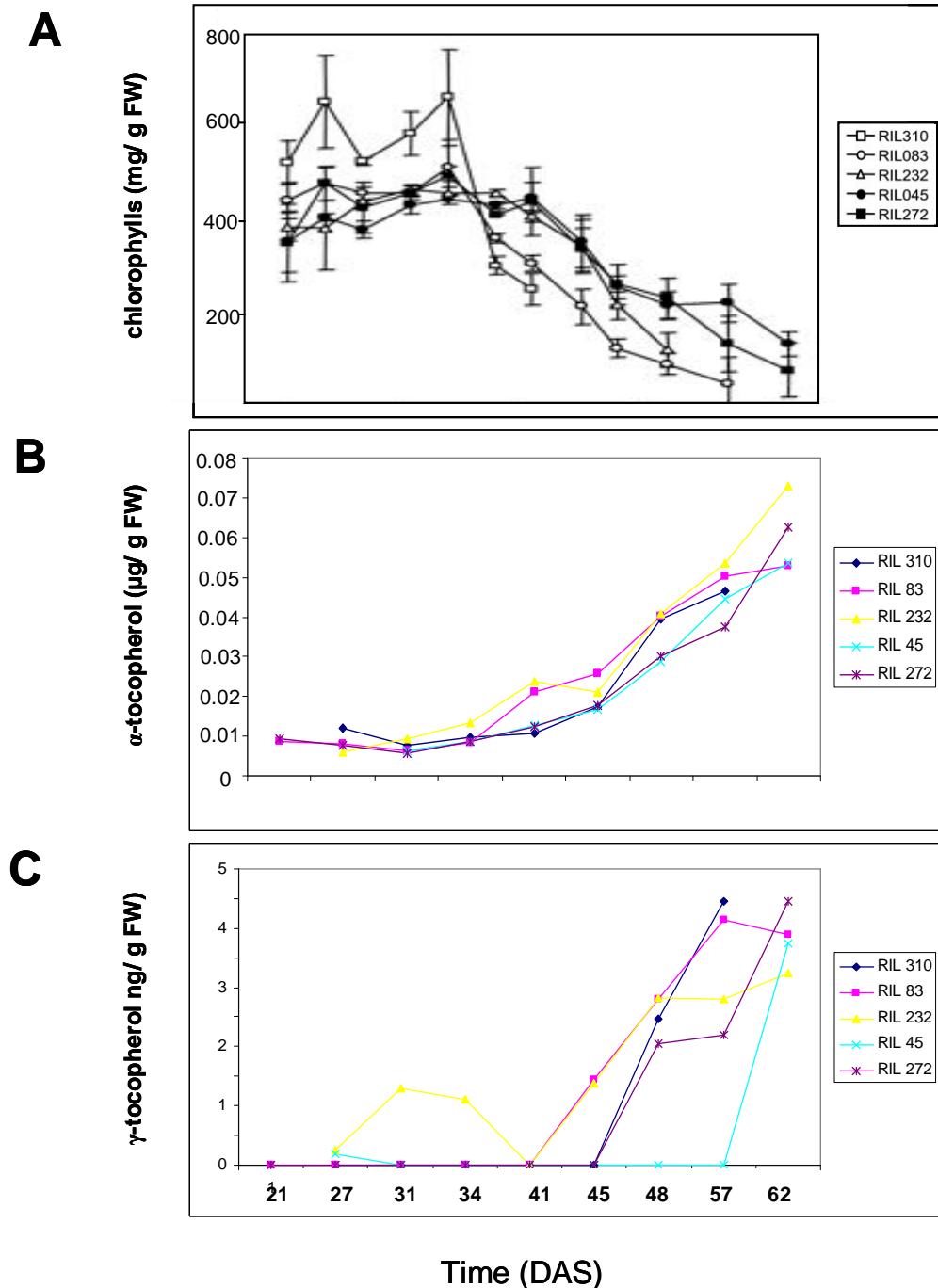
As previously shown in this work for the tobacco system, the senescence-associated accumulation of  $\alpha$ -tocopherol increased slightly and was shown to be dependent on leaf age, whereas the accumulation of  $\gamma$ -tocopherol occurs transiently at later stages of senescence.

In order to examine whether these findings are also valid in another plant model system with irregular senescence behaviour, five RILs of *Arabidopsis thaliana* were investigated. RILs are the result of a cross between the two different wild types Shadhara and Bay-0 that are genetically quite distant from each other. Tocopherols were measured in five RILs exhibiting different onsets of leaf senescence (from early senescing to late senescing) in a developmental time course. As plant material, the first six emerging leaves of the rosette were used.

$\alpha$ -tocopherol accumulation increased slightly and constantly with leaf age in every line (Fig. 3.15 B), regardless of the differences in the onsets of senescence in the RILs as indicated by the chlorophyll content (Fig. 3.15 A). These results demonstrated that the age-dependent accumulation of  $\alpha$ -tocopherol is not only occurring in the tobacco leaves, but also in rosette leaves of five RILs of *Arabidopsis thaliana*. However, in contrast to the tobacco system, no decrease at the end of development was evident (Fig. 3.3; Fig. 3.4; Fig. 3.15 B). This can be explained by the fact, that the tocopherol extract measured in tobacco was derived from one single leaf and the extract analysed in the RILs was a mixture of six leaves what makes leveling effects possible.

While the late senescing lines RIL 45 und RIL 272 reached their maximum levels very late at DAS 62, the early senescing lines RIL 310 and RIL 83 already exhibited their maximum peaks at DAS 57 (Fig. 3.15 C). The earlier senescing RIL 232 showed a transient peak from DAS 31 to 34, when senescence was still not initiated, but the major accumulation in RIL 232 started fairly early in WAS 45 (Fig. 3.15 C). It is obvious, that early senescing lines showed an early accumulation of  $\gamma$ -tocopherol, whereas the late senescing lines showed a late accumulation.

These results suggest that  $\gamma$ -tocopherol might play a role that is associated to late senescence processes, while  $\alpha$ -tocopherol could be responsible for more global processes such as the remobilisation of nutrients.

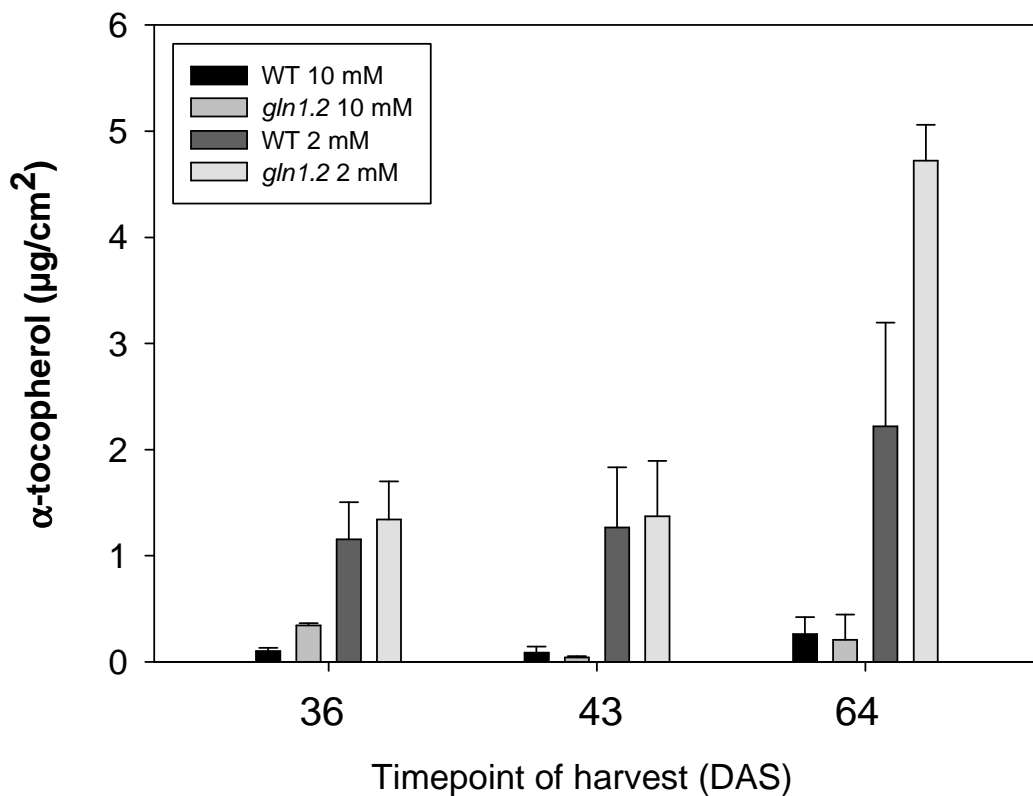


**Fig. 3.15** Content of tocopherols in rosette leaves of *Arabidopsis thaliana* RILs. (A) The values for the content of chlorophylls were taken from Diaz *et al.*, 2005. The contents of  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol (C) were measured by HPLC in leaf material from the first six emerging leaves of five recombinant inbred lines (RIL310, RIL83, RIL232, RIL45, RI 272) in DAS (days after sowing).

### 3.3 The effect of the knock-out of the gene *gln1.2* on tocopherol content in *Arabidopsis thaliana*

#### 3.3.1 Tocopherol content in rosette leaves

Studies with tobacco indicated that  $\alpha$ -tocopherol might play a role in remobilisation processes. To further test this hypothesis, tocopherols were measured in the *gln1.2*-knock-out mutant of *Arabidopsis thaliana*, which lack a functional *gln1.2* gene that encodes the cytosolic glutamine synthase GS1. In contrast to the four other isoforms (*gln1.1*, *gln1.3*, *gln1.4*, *gln1.5*) existing in *Arabidopsis thaliana*, *gln1.2* expression is up-regulated during leaf senescence. The *gln1.2*-knock-out mutant was shown to be affected in remobilisation processes as demonstrated by a delay in flowering, a slower stem growth and a higher accumulation of total amino acids compared to the wild type (Master thesis of Laure Gaufichon, 2006).



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**Fig. 3.16 Developmental changes in the  $\alpha$ -tocopherol content of the *gln1.2*-knock-out mutant of *Arabidopsis thaliana*.** The graph shows the  $\alpha$ -tocopherol content (in  $\mu\text{g/g}$  FW) in the first six rosette leaves of the *gln1.2*-knock-out mutant and the wild type grown under 10 mM or 2 mM nitrogen. The measurements were performed at DAS 36 and DAS 43, both representing the mature leaf, and at DAS 64, representing the senescent leaf. All values are the mean of three independent measurements.

