

**The Induction and Regulation of Epidermal  
Flavonoid Accumulation at Low Temperature  
in *Arabidopsis thaliana***

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## Abbreviations

*	statistically significantly different ( $P < 0.05$ )
**	highly statistically significantly different ( $P < 0.01$ )
***	very highly statistically significantly different ( $P < 0.001$ )
<i>A. thaliana</i>	<i>Arabidopsis thaliana</i>
AA	amino acid
ABA	abscisic acid
ArKr	argon and krypton lasers as light sources in CLSM (488 nm)
ArUV	argon and UV lasers as light sources in CLSM (364 nm)
ATP	adenosine triphosphate
AU	absorption units
BG	blue-green
B	blue
bp	base pair(s)
bZIP	basic leucine zipper (domain)
C3HC4	C3HC4-type cysteine spacing in zinc ion binding RING finger proteins
CaM	calmodulin
cc	<i>cry1cry2</i> deletion mutant of <i>A. thaliana</i> Col
CCCH	cystein-x8-cystein-x5-cystein-x3-histidine
cDNA	coding DNA
CHI	chalcone flavanone isomerase
CHS	chalcone synthase
CLSM	confocal laser scanning microscopy
Col	<i>Arabidopsis thaliana</i> ecotype Columbia
comp.	composition
CONSTANS	flowering-time gene in <i>Arabidopsis</i> , encodes a zinc finger protein
COR	cold regulated
cry	cryptochrome (blue light sensor)
Cy3	fluorophore with excitation at 550 nm, emission at 570 nm (green)
Cy5	fluorophore with excitation at 649 nm, emission at 670 nm (red)
Cyt b6/f	cytochrome b6/f complex
d	days
DAB	diaminobenzidin
DAF	2', 7'-dichlorofluorescein diacetate
DFR	dihydro flavonol synthase
DIG	digoxigenin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRE-CRT	drought and low temperature stress responsive element
dUTP	deoxyuridine triphosphate
E.R.	endoplasmatic reticulum

ESI-MS	electrospray ionisation – mass spectroscopy
EST	expressed sequence tag
etc.	et cetera
ETR	electron transport rate
Fd	ferredoxin
F3H	flavanone-3-hydroxylase
F3'H	flavonol-3'-hydroxylase
Fm	maximum (chlorophyll-) fluorescence
FS	flavonol synthase
Fv	variable (chlorophyll-) fluorescence
FW	leaf fresh weight (in mg)
glc	glucose residue, glucopyranoside
glyc.	glycosides
G-protein	guanine nucleotide binding protein (a GTPase)
HCA	hydroxy cinnamic acid
HindIII	nuclease from <i>Haemophilus influenzae</i>
HPLC	high performance liquid chromatography
HR	hypersensitive response
i.e.	id est (latin = that is to say)
Int. Std.	internal standard (Naringenin)
kb	kilo base = 1000 bases
$\lambda$	lambda, symbol for wavelength
LHY	late elongated hypocotyl, a circadian-clock associated, myb-related gene
M	molar (mol/l)
min.	minute
MOPS	3-(N-morpholine) propanesulfonic acid
mRNA	messenger RNA
MS	mass spectroscopy
NAD	nicotinamide adenine dinucleotide
NADP(H)	nicotinamide adenine dinucleotide phosphate
Nat. A	Naturfarbstoffreagenz A
NBT	nitrotetrazolium blue chloride
NOS	nitric oxide synthase
ns	not (statistically) significant (different)
OMT	O-methyl transferase
P	level of significance (P value of statistical tests)
PAL	phenylalanine ammonia-lyase
PAM	pulse amplitude modulated fluorometer
PAR	photosynthetically active radiation (400 – 700 nm)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
phot	phototropin (blue light sensor)
pp	<i>phot1phot2</i> deletion mutant of <i>A. thaliana</i> Ws
PR	pathogenesis related



PS I	photosystem I
PS II	photosystem II
psETC	photosynthetic electron transport chain
Q <sub>A</sub>	plastoquinone; the first electron acceptor in psETC
1-q <sub>L</sub>	reduction state of plastoquinone
R <sup>2</sup>	correlation coefficient
rha	rhamnose residue, rhamnopyranoside
RING finger	class of ubiquitin-protein ligases with zinc ion binding RING domain
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
RT	reverse transcriptase
Rubisco	ribulose-bisphosphate carboxylase oxygenase
<i>S. tuberosum</i>	<i>Solanum tuberosum</i>
SD	short-day
SEM	standard error of the mean
SIN-1	3-morpholinopyrrolidine-hydrochloride
SNAP	S-nitroso-N-penicillamin
SNP	sodium nitroprusside
SOD	superoxide dismutase
SP6	universal primer that binds to SP6 promoter region
T7	universal primer that binds to T7 promoter region
Tair	the <i>Arabidopsis</i> information resource
TCA cycle	tricarboxylic acid cycle (Krebs cycle)
temp.	temperature
TF	transcription factor
T <sub>m</sub>	melting temperature
transmitt.	transmittance
tRNA	transfer RNA
UDP	uridine diphosphate
UV-A	ultraviolet A radiation (320 – 400 nm)
UV-B	ultraviolet B radiation (280 – 320 nm)
vs.	versus
VTE, vte	vitamin E deficient
Ws	<i>Arabidopsis thaliana</i> ecotype Wassilewskija
WT	wildtype
ZBM	Zentrum für Biochemie und Molekularbiologie

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## I. Introduction

Flavonoids in fruits and vegetables are an important part of the daily diet of humans. Research on flavonoids has recently intensified as more and more studies suggest a potential beneficial impact of this group of compounds on human health due to their antioxidative, anticarcinogenic and antimicrobial properties *in vitro* (see review of Pietta 2000). They possess a high antioxidative activity due to two hydroxy groups situated at the second benzene ring. The flavonol quercetin is the most promising phenylpropanoid as it showed the highest antioxidative capacity measured in the Trolox Equivalent Antioxidative Capacity test (TEAC test) (Rice-Evans et al. 1996). Similar high TEAC values were reached by the anthocyanin cyanidin. The antioxidant efficacy of flavonoids *in vivo* is less well documented due to the limited knowledge on the antioxidative activity of its metabolites. A small fraction of the flavonoids taken up via the diet appears to be absorbed as aglycon or as glycoside while the main fraction is degraded to different phenolic acids. Both fractions might display an *in vivo* antioxidant activity which results in a sparing effect on  $\alpha$ -tocopherole and  $\beta$ -carotene. The antioxidative capacity is explained by their ability to quench reactive oxygen species (ROS), which develop through many cellular processes in animals and humans. Flavonoids and HCAs are predicted to protect specifically against coronary heart disease (see Zutphen elderly study by Hertog et al. 1993) and arteriosclerosis and may also decelerate aging processes (Middleton et al. 2000). Because of the potential beneficial impact on human health of flavonoids, it is desirable to investigate the factors which determine flavonoid content in plants in order to increase the flavonoid content in the human diet. But first of all, flavonoids are synthesized for the plants themselves in order to fulfil many ecological and physiological functions and are not build up especially for humans. They have a role in growth, development, reproduction (attraction of pollinating insects) and protection against harmful UV radiation. Some are involved in pathogen attack and are antifungal in leaves, others have antimicrobial, insecticidal property or oestrogenic activity (Dixon & Paiva 1995, Harborne & Baxter 1999).

Plants are exposed to a plethora of unfavourable environmental conditions during their life span. Since they cannot move away, they have to acclimate to them in order to survive. During acclimation two different strategies exist. One is a periodic or spatial escape behaviour, e. g. through the development of long-term stages. The other strategy is resistance, by which the unfavourable condition can be survived through tolerance or avoidance. In order to resist harmful UV-B radiation many plants accumulate in their epidermis UV-absorbing phenylpropanoids such as flavonoids for screening UV-B radiation (Hahlbrock & Scheel 1989).

### General characteristics of flavonoids and HCAs

Flavonoids are structurally polyphenols, which consist of two aromatic benzene rings joined by an oxygen containing heterocycle (figure I.1). This structure is called flavan. Different combinations of oxygens, hydroxyl and methyl groups attached to the benzene ring create the various classes of flavonoids. In planta they are present usually as glycosides with molecules of rhamnose and/ or glucose attached to the aglycon (glucopyranoside and rhamnopyranoside moieties). Flavonoids are mostly invisible for the human eye and absorb both, UV-A and UV-B radiation.

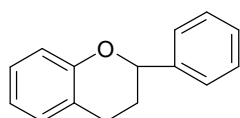


Figure I.1: Structure of flavan, which is the basis for various classes of flavonoids (from Richter 1998).