The *gln1.2*-knock-out mutant and the corresponding wild type were grown on soil supplemented with 10 mM (normal growth conditions) or 2 mM (nutrient deficiency) nitrate. The measurements were performed with material from the whole rosette at WAS 36 and WAS 43 representing the mature leaf, and at WAS 64 representing senescent leaves.

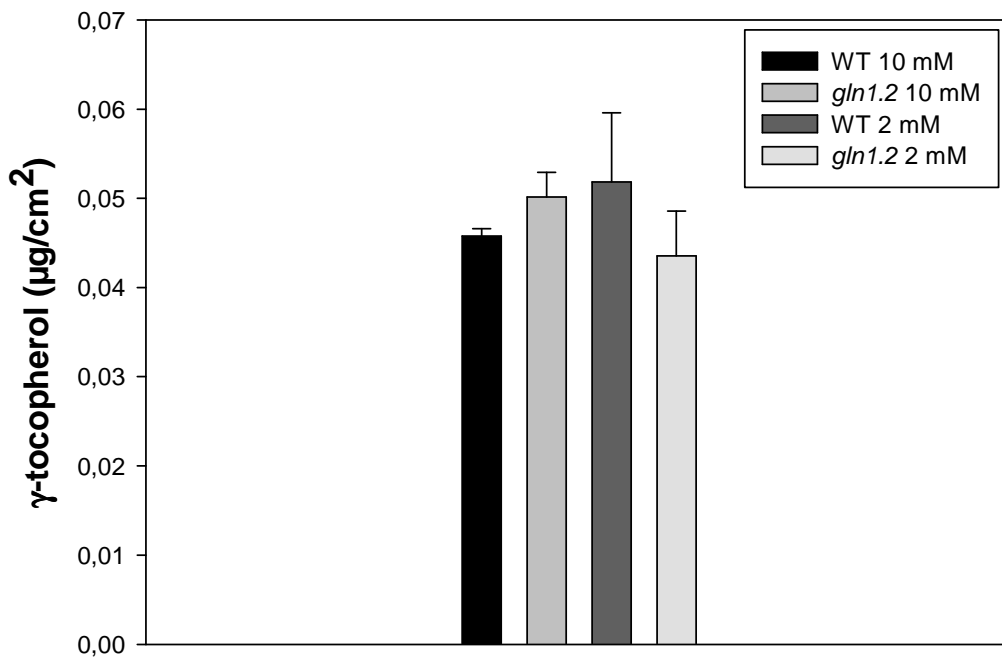
The level of  $\alpha$ -tocopherol was very low in mature leaves (WAS 36 and WAS 43) as well as senescent leaves (WAS 64) of the wild type and the mutant when grown under 10 mM nitrate (Fig. 3.16). However,  $\alpha$ -tocopherol was significantly increased, when wild type and mutant plants are grown under 2 mM nitrate. The increase in  $\alpha$ -tocopherol content was less pronounced in mature leaves (WAS 36 and WAS 43) and more in senescent leaves (WAS 64), particularly those of the mutant. The results for the measurement of  $\gamma$ -tocopherol are not presented here, since they were not detectable.

### **3.3.2 Tocopherol content of seeds**

As a control  $\gamma$ -tocopherol representing the major tocopherol form found in seeds (see chapter 1.2.4) was measured in seeds of the *gln1.2*-knock-out mutant and the wild type when both were grown under 2 mM or 10 mM nitrogen. Unexpectedly, there are neither significant differences between the wild type and the *gln1.2*-knock-out mutant nor significant differences between the seeds subjected to different growth conditions with either 2 mM or 10 mM nitrate (Fig. 3.17).

In conclusion, while the growth condition and the disturbance of remobilisation caused by the knock-out of the *gln1.2* gene had a significant impact on the  $\alpha$ -tocopherol content in the leaf, no changes in  $\gamma$ -tocopherol content were detectable in the seed.





**Fig. 3.17**  $\gamma$ -tocopherol content in seeds of the *gln1.2*-knock-out mutant of *Arabidopsis thaliana*. The graph shows the content of  $\gamma$ -tocopherol (in  $\mu\text{g}/\text{g}$  FW) in seeds of the *gln1.2*-knock-out mutant and the wild type grown under 10 mM or 2 mM nitrate. All values are the mean of four independent measurements.

### 3.4 Tocopherol content in the *Festuca/Lolium* stay-green mutant

According to the time course experiment on *SAG12-IPT* tobacco grown under normal growth conditions, the accumulation of tocopherols did not appear to be a passive consequence of the chlorophyll degradation, as the amount of degraded chlorophyll is about 10 fold higher than the amount of accumulating tocopherols. So the idea that tocopherol biosynthesis functions as a kind of ‘waste bin’ for chlorophyll broken down during the progression of leaf senescence was not supported here. However, the results of the *SAG12-IPT* tobacco time course could not rule out that the accumulating tocopherol uses the reactivated phytol coming from the breakdown of chlorophylls.

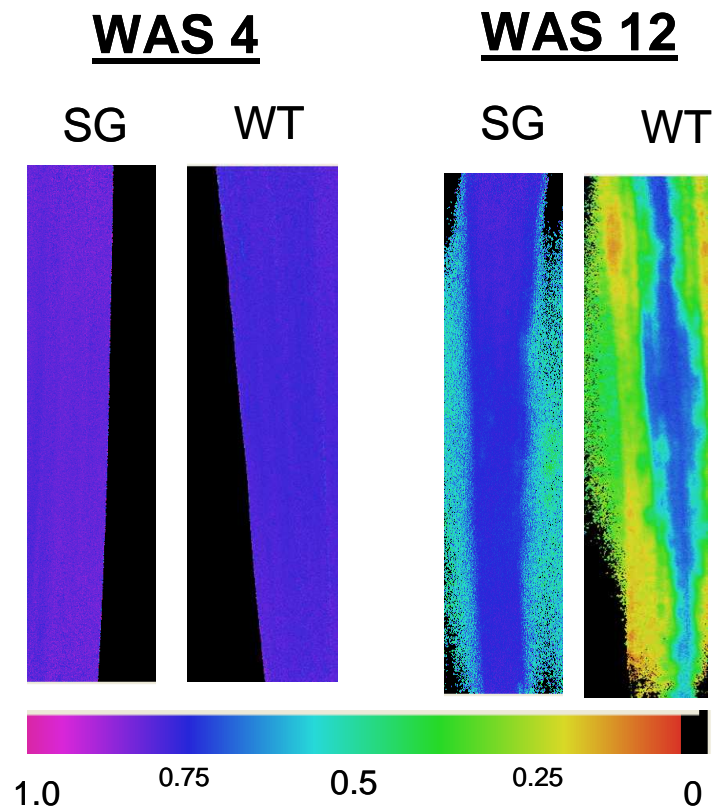
To investigate whether tocopherols are still able to accumulate when there is no phytol from chlorophyll degradation available, the  $\alpha$ - and  $\gamma$ -tocopherol

and chlorophyll contents were measured in leaf number one of the *Festuca/Lolium* stay-green mutant and wild type in WAS 4 and WAS 12, representing young and old leaf, respectively. This mutant exhibits a disturbance in pigment metabolism caused by the blockage of the ring opening step in chlorophyll breakdown (Thomas *et al.*, 2002). Although the ring opening step occurs later than phytol elimination from the chlorophyll molecule by the chlorophyllase, a progressive accumulation of the upstream metabolite chlorophyll can be observed (Thomas, *et al.*, 2002).

As further senescence parameters, the maximum PSII efficiency (Tab. 3.2; Fig. 3.18) and the SPAD value (Tab. 3.3) were measured. As expected, the values for  $F_v/F_m$ , which represent the maximum PSII efficiency of as well as the SPAD values decreased in the wild type from WAS 4 to WAS 12 (Tab. 3.2; Fig. 3.18, Tab. 3.3). In contrast, the stay-green mutant showed no significant decrease of  $F_v/F_m$  or SPAD (Tab. 3.2; Fig. 3.18; Tab. 3.3). Fig. 3.19 presents the values for chlorophylls and tocopherols. In the wild type, chlorophylls decreased and  $\alpha$ - and  $\gamma$ -tocopherol accumulated from WAS 4 to WAS 12. By contrast, the stay-green mutant showed a significant increase in  $\alpha$ - and  $\gamma$ -tocopherol contents, although no degradation of chlorophylls was observed. Evidently chlorophyll degradation is no prerequisite for the accumulation of tocopherols.

**Tab. 3.2 Measurement of the maximal efficiency in a young and an old leaf of the *Festuca/Lolium* stay-green mutant.**  $F_v/F_m$  was measured by Imaging-PAM in leaf number one of the *Festuca/Lolium* stay-green mutant and in the wild type. Measurements of  $F_v/F_m$ , representing the maximum PSII efficiency, were performed in WAS 4 and WAS 12. Mean values of measurements from six independent areas of interest equally distributed over the whole leaf blade (SD is given in brackets) are stated.

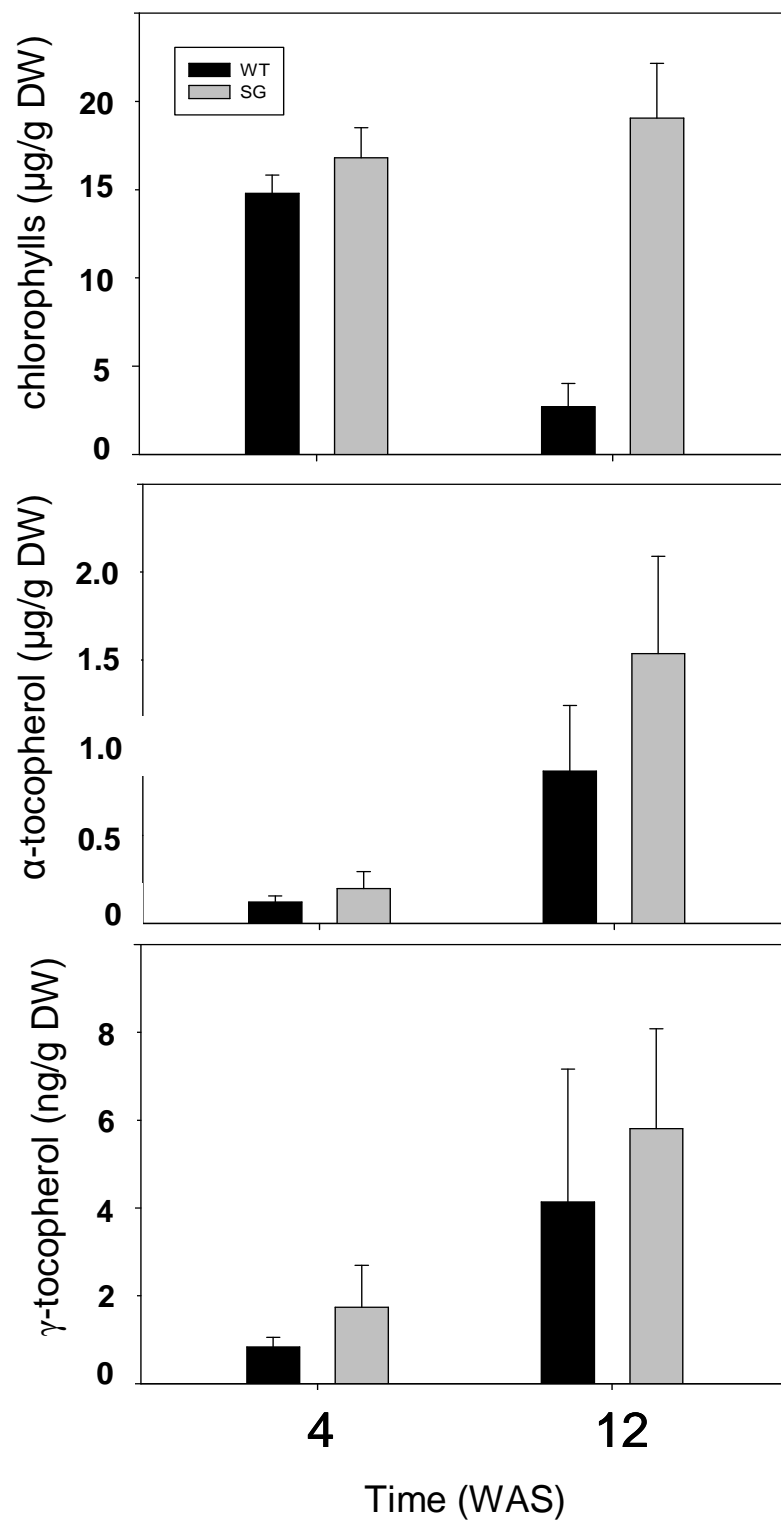
Age of leaf	Wild type	Stay green
WAS 4	0.79 (0.005)	0.80 (0.009)
WAS 12	0.45 (0.175)	0,72 (0.057)



**Fig. 3.18 Image of the Imaging-PAM.** Leaf number one of the *Festuca/Lolium* stay-green mutant (SG) and of the wild type (WT) were analysed in WAS 4 and WAS 12. Blue areas indicate a high value for  $F_v/F_m$ , whereas yellow and green areas show a more or less decreased value for  $F_v/F_m$ , respectively. The values for the measurement with the Imaging-PAM are given in table 3.2 A.

**Tab. 3.3 Measurement of SPAD.** SPAD values representing the relative chlorophyll content were measured in leaf number one of the *Festuca/Lolium* stay-green mutant and in the wild type. Measurements were performed in WAS 4 and WAS 12. Values are the mean of about 30 measurements equally distributed over the whole leaf blade of all leaves taken for further analyses (SD is given in brackets).

Age of leaf	Wild type	Stay green
WAS 4	33.50 (3.30)	35.73 (3.24)
WAS 12	14.44 (6.97)	45,765 (5.00)

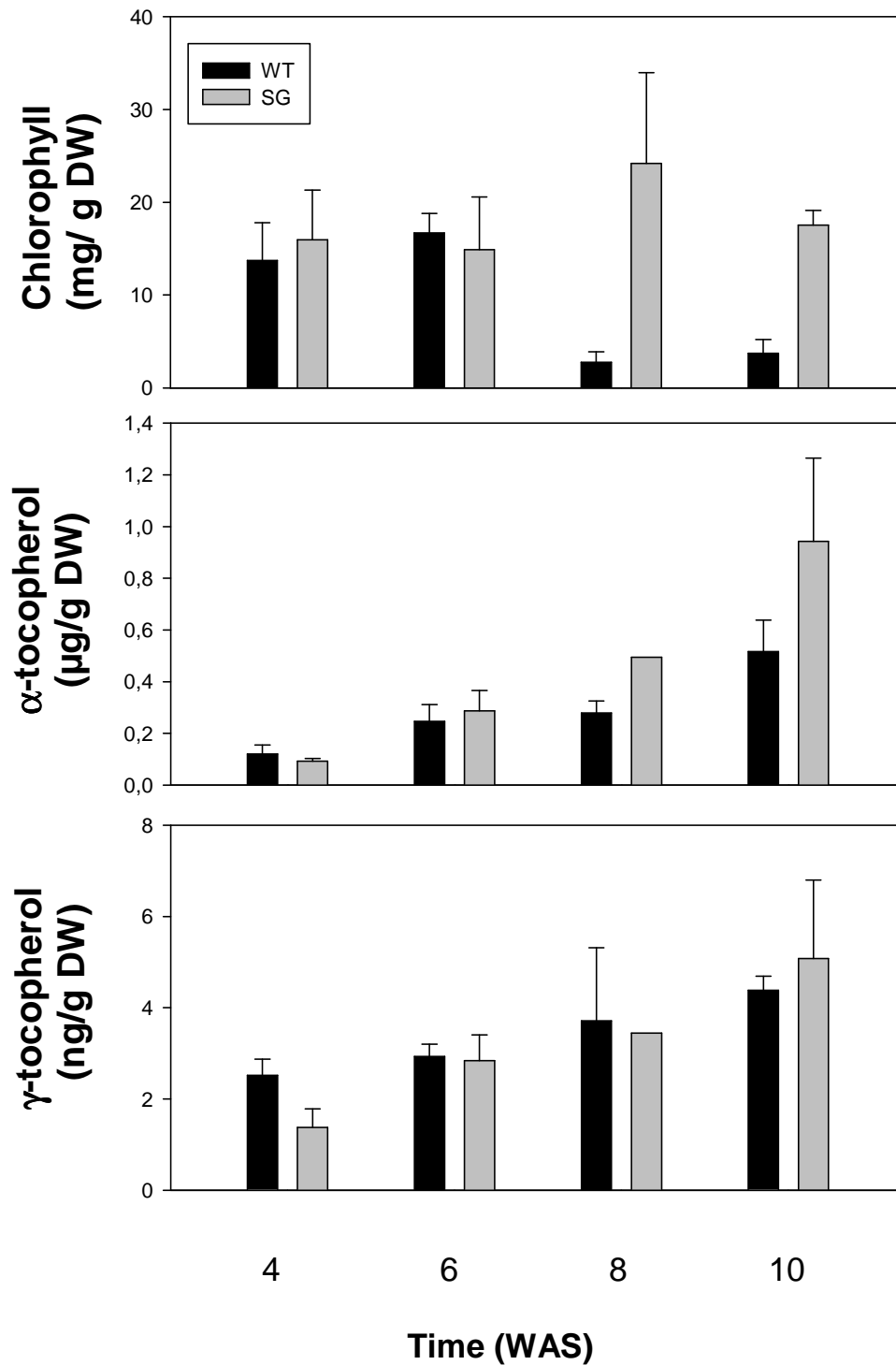


**Fig. 3.19** Content of chlorophylls and tocopherols in young and senescent leaves of the *Festuca/Lolium* stay-green mutant. Chlorophylls (A),  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol (C) were determined in leaf number one of the *Festuca/Lolium* stay-green mutant (grey bars) and in the wild type (black bars). Measurements were performed in WAS 4 and WAS 12, representing a young and a senescent leaf, respectively. All values are the result of six independent measurements with leaves from six different plants.

To confirm these results, a more accurate time course experiment was performed including four different time points (WAS 4, WAS 6, WAS 8, WAS 10). In the stay-green mutant, the maximum PSII efficiency remained constant over the whole time course, whereas the wild type showed a significant decrease in WAS 10 (Tab. 3.4).  $\alpha$ - and  $\gamma$ -tocopherol accumulated in the wild type and the stay-green mutant, although the stay-green mutant showed no degradation of chlorophylls (Fig. 3.20). Interestingly, the  $\alpha$ -tocopherol content in WAS 10 showed a greater increase in the mutant than in the wild type (Fig. 3.20 B), while the  $\gamma$ -tocopherol content was nearly the same in both in WAS 10 (Fig. 3.20 C). This indicates that the two forms of tocopherol might have different functions.

**Tab. 3.4: Development-dependent changes of maximal PSII-efficiency in the *Festuca/Lolium* stay-green mutant.**  $F_v/F_m$  was measured by Imaging-PAM in leaf number one of the *Festuca/Lolium* stay-green mutant and in the wild type. Measurements of  $F_v/F_m$ , representing the maximal efficiency of PSII, were performed in WAS 4, 6, 8 and WAS 10. Values are the mean of nine independent areas of interest equally distributed over the whole leaf blade (SD is given in brackets).

<b>Age of leaf</b>	<b>Wild type</b>	<b>Stay-green</b>
WAS 4	0.761 (0.009)	0.785 (0.007)
WAS 6	0.807 (0.005)	0.790 (0.012)
WAS 8	0.713 (0.066)	0.755 (0.034)
WAS 10	0.395 (0.362)	0.787 (0.021)



**Fig. 3.20 Chlorophylls and tocopherols content in young and senescent leaves of the *Festuca/Lolium* stay-green mutant.** Chlorophylls (A),  $\alpha$ -tocopherol (B) and  $\gamma$ -tocopherol (C) were determined in leaf number one of the *Festuca/Lolium* stay-green mutant (grey bars) and in the wild type (black bars). Measurements were performed in WAS 4, 6, 8 and 10. All values are the result of three independent measurements with leaves from three different plants.

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## 4 Discussion

Since their discovery by Evans and Bishop in 1922, tocopherols have been investigated to a great extent in several fields of science such as medical and pharmaceutical sciences, human and animal nutrition, biochemistry, botany and microbiology. For a long time, research mainly focussed on effects and mechanisms of tocopherols in human beings, although these secondary metabolites are exclusively synthesised by oxygenic and photosynthetic organisms: plants, algae and some cyanobacteria. However, in the late 1970s and early 80s, plants and cyanobacteria became crucial to the elucidation of the biosynthetic pathway for tocopherols (DellaPenna, 2005). Nevertheless, only over the last decade genes and proteins involved in tocopherol biosynthesis were finally isolated and characterised and higher plant biology contributed strongly to the body of knowledge (DellaPenna, 2005). Most studies on the function of tocopherols in higher plants typically considered the antioxidant properties and functions related to antioxidants, such as photoprotection and reduction of lipid peroxidation by reduction of lipid peroxy radicals to their corresponding hydroperoxides (Li *et al.*, 2008). The idea that tocopherols, especially  $\alpha$ -tocopherol, could also play a role in cellular signaling and regulation of higher plants was originally derived from animal studies, but only few data demonstrated these non-antioxidant functions in photosynthetic organisms (Li *et al.*, 2008).

Although it is known since 1989 that tocopherols accumulate during leaf senescence (Rise *et al.*, 1989; Chrost *et al.*, 1999; Dertinger *et al.*, 2003; Falk *et al.*, 2003) the specific functions of  $\alpha$ - and  $\gamma$ -tocopherol during that developmental process remain unclear until today. Tocopherol accumulation was mainly considered an inevitable consequence of chlorophyll breakdown because phytol released from chlorophyll catabolism can be employed for tocopherol biosynthesis (Rise *et al.*, 1989; Dörmann, 2007). To my knowledge, the specific functions of  $\alpha$ - and  $\gamma$ -tocopherol during leaf senescence or common regulatory mechanisms between the senescence syndrome and the metabolism of tocopherols have not been considered so far.

#### 4.1 The relationship between tocopherol accumulation and chlorophyll breakdown

Chlorophyll is the most abundant photosynthetic pigment in higher plants. During leaf senescence, chlorophyll is hydrolysed, resulting in the release of free phytol (Ischebeck *et al.*, 2006). As early as 1989, when Rise *et al.* (1989) detected tocopherol accumulation in senescing leaves of several higher plants, it was hypothesised that the phytol used for tocopherol biosynthesis is derived from chlorophyll breakdown and that the accumulation might simply be a passive consequence of chlorophyll catabolism (Dörmann, 2007). It was not considered whether tocopherol might have any direct function during leaf senescence. At this time it was also not known whether tocopherols accumulate when chlorophylls are not degraded.

In the present study, experiments on stay-green organisms such as the *SAG12-IPT* tobacco or the *Festuca/Lolium* stay-green mutant were used to investigate the relevance of chlorophyll degradation for tocopherol accumulation.

The time course experiment on *SAG12-IPT* tobacco grown under normal growth conditions indeed showed that chlorophyll degradation was delayed in the *SAG12-IPT* line compared to the control, but nevertheless the chlorophyll content (Fig. 3.2 B), and the tocopherol content, especially  $\alpha$ -tocopherol (Fig. 3.3 A), changed in an antiproportional manner during senescence. These findings do not contradict the hypothesis that free phytol, possibly derived from chlorophyll breakdown, serves as a substrate for tocopherol biosynthesis (Rise *et al.*, 1989; Ischebeck *et al.*, 2006; Dörmann, 2007).

Although it seems likely, that the phytol for tocopherol biosynthesis is derived from chlorophyll catabolism, it remained unclear whether chlorophyll breakdown is really a mandatory prerequisite or the explicit reason for tocopherol accumulation. However, if tocopherol accumulation just represents a cellular mechanism to deposit phytol derived from the chlorophyll degradation in chloroplasts, the efficiency of that mechanism would be quite low, because the amount of chlorophylls degraded during the time course performed with *SAG12-IPT* tobacco is about ten fold higher



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than the amount of accumulated tocopherol. Taking this into account, tocopherol accumulation during leaf senescence might be more than a deposit for phytol released from chlorophyll catabolism. Moreover, the isoprenoid phytol moiety represents only one part of the tocopherol molecule. The hydrophilic head group derived from homogentisate also needs to be synthesised for tocopherol biosynthesis, and it seems unlikely that plants undergo such an energy consuming process merely to provide a storage opportunity for phytol released during chlorophyll breakdown.

Apart from the kinetics approach where the temporal changes in one and the same leaf were analysed, tocopherols were measured in a young and an old leaf derived from the top and the bottom of the same plant, respectively. The observation that  $\alpha$ -tocopherol accumulated in the old, but not senescent *SAG12-IPT* leaf which showed no decrease in chlorophyll content (Fig. 3.12 B; 3.12 C), indicates that chlorophyll breakdown is not mandatory.

To investigate the importance of chlorophyll breakdown for tocopherol accumulation in leaves of higher plants further, a regreening experiment with yellow, senescent tobacco leaf discs was performed (Fig. 3.5). The regreening of the tobacco leaf discs was obtained by exogenous application of the phytohormone cytokinin. This experiment clearly demonstrated that tocopherols also accumulate in senescent leaf discs (Fig. 3.6 B; Fig. 3.6 C), when chlorophyll is not degraded, but even synthesised *de novo* (Fig. 3.6 A). Since chlorophyll metabolism serves as a sink for phytol under these conditions and a competition between chlorophyll and tocopherol synthesis for phytol takes place, it is possible that phytol is not the limiting factor. Furthermore, it appears that chlorophyll degradation is not the only phytol source in senescing leaves.

In addition to the studies carried out on tobacco leaves, tocopherol accumulation was investigated in the cosmetic stay-green *Festuca/Lolium* mutant, which exhibits a block of chlorophyll breakdown (Fig. 3.19; Fig. 3.20). The results showed that  $\alpha$ -tocopherol as well as  $\gamma$ -tocopherol accumulated, although the amount of chlorophyll was constant or at the very

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least did not decrease. Interestingly, the *Festuca/Lolium* mutant was described as a cosmetic stay-green organism exhibiting a block in chlorophyll breakdown accompanied by a fairly normal decrease in photosynthetic capacity (Howard *et al.*, 2002). This seems to contradict the measurements of maximum PSII activity performed in this work. The  $F_v/F_m$  value did not decrease significantly as observed in the wild type (Fig. 3.18; Tab. 3.2; Tab. 3.4). However, the determination of the maximum PSII activity is not the only parameter for photosynthetic activity. CO<sub>2</sub> up-take might be a more suitable approach to show that photosynthetic capacity diminishes, as would have been expected for a cosmetic stay-green organism.

The finding that tocopherols accumulated even though chlorophyll was not degraded was also observed in the kinetics experiment on RILs of *Arabidopsis thaliana*. Late senescing lines already showed the onset of tocopherol accumulation ( $\alpha$ -tocopherol) even when chlorophyll degradation and leaf yellowing had not yet started (Fig. 3.15).

In summary, although the phytol moiety used for tocopherol synthesis might be derived from chlorophyll degradation, chlorophyll breakdown does not seem to be the reason for tocopherol accumulation during leaf senescence as tocopherols also accumulate when chlorophylls are not degraded. These conclusions were supported by the time course experiment on the *SAG12-IPT* tobacco grown under normal growth conditions, the regreening experiment, the measurement of tocopherols in the gradient of tobacco leaf age, the analysis of the *Lolium/Festuca* stay-green mutant and the kinetics performed with RILs.

## 4.2 Specific functions of $\alpha$ -tocopherol

Many functions of  $\alpha$ -tocopherol are due to its antioxidant properties, but this work intended to elucidate further non-antioxidant functions. To shed light on the functions of  $\alpha$ -tocopherol during the last phase of leaf development, a more detailed analysis of senescence-related accumulation with regards to

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its onset, duration and extent was necessary. Therefore, the tocopherol content in *SAG12-IPT* tobacco and a corresponding control line were measured over a specific time course, both grown under normal growth conditions (Fig. 3.3 A, B; Fig. 3.4 B, C). In both lines, the accumulation of  $\alpha$ -tocopherol increased in a fairly leaf age-dependent manner on a  $\mu\text{g}$  scale. Of course, an age-dependent increase includes an increase in senescence and it has to be emphasised that the age-dependent increase had its maximum during this later phase of leaf development. The age-dependency of  $\alpha$ -tocopherol accumulation observed here is consistent with the  $\alpha$ -tocopherol content in leaves of the *SAG12-IPT* line reported by Dertinger *et al.* (2003). However, in that article the accumulation of  $\alpha$ -tocopherol was mainly discussed with regard to the antioxidative and membrane stabilising functions of  $\alpha$ -tocopherol. Moreover, sampling was carried out for only a few times, which did not allow the observation that the  $\alpha$ -tocopherol content in the leaf of the *SAG12-IPT* line decreased significantly at the time when the leaf of the control was shortly before dying (Fig. 3.3 A; Fig. 3.4 B). This indicates that the development of both lines was identical until the level of  $\alpha$ -tocopherol increased again as a consequence of the senescence-specific activation of the *SAG12*-promoter in the *SAG12-IPT*-line. Correspondingly, the expression of the bacterial *ipt1* gene showed no significant induction until tocopherol content increased again in the *SAG12-IPT*-line (Fig. 3.8 A).

The findings from the time course experiment that  $\alpha$ -tocopherol accumulates in a leaf age-dependent manner was reinforced by the measurement of  $\alpha$ -tocopherol in a young and an old leaf derived from the top and the bottom of the same plant, respectively (Fig. 3.12 C).  $\alpha$ -tocopherol accumulated in the old leaf of the *SAG12-IPT* line as well as in the old leaf of the control, though chlorophyll was only degraded in the control, whereas the old leaf of the *SAG12-IPT* line was clearly not senescing due to the effect of endogenously produced cytokinin (Fig. 3.12 C).

When  $\alpha$ -tocopherol was measured in a developmental time-course of five *Arabidopsis thaliana* RILs exhibiting different onsets of leaf senescence

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(Fig. 3.15), the amount of  $\alpha$ -tocopherol increased with leaf age and was not dependent on the onset of leaf senescence. This finding is in accordance with the results obtained with tobacco.

Following the characterisation of  $\alpha$ -tocopherol with respect to its onset, duration and extent, the functional analysis was subsequently focussed. To date investigations into the functions of  $\alpha$ -tocopherol were mainly concerned with its antioxidant properties and especially  $\alpha$ -tocopherol was shown to play a crucial role in the chemical scavenging of singlet oxygen produced by the triplet states of chlorophylls in the PSII reaction centre (Kruk *et al.*, 2005). Therefore,  $\alpha$ -tocopherol is considered part of the ROS modulation system (Foyer and Noctor, 2005). Studies on oxidative stress in outdoor grown *vte1* and *vte4* knock-out mutants of *Arabidopsis thaliana* lacking total tocopherol and  $\alpha$ -tocopherol, respectively, demonstrated, that  $\gamma$ -tocopherol might also have a function in preventing the oxidation of cellular components (Semchuk *et al.*, 2009). Although  $\alpha$ -tocopherol is the major form of tocopherol in the leaf, many studies suggest, that  $\gamma$ -tocopherol is at least partially able to overtake the antioxidant functions of  $\alpha$ -tocopherol, if  $\alpha$ -tocopherol biosynthesis is prevented somehow (Bergmüller *et al.*, 2003; Meada *et al.*, 2006; Kobayashi and DellaPenna, 2008; Semchuk *et al.*, 2009).

However, the time course on the *Festuca/Lolium* mutant showed that the accumulation of  $\alpha$ -tocopherol, but not that of  $\gamma$ -tocopherol, was significantly enhanced in senescent leaves of the stay-green mutant compared to the corresponding wild type (Fig. 3.20). This indicates that  $\alpha$ - and  $\gamma$ -tocopherol might have different functions, and it is in accordance with this postulation that  $\alpha$ -tocopherol in particular is crucial for the protection of PSII and D1 protein turnover, especially under strong light (Krieger-Liszkay and Trebst, 2006). In the *Festuca/Lolium* mutant, the accumulation of  $\alpha$ -tocopherol obviously represents a stress response induced by excess amounts of chlorophyll giving rise to an increased level of chlorophyll in the triplet state under conditions of photooxidative stress.

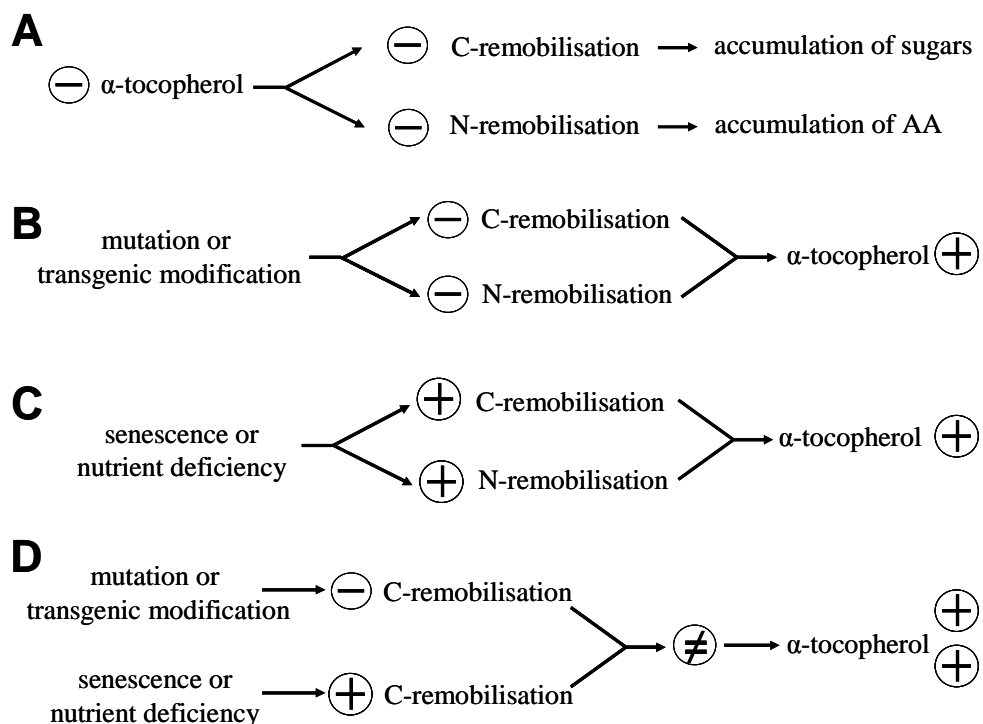
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However, several studies on tocopherol-deficient plants support the idea that  $\alpha$ -tocopherol might have a function that is not related to its antioxidant properties. The *sxd1*-mutant of maize (Russin *et al.*, 1996) and the transgenic *vte1*-RNAi-potato (Hofius *et al.*, 2004), both deficient in tocopherols, are characterised by an export block of carbohydrates in source leaves resulting in the accumulation of soluble sugars and starch. In addition, it was reported that the tocopherol-deficient *vte2*-RNAi-tobacco accumulates amino acids in senescent leaves (Abasi *et al.*, 2009). These findings suggest that a decrease of  $\alpha$ -tocopherol content causes a decline of carbohydrate and nitrogen remobilisation (Fig. 4.1 A). Obviously,  $\alpha$ -tocopherol serves as a factor that is highly relevant for the proper remobilisation of nutrients.

The time course experiment on *SAG12-IPT* tobacco grown under normal growth conditions revealed that the *SAG12-IPT* line showed an increased level of  $\alpha$ -tocopherol compared to the control line. The maximum value of the control was just approximately 70% of the maximum value observed in the *SAG12-IPT* tobacco (Fig. 3.3 B). If  $\alpha$ -tocopherol is crucial for remobilisation processes as postulated above, it makes sense to hypothesise that the increased accumulation in leaves of *SAG12-IPT* line is a response to a retarded remobilisation of nutrients occurring as a consequence of an increased cytokinin-mediated sink strength which is caused by mutation or transgenic modification (Fig. 4.1 B). From that point of view,  $\alpha$ -tocopherol could be considered as a remobilisation-promoting factor. That the *SAG12-IPT* tobacco is retarded in remobilisation processes was already described in the introduction (see chapter 1.1.5). In this work, the disturbance of remobilisation processes in the *SAG12-IPT* tobacco was indicated by the high content of soluble sugars (Fig. 3.7) as already described by Wingler *et al.* (1998), the low expression level of the *gs1* gene (Fig. 3.8 D) and the constant expression of the genes *rbcS* and *cab* (Fig. 3.8 C). Moreover, the observations that the expression of *rbcL* was increased throughout the time course (Fig. 3.8 C) and that the expression of *rbcL* was in contradiction to the amount of Rubisco protein detected by Western blot analysis (Fig. 3.9)

strongly suggested that remobilisation is impaired in the *SAG12-IPT* tobacco.

To test the hypothesis that a plant with reduced remobilisation shows an increased  $\alpha$ -tocopherol content,  $\alpha$ -tocopherol was measured in mature and senescent rosette leaves of the *gln1.2* knock-out mutant and the corresponding wild type of *Arabidopsis thaliana* (Fig. 3.16). The *gln1.2* knock-out mutant is affected in remobilisation processes as demonstrated by a delay in flowering, a slower stem growth and a higher accumulation of total amino acids compared to the wild type (Master thesis of Laure Gaufichon, 2006). The *gln1.2* knock-out mutant showed an extremely high  $\alpha$ -tocopherol content in senescent rosette leaves when the plants were grown under low nitrogen conditions (Fig. 3.16).



**Fig. 4.1: Relationship between the content of  $\alpha$ -tocopherol and the remobilisation of carbohydrates and nitrogen.** (A) A decrease in  $\alpha$ -tocopherol content causes a decrease of C- and N-remobilisation. Subsequently an accumulation of sugars or amino acids (AA) occurs. (B) If a mutation or transgenic modification causes a decrease of C- and N-remobilisation, the  $\alpha$ -tocopherol content is increased. (C) If a plant enters senescence or is grown under nutrient deficiency, remobilisation is increased. Subsequently the content of  $\alpha$ -tocopherol is increased. (D) If a plant with a mutation or transgenic modification shows a decrease in remobilisation, but in parallel an enhanced need to remobilize due to senescence or nutrient deficiency, this contradiction is counteracted by a strongly increased  $\alpha$ -tocopherol level. + indicates an increase, ++ a strong increase, - a decrease and  $\neq$  opposing effects.

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The experiment on the *gln1.2* knock-out mutant supported the hypothesis that an organism, which shows a decrease in remobilisation due to mutation or transgenic modification, exhibits an increased level of  $\alpha$ -tocopherol (Fig. 4.1 B). The observation that this prominent value for  $\alpha$ -tocopherol only appeared during senescence and under low nitrogen conditions can be explained by the senescence-associated expression of the *gln1.2* gene and the enhanced need for remobilisation, respectively.

The findings that plants deficient in  $\alpha$ -tocopherol show a disturbance in C- and/ or N-remobilisation (Fig. 4.1 A) and that plants with a decreased C- and/ or N-remobilisation which is caused by mutation or transgenic modification, show an increased content of  $\alpha$ -tocopherol (Fig. 4.1 B), support the basic hypothesis that  $\alpha$ -tocopherol accumulation is linked to remobilisation. So, if the age-dependent  $\alpha$ -tocopherol increase, that also takes place and even reaches its maximum during senescence, is related to an enhanced remobilisation, the  $\alpha$ -tocopherol level should also be increased in other situations of remobilisation that are senescence-independent. Therefore, the *SAG12-IPT* tobacco and the control were grown under nutrient deficiency, as under this condition the inner-organismic remobilisation and recycling of nutrients is increased (Schulze *et al.*, 1994; Diaz *et al.*, 2008). Accordingly, leaves of the *SAG12-IPT* tobacco and of the corresponding control tobacco showed a dramatic increase in  $\alpha$ -tocopherol levels throughout their development when grown under nutrient deficiency (Fig. 3.11; Tab. 3.1). Long before the usual onset of leaf senescence, the content of  $\alpha$ -tocopherol was about 10-fold higher in the leaves of both tobacco lines when grown under normal growth conditions (Tab. 3.1) compared to plants grown under nutrient deficiency conditions. The amount of  $\gamma$ -tocopherol was even increased up to 60-fold. These results support the idea that plants with an increased need to remobilise due to senescence or nutrient deficiency have an increased level of tocopherols (Fig. 4.1 C).

To further test whether this is also the case in other plant species,  $\alpha$ -tocopherol was measured in the *gln1.2* knock-out mutant and the corresponding wild type, both grown on 2 mM (nutrient deficiency) and 10 mM (optimal concentration) nitrogen. As expected, the mutant and the wild

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type plants grown under 2 mM nitrogen showed an up to 10-fold higher level of  $\alpha$ -tocopherol compared to plants grown under normal growth conditions such as 10 mM nitrogen (Fig. 3.16). The observation that the  $\alpha$ -tocopherol level is higher in senescent leaves is consistent with the enhanced need to remobilise in this stage of development (Fig. 3.16). Therefore, this experiment indicated that plants with an increased need to remobilise, for example during senescence or under nutrient deficiency, exhibit a higher tocopherol content (Fig. 4.1 C).

For comparison, the tocopherol content was also analysed in the seeds of the *gln1.2*-knock-out mutant and the corresponding wild type as seed filling is dependent on remobilisation. It should be noted that  $\gamma$ -tocopherol was measured and not  $\alpha$ -tocopherol, since this is the major form of tocopherol in seeds. There were neither significant differences between the wild type and the *gln1.2* knock-out mutant nor between those grown under different conditions, i.e. with 2 mM or 10 mM nitrogen (Fig. 3.17). As the content of  $\gamma$ -tocopherol was even increased up to 60-fold in leaves of tobacco grown under nutrient deficiency, it is possible, that  $\gamma$ -tocopherol is at least partially able to take over the remobilisation-related functions of  $\alpha$ -tocopherol.

The experiments on the *SAG12-IPT* tobacco and the *gln1.2* knock-out mutant not only showed that plants with decreased remobilisation of nutrients, due to a mutation or a transgenic modification, showed a higher level of  $\alpha$ -tocopherol (Fig. 4.1 B). It was also demonstrated, that  $\alpha$ -tocopherol increases when plants showed an enhanced need to remobilize during senescence or under nutrient deficiency; Fig. 4.1 C). Interestingly, both plant systems indicated that the  $\alpha$ -tocopherol level is especially high, when both aspects are combined (Fig. 4.1 D). If a mutant or transgenic line shows a decrease in remobilisation, but is at the same time forced to remobilize due to nutrient deficiency, these opposing effects are counteracted by a very strong increase of  $\alpha$ -tocopherol content (Fig. 4.1 D). In the *SAG12-IPT* tobacco, the level of  $\alpha$ -tocopherol was already higher under normal growth conditions than in the wild type (Fig. 3.3; Fig. 3.4). This increased further when grown under nutrient deficiency (Fig. 3.11; Tab. 3.1). Accordingly, in the *gln1.2*-knock-out mutant and the



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corresponding wild type  $\alpha$ -tocopherol level were significantly increased due to growth under nutrient deficiency, but as the *gln1.2*-knock-out mutant additionally exhibited a decline in remobilisation during senescence, the value was extremely pronounced in that phase of development (Fig. 3.16).

In conclusion, the results of this work strongly suggest that  $\alpha$ -tocopherol levels and nutrient remobilisation are linked and that  $\alpha$ -tocopherol serves as a remobilisation-promoting factor. The  $\alpha$ -tocopherol content is increased when normal plant remobilisation is hindered (Fig. 4.1 B) and also when a plant exhibits an increased need to remobilise, for instance during leaf senescence or under nutrient deficient growth conditions (Fig. 4.1 C). If a plant with retarded remobilisation is grown on nutrient deficiency in parallel, a synergistic effect causes a very strong increase in  $\alpha$ -tocopherol content (Fig. 4.1 D) These three findings are in accordance with the observation that tocopherol-deficient plants show impaired remobilisation (Fig. 4.1 A). In the introduction the five major classes of SAGs were described (see chapter 1.1.2): genes for macromolecule degradation, nutrient recycling and remobilisation, defence and cell rescue mechanisms, transcriptional regulation and signal transduction. Due to the results of this work, the senescence-associated *hpd* gene of tocopherol biosynthesis should be classified as SAG related to nutrient recycling and remobilisation. This is also supported by the observation that the expression level of the *hpd* gene, which is correlated to the amount of  $\alpha$ -tocopherol, and the marker genes for N-remobilisation *gs1* and *gdh* exhibited a similar expression pattern (Fig. 3.8), and that the  $\alpha$ -tocopherol accumulation reached its maximum at a time point during leaf senescence where mainly remobilisation processes take place.

Although a relationship between  $\alpha$ -tocopherol accumulation and remobilisation is strongly suggested in this work, it remains unclear, which signals and molecular mechanisms are responsible for this interplay. Possibly,  $\alpha$ -tocopherol acts as a regulator or sensor of cellular signals by affecting the level of sugars, directly or indirectly (Li *et al.*, 2008). Tocopherol may play a role as a signal molecule participating in

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chloroplast-to-nucleus signal transduction pathways (Li *et al.*, 2008). These signal cascades may be associated with the regulation of carbohydrate metabolism. Since carbohydrate metabolism and nitrogen metabolism are dependent on each other, an impact on the remobilisation of nitrogen is not unlikely (Fritz *et al.*, 2006; Li *et al.*, 2008).

Therefore, it is possible that tocopherols are directly or indirectly involved in the remobilisation of proteins from the chloroplast. The fact that  $\alpha$ -tocopherol is able to affect membrane integrity and fluidity is well-established (Munné-Bosch and Allegre, 2002). This poses the question whether  $\alpha$ -tocopherol regulates the generation and formation of membrane vesicles such as SAVs or RCBs, which are probably involved in the degradation and remobilisation of chloroplast proteins (Krupinska, 2006). In fact, the biosynthesis of tocopherols requires the breakdown of the aromatic amino acid tyrosine as homogentisate - a key molecule of tocopherol biosynthesis (see chapter 1.2.4) - is produced from the tyrosine aromatic amino acid catabolite *p*-hydroxyphenylpyruvate (Li *et al.*, 2008). Maybe the release of tyrosine and its breakdown during protein degradation promotes the biosynthesis of  $\alpha$ -tocopherol, which serves in the subsequent remobilisation as a factor influencing the membrane integrity and fluidity of the vesicles mentioned above.

Furthermore,  $\alpha$ -tocopherol might play a role in phloem loading. It was reported that a rapid reduction of photoassimilate export in the *vte2* mutant of *Arabidopsis thaliana* takes place under low-temperature. In parallel, callose deposition exclusively occurs in phloem parenchyma transfer cell walls adjacent to the companion cell/sieve element complex (Maeda *et al.*, 2006). However, the analysis of tocopherols in veinal and interveinal areas of tobacco leaf (data not shown) revealed no differences between both types of tissues as is expected, if tocopherols were be involved in phloem loading.

### **4.3 Specific functions of $\gamma$ -tocopherol**

In the past, plant biology research as well as the investigations in the scientific fields of medicine and human nutrition mainly addressed the antioxidant properties of  $\alpha$ -tocopherol, whereas the role of  $\gamma$ -tocopherol was

barely investigated. However, a few recent studies on  $\gamma$ -tocopherol and its functions in higher plants have emerged.

At the start of this study it was thought that  $\gamma$ -tocopherol could be a scavenger of the senescence-modulating signal molecule nitric oxide (NO), what is likely as  $\gamma$ -tocopherol possesses a free C-5 position at the chromanol ring (Wagner *et al.*, 2004). Furthermore, the nitration of  $\gamma$ -tocopherol was demonstrated for seeds of *Arabidopsis thaliana*, tobacco and rapeseed and the resulting adduct was present in the leaf tissue of the *vte4* mutant of *Arabidopsis thaliana* (Desel *et al.*, 2007). Following the analysis of tocopherols in the leaf age gradient of *SAG12-IPT* tobacco, which indicated that the accumulation of  $\gamma$ -tocopherol is senescence-dependent (Fig. 3.12 C) and the decrease of NO age-dependent (Fig. 3.12 D), this initial hypothesis was not investigated further. In addition, the 5-nitro- $\gamma$ -tocopherol adduct was not detectable in leaves of tobacco even when grown under nutrient-deficiency (Dr. Christine Desel, personal communication), which resulted in a content of  $\gamma$ -tocopherol that is up to 100-fold higher compared to leaves derived from tobacco grown under normal growth conditions (Tab. 3.1).

Although it is known from studies on senescing barley or tobacco leaves that the content of  $\gamma$ -tocopherol increases up to 10-fold in comparison to young leaves (Chrost *et al.*, 1999; Falk *et al.*, 2003), specific functions of  $\gamma$ -tocopherol during leaf senescence have not been considered until now. As stated in the introduction leaf senescence can be divided into four stages, namely induction, initiation, reorganisation and termination (see chapter 1.1.1). To elucidate the function of  $\gamma$ -tocopherol during leaf senescence the accumulation of  $\gamma$ -tocopherol was analysed during these four stages. In contrast to the more or less age-dependent accumulation of  $\alpha$ -tocopherol, the accumulation of  $\gamma$ -tocopherol was found to be transient and restricted to the later stages of senescence (Fig. 3.3 B; Fig. 3.4 C). This was underlined by the observation that the accumulation of  $\gamma$ -tocopherol was accompanied by an increased expression level of the genes *cp1* and *pr1b*, which are known to be involved in protein degradation during the reorganisation stage and processes of programmed cell death in the terminal stage of leaf senescence, respectively (Fig. 3.8 D; Beyene *et al.*, 2006; Wingler *et al.*, 2005).

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Furthermore, the senescence-associated  $\gamma$ -tocopherol accumulation was measured in a young and an old leaf derived from the top and the bottom of the same plant, respectively (Fig. 3.12 D). In contrast to  $\alpha$ -tocopherol which accumulated in the old, but non-senescent leaf of the *SAG12-IPT* tobacco as well as in the old, senescing leaf of the control plant, the accumulation of  $\gamma$ -tocopherol was only detectable in the old leaf of the control plant, which was undergoing senescence as indicated by a significant decrease in chlorophyll content (Fig. 3.12 B, D).

Moreover, the spatial distribution of  $\gamma$ -tocopherol in senescing tobacco leaves of the *SAG12-IPT* line and the control line was determined. In the control plants that exhibited green, yellow and brown tissue in one and the same leaf, the content of  $\gamma$ -tocopherol was significantly increased in yellow and brown tissue providing further evidence that the accumulation of  $\gamma$ -tocopherol is restricted to later stages of senescence, in particular to the processes of cell death that occur in the terminal stage. The senescent leaf of *SAG12-IPT* tobacco also contained green and brown tissue. However, the leaf of that transgenic tobacco contained white, bleached tissue instead of yellow tissue. The occurrence of white, bleached tissue in the *SAG12-IPT* line may be due the fact, that chlorophylls are not degraded to NCCs that are mobilised to the vacuole for storage. Instead, chlorophylls remain intact until photooxidative stress causes bleaching and hence the white color. The measurement of  $\gamma$ -tocopherol in the leaf of the *SAG12-IPT* line showed that the accumulation is exclusively restricted to brown tissue. These results are in accordance to those from the time course experiment: the accumulation of  $\gamma$ -tocopherol is related to later stages of senescence, especially to the processes of cell death occurring in the terminal stage as indicated by increased values for  $\gamma$ -tocopherol in yellow and brown tissue of the control and an increased content in brown tissue of the *SAG12-IPT* line. Both lines thus have in common, that  $\gamma$ -tocopherol is mainly found in brown and nearly parched tissue. The observation that the yellow tissue of the control, but not the white tissue of the *SAG12-IPT* line, contained a high content of  $\gamma$ -tocopherol, underlines that the senescence expires differentially in both lines. This is in accordance with the finding that the *SAG12-IPT* tobacco

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progresses directly into cell death without previous remobilisation of nutrients (Wingler *et al.*, 2005).

It has to be mentioned, that the time course experiment on the five RILs of *Arabidopsis thaliana* also showed a senescence-specific accumulation of  $\gamma$ -tocopherol. Early-senescing lines exhibited an early onset of  $\gamma$ -tocopherol accumulation, whereas late-senescing lines correspondingly started to accumulate  $\gamma$ -tocopherol later (Fig. 3.15). Interestingly, the increase was not transient, but  $\gamma$ -tocopherol level was elevated until the end of leaf development.

In summary, the results of the experiments described above strongly suggest that the accumulation of  $\gamma$ -tocopherol is senescence-specific.  $\gamma$ -tocopherol accumulation is restricted to later stages of leaf senescence implicating a function in the regulation of the terminal processes of cell death, regardless whether cell death occurs via PCD as in the control or necrosis as in the *SAG12-IPT* line. Therefore, the accumulation of  $\gamma$ -tocopherol appears to be a suitable marker for senescence-related cell death. Though it cannot be excluded, that  $\gamma$ -tocopherol has no function in cell death and that its accumulation is simply the result of a low activity of  $\gamma$ -TMT, such a possibility seems unlikely since  $\gamma$ -tocopherol was shown to induce apoptosis and cell death in two different human colon cancer cell lines (Campbell *et al.*, 2006). DNA laddering, a well-known trait of apoptosis in mammalian cells, was only evident in samples treated with  $\gamma$ -tocopherol, but not the  $\alpha$ -tocopherol-treated samples in both colon cancer cell lines tested (Campbell *et al.*, 2006). The molecular mechanisms leading from tocopherol activity to cell death are currently heavily discussed. In human breast cancer cells, for instance,  $\gamma$ -tocopherol induces apoptosis via mediating an apoptotic signalling pathway (Yu *et al.*, 2008). Treatment of malignant cells with  $\gamma$ -tocopherol increased mRNA, protein and cell surface expression of a death receptor (DR5; Yu *et al.*, 2008). Of course, this type of protein has not been identified in the whole plant kingdom so far. However,  $\gamma$ -tocopherol seems only to affect cells that are destined to undergo cell death like the colon cancer cells mentioned above, while normal colon cells stay unaffected

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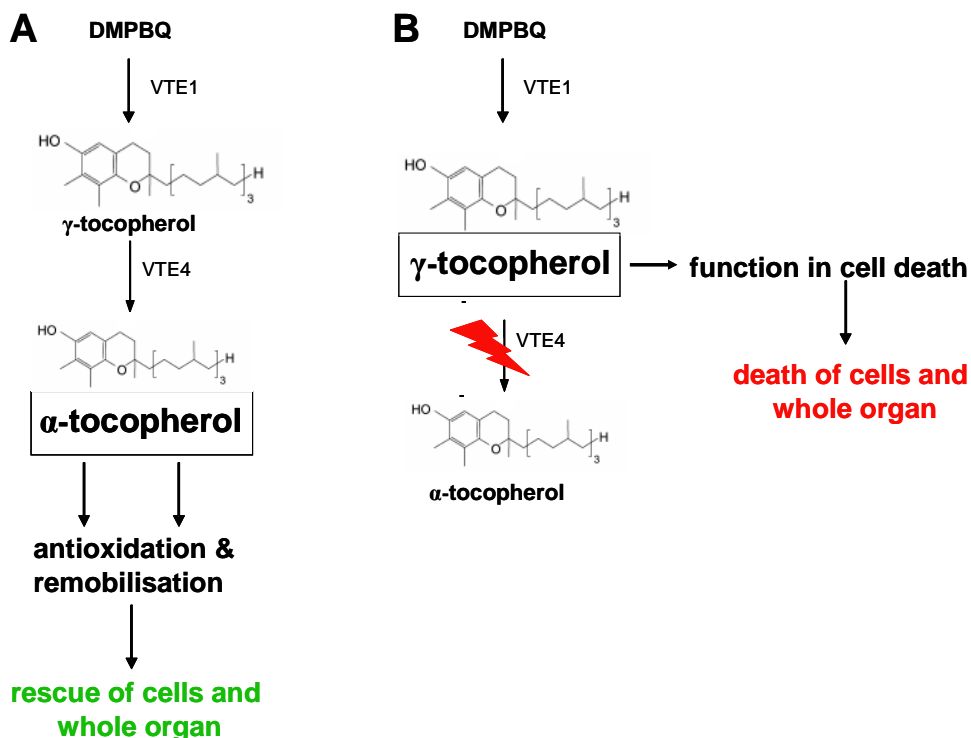
(Campbell *et al.*, 2006). This is supported by the results of the present study that  $\gamma$ -tocopherol induces cell death only in senescent cells, which are already destined to die. Accordingly, cells that are not senescent show no accumulation of  $\gamma$ -tocopherol. Whilst senescent plant cells and human cancer cells are not directly comparable, the underlying mechanisms including the activity of  $\gamma$ -tocopherol may well be similar. If senescent tissue is to a plant what cancer is to a human being... who knows?

#### 4.4 Conclusions

The functions of  $\alpha$ -tocopherol and  $\gamma$ -tocopherol during leaf ageing and leaf senescence as determined in this study are summarised by the model presented in Fig. 4.2. As long as the leaf undergoes tocopherol biosynthesis up to the final end product, namely  $\alpha$ -tocopherol which is the major form of tocopherol in leaves,  $\alpha$ -tocopherol assumes specific functions in the antioxidative network, such as scavenging of singlet oxygen in the PSII reaction centre or the prevention of lipid peroxidation. In addition, a specific function as a remobilisation promoting factor is suggested by this work. Both its antioxidant function and its role in remobilisation result in the maintenance and rescue of cells and of the whole organ (Fig. 4.2 A). By contrast, if tocopherol biosynthesis would be disturbed at the conversion from  $\gamma$ -tocopherol to  $\alpha$ -tocopherol, a temporary accumulation of  $\gamma$ -tocopherol occurs, which might be involved in the switch from remobilisation and antioxidant protection to programmed cell death (Fig. 4.2 B). According to that model, the accumulation of  $\gamma$ -tocopherol may serve as a marker for cell death. The accumulation of  $\alpha$ -tocopherol is age-dependent and a possible marker for senescence-related remobilisation. This idea is supported by the fact, that the accumulation also occurs during remobilisation events that are not restricted to senescence, as demonstrated by the high values in plants grown under nutrient deficiency.

Of course, this model generates many open questions, especially about the underlying mechanisms of regulation, and without doubt further investigations are necessary to fully elucidate the interplay of senescence and tocopherols.

First and foremost ROS, the phytohormone ethylene and the growth regulators jasmonate and salicylic acid should be considered as potential mediators between the senescence syndrome and the biosynthesis of tocopherols, as these molecules are known to play a role in both processes. Although the results of this work suggest that the breakdown of chlorophyll has no modulating impact on the tocopherol biosynthesis, it is quite likely that other biosynthetic and signalling pathways regulate the biosynthesis of tocopherols. The senescence-associated expression of the *hgo* gene (Fig. 3.8 B) provides evidence that the tocopherol biosynthesis pathway is not only regulated by the biosynthetic pathway of plastoquinone, as shown in studies on the cyanobacterium *Synechocystis sp.* PCC6803 (Dähnhardt *et al.*, 2002). The HGO-pathway, which converts homogentisate to the end products fumarate and acetoacetate, also seems to have a modulating impact on the biosynthesis of tocopherols.



**Fig. 4.2 Hypothetical model to summarise the functions of tocopherols during leaf aging and leaf senescence.** (A) The end product of tocopherol biosynthesis is  $\alpha$ -tocopherol, which has functions in the antioxidative network and in remobilisation. This results in the rescue of the cells and the whole organ. (B) Tocopherol biosynthesis is disturbed at the conversion from  $\gamma$ -tocopherol to  $\alpha$ -tocopherol. As a consequence,  $\gamma$ -tocopherol accumulates and participates in cell death processes resulting in the death of the cells and the whole organ.

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The generation and characterisation of a transgenic *SAG12-vte4*-tobacco line, which silences *vte4* exclusively during senescence, would shed new light into the senescence- and age-related regulation of tocopherol biosynthesis and the model proposed in Fig. 4.2.

Finally, the important finding of this work that tocopherol content is dramatically increased when plants are grown under nutrient deficiency is emphasized once more. This is certainly of particular relevance to agriculture. In order to enrich the content of tocopherols in leafy vegetables such as lettuce, it will not be necessary to modify plants genetically. Instead, the simple act of reducing nutrients in the soil would suffice to generate plants with a much higher content of tocopherols, which are so vital to human health and nutrition! Although the impact of nutrient deficiency on the content of tocopherols has been demonstrated for the leaf organ, the tocopherol content in seeds was not found to be dependent on the growth conditions. Therefore, with respect to agricultural and nutritional aspects, it needs to be investigated further whether the growth under nutrient deficiency has an influence on the tocopherol content in fruits as these represent a very crucial component of the human diet.



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## 5 Abstract

Although it is well-known that tocopherols are important for human nutrition and health, their functions in plants are not well understood until now. As  $\alpha$ - and  $\gamma$ -tocopherol accumulate in senescent leaves of higher plants, specific functions of  $\alpha$ - and  $\gamma$ -tocopherol during leaf senescence were investigated.

To test the hypothesis, that tocopherol accumulation is an inevitable passive consequence of chlorophyll breakdown, tocopherols and chlorophylls were measured in stay-green organisms such as the transgenic *SAG12-IPT* tobacco and the *Festuca/Lolium* mutant. These analyses revealed that chlorophyll breakdown is no mandatory prerequisite for the accumulation of tocopherols, though it is possible that phytol used for tocopherol synthesis is derived from the chlorophyll catabolism.

Experiments on leaves of the *SAG12-IPT* line and on rosette leaves of RILs of *Arabidopsis thaliana* demonstrated that the accumulation of  $\alpha$ -tocopherol is dependent on leaf age. The measurement of  $\alpha$ -tocopherol in the *SAG12-IPT* tobacco and the *gln1.2* knock-out mutant of *A. thaliana*, both affected in nutrient remobilisation, elucidated that plants with an impairment of remobilisation exhibit an increased level of  $\alpha$ -tocopherol. Furthermore, the tocopherol content was dramatically increased when plants were forced to remobilise by growing them under nutrient deficiency. These results indicate, that the accumulation of  $\alpha$ -tocopherol plays a crucial role for the remobilisation, not only during senescence.

The measurement of  $\gamma$ -tocopherol in leaves of *Arabidopsis thaliana* RILs and the *SAG12-IPT* line showed, that  $\gamma$ -tocopherol accumulation is restricted to later stages of the senescence. Gene expression analysis and the observation that  $\gamma$ -tocopherol content is significantly elevated in brown, senescent leaf tissue provide evidence, that  $\gamma$ -tocopherol is involved in cell death processes. Therefore,  $\gamma$ -tocopherol accumulation is discussed as a marker for the last phase of senescence which is characterised by cell death.

## 6 Zusammenfassung

Tocopherole sind wichtig für die menschliche Ernährung und Gesundheit, ihre Funktionen in Pflanzen sind hingegen bis heute nahezu unverstanden. Es war bekannt, dass  $\alpha$ - und  $\gamma$ -Tocopherol während der Blattseneszenz Höherer Pflanzen akkumulieren. Aufgabe der vorliegenden Arbeit war daher, mögliche Funktionen von  $\alpha$ - und  $\gamma$ -Tocopherol während dieser letzten Phase der Blattentwicklung aufzuzeigen und zu prüfen.

Zunächst sollte geklärt werden, ob die Akkumulation der Tocopherole während der Seneszenz durch einen erhöhten Chlorophyllabbau bedingt wird. Hierzu wurden Tocopherole und Chlorophylle in *stay-green* Pflanzen quantifiziert, die sich durch eine verzögerte oder ausbleibende Degradierung von Chlorophyllen auszeichnen. Diese Analysen an der *SAG12-IPT* Tabaklinie als auch an der *Festuca/Lolium* Mutante zeigten eindeutig auf, dass Tocopherole auch akkumulieren, wenn kein gleichzeitiger Abbau von Chlorophyllen stattfindet. Es ist jedoch nicht ausgeschlossen, dass der Phytolrest, der zur Biosynthese von Tocopherolen benötigt wird, aus dem Chlorophyllabbau stammt.

Ferner wurde durch Untersuchungen an Blättern der *SAG12-IPT* Tabaklinie sowie an Rosettenblättern von *Arabidopsis thaliana* RILs demonstriert, dass die Akkumulation von  $\alpha$ -Tocopherol mit steigendem Blattalter zunimmt. Die gemessenen  $\alpha$ -Tocopherolgehalte in der *SAG12-IPT* Tabaklinie und der *gln1.2* Mutante von *Arabidopsis thaliana* lassen weiterhin vermuten, dass Pflanzen, die eine Störung bezüglich der Remobilisierung von Nährstoffen aufweisen,  $\alpha$ -Tocopherol vermehrt anreichern. Ein Zusammenhang zwischen der Akkumulation von  $\alpha$ -Tocopherol und der Remobilisierung von Nährstoffen wurde zudem dadurch bestätigt, dass Pflanzen, die aufgrund von Nährstoffmangel vermehrt remobilisieren müssen, ebenfalls stark erhöhte Gehalte an Tocopherole besitzen.

Die Quantifizierung von  $\gamma$ -Tocopherol in Blättern der *SAG12-IPT* Tabaklinie sowie in Rosettenblättern der *A. thaliana* RILs zeigten auf, dass die Akkumulation von  $\gamma$ -Tocopherol auf eine späte Phase der Blattseneszenz beschränkt ist. Auch konnte bestätigt werden, dass die Expression von Genen, die an der  $\gamma$ -Tocopherolbiosynthese beteiligt sind, in braunem,

nahezu totem Blattgewebe besonders hoch ist. Diese Daten weisen darauf hin, dass  $\gamma$ -Tocopherol eine Bedeutung für Zelltodprozesse besitzt, welche die die Blattseneszenz terminieren. Hohe Gehalte an  $\gamma$ -Tocopherol sind demnach ein Kennzeichen der letzten Phase der Seneszenz.

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## 8 Curriculum vitae

### 1 Personal information

**Date of birth:** 11.03.1977

**Place of birth:** Erbach / Odenwald

**Nationality:** German

**Marital status:** unmarried



### 2 School education

**1996** Obtaining of general qualification for university entrance at the Karl-Theodor von Dalberg-Gymnasium in Aschaffenburg (Bavaria)

### 3 Alternative civilian service

**01.09.1996 – 31.09.1997**

Alternative civilian service at the Sozialstation in Höchst / Odenwald (mobile nursing service)

### 4 Studies at university

**01.10.1997 – 31.03.2004**

Studies at the Johann-Wolfgang-von-Goethe-University in Frankfurt / Main in order to become high-school teacher for biology and chemistry

**01.04.2000 – 25.10.2004**

Studies & diploma in biology at the Johann-Wolfgang-von-Goethe-University in Frankfurt / Main; focus of studies: genetics of plants & fungi, biochemistry & physiology of plants and (inorganic) chemistry; diploma thesis in plant genetics "Analysis of senescence-associated changes of copper metabolism in *Arabidopsis thaliana*", supervisor: Prof. Dr. H. D. Osiewacz

### 5 Work experience

**Winter 2002 / 2003 (as undergraduate)**

Supervising the practical course "Organic chemistry for biologists"

**Summer 2003 & summer 2004 (as undergraduate)**

Supervising the practical course "Developmental physiology of plants for biologists"

**16.11.2004 – 31.09.2005**

Position as graduate assistant of Prof. Dr. H. D. Osiewacz  
(Research group for developmental biology & biotechnology at the Botanical Institute of the Johann-Wolfgang-von-Goethe-University in Frankfurt / Main)

**01.10.2005 up to date**

PhD position in the research group of Prof. Dr. K. Krupinska (Department for Plant Cell Biology at the Botanical Institute of the Christian-Albrechts-University in Kiel) and member of the DFG-funded Graduiertenkolleg 820 "Natural Antioxidants- Effects in Plants, Foods, Animals and Humans"

**Summer 2007**

Supervizing a three month, DAAD-funded internship of an American undergraduate student

**01.04.2008 – 30.09.2008**

Marie-Curie funded internship in the laboratory of Dr. Céline Masclaux-Daubresse, Nitrification azotée des plantes (NAP) at INRA in Versailles / France

**6 Qualifications & competences****6.1 Molecular biology**

- Isolation of nucleic acids from plants & fungi
- Southern Blot Analysis, Northern Blot Analysis, Western Blot Analysis
- PCR, semi-quantitative RT-PCR, Real-Time-PCR
- Cloning in *E. Coli*
- Generation of transgenic RNAi-plants in *A. thaliana*

**6.2 Physiology & biochemistry**

- Isolation of carotenoids, chlorophylls and tocopherols from plant tissue
- Assays to detect ROS in plant tissues
- Measurement of photosynthetical parameters by imaging-PAM
- Assays to measure soluble sugars, amino acids and  $\text{NH}_4^+$

**6.3 Instrumental analytics**

HPLC to characterize tocopherols and carotenoids

**6.4 Languages**

Mother language: German

Foreign languages: English (very good), Latin (very good), French (moderate)

**6.5 Mobility**

Drivers licence Class 3 (own car at hand)

## 6.6 Computer skills

Microsoft Word, Excel, Powerpoint, Sigma Plot, Corel Draw, Adobe Photoshop, Clone Manager, Imaging-PAM-Software, HPLC-Software von Shimadzu

## 6.7 Knowledge derived from the studies in order to become high school teacher

Good knowledge in inorganic, organic & physical chemistry, basics in psychology & pedagogy

## 6.8 Participation in congresses, workshops and courses for further training

- “Botanical Congress 2004” in Braunschweig / Germany:  
poster & talk in the satellite-meeting “Molecular mechanisms of ageing and senescence processes in plants” (as undergraduate)
- Congress for “Oxygen metabolism, ROS & redox signalling in plants 2005” in Bristol / GB
- Workshop for “Communication & Presentation” in Salzac / Germany, 2006
- Practical course for microscopy (CLSM) at the ZM in Kiel / Germany, 2006
- Congress for “Plant genetics” in Kiel / Germany, 2006
- “4<sup>th</sup> SPPS PhD Student Conference” in Lycksele / Sweden 2006:  
talk & winner of the “Auditorial Talk Award”
- Practical course for Real-Time-PCR by Applied Biosystems in Hannover, 2006
- “3<sup>rd</sup> European Workshop on Plant Senescence 2007” in Salzac / Germany:  
poster
- Supervising a three month, DAAD-funded internship of an American undergraduate student in summer 2007
- “Botanical Congress 2007” in Hamburg / Germany

## 6.9 Membership in biological societies

- Scandinavian Plant Physiology Society SPPS
- German Botanical Society (Section Physiology & Molecular Biology of Plants)

## 7 Hobbies

**7.1 Sports:** tennis, volleyball, alpine skiing

**7.2 Other activities:** travelling, dancing and listening to trance music



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## 10 Erklärung

Hiermit erkläre ich an Eides statt, dass ich bis zum heutigen Tage weder an der Christian-Albrechts-Universität zu Kiel noch an einer anderen Hochschule ein Promotionsverfahren endgültig nicht bestanden habe oder mich in einem entsprechenden Verfahren befinde.

Weiterhin versichere ich an Eides statt, dass ich die Inanspruchnahme fremder Hilfen aufgeführt habe, sowie, dass ich die wörtlich oder inhaltlich aus anderen Quellen entnommenen Stellen als solche gekennzeichnet habe. Diese Abhandlung ist nach Inhalt und Form -abgesehen von der Beratung durch meine Betreuerin- meine eigene Arbeit.

Zudem erkläre ich, dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

Kiel, den

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Sascha O. Ludwig