Another group of phenolic substances, the structurally simpler hydroxy cinnamic acids (HCAs) like sinapic acid or ferulic acid are strongly UV-B absorbing and less UV-A absorbing (Li et al. 1993, Landry et al. 1995). They consist of only one aromatic benzene ring with one attached carbonic acid and are often also glycosidised with sugars (Richter 1998).

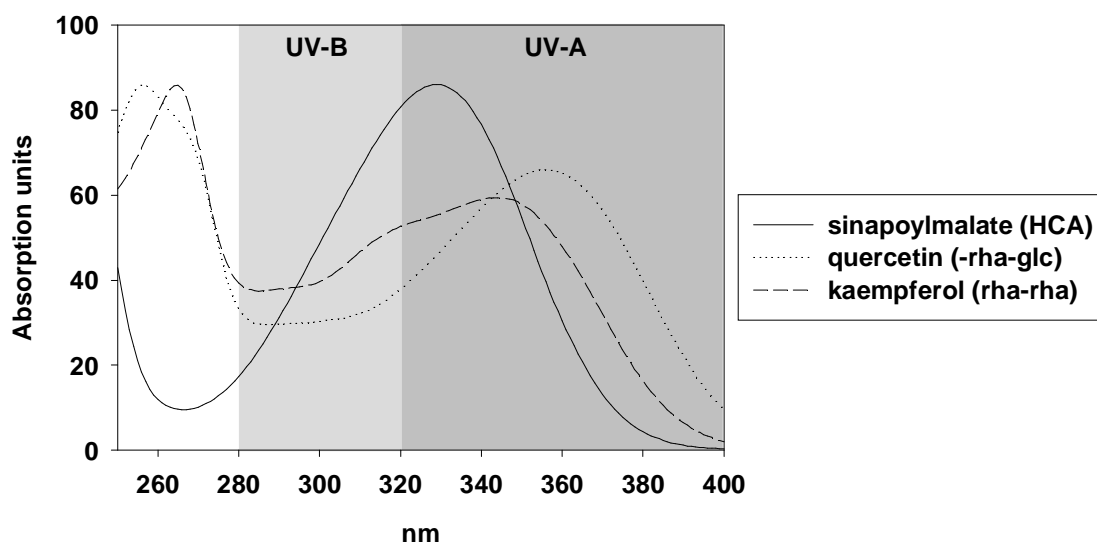


Figure I.2: Exemplary absorption spectra of sinapoylmalate, the main HCA found in *Arabidopsis (A.) thaliana*, and two flavonol glycosides, quercetin-rha-glc and kaempferol-rha-rha (rha = rhamnopyranoside, glc = glucopyranoside), which were also found in *A. thaliana*. The spectra were taken online during HPLC analysis (see method section III.3.2).

In contrast, flavonoids like flavones and flavonols to which quercetin and kaempferol belong, have a strong absorbance in the UV-A and UV-B region (figure I.2). To the group of flavonoids also belong the blue and red coloured anthocyanins which are not UV-shielding.

### *The biosynthesis of flavonoids and its regulation*

The biosynthetic pathway leading to flavonoid accumulation was under intensive research and was elucidated in the early '90s of the last century (Hahlbrock & Scheel 1989, Shirley et al. 1995, Weisshaar & Jenkins 1998, Winkel-Shirley 2001).

Flavonoid biosynthesis is localised in the cytoplasm at the endoplasmatic reticulum (E.R.) and at electron-dense particles in multi-enzyme-complexes. Since recently it is assumed that flavonoids are also synthesized in the nucleus as enzymes of flavonoid bioynthesis were detected in this organelle (Saslowsky et al. 2005). Flavonoids synthesized in the cytoplasm are transported as glycosides into the vacuole of the same cell and presumably also into the nucleus (Schmelzer et al. 1988, Goodman et al. 2004).

The pathway starts with the synthesis of the phenylalanine precursor arogenate from erythrose-4-phosphate and phosphoenolpyruvate, which is a product of the shikimate pathway. Phenylalanine is converted to cinnamic acid by the first important key enzymes of flavonoid biosynthesis, the phenylalanine ammonia-lyase (PAL). Cinnamic acid is converted to p-coumaric acid by cinnamic-acid-4-hydroxylase. P-coumaric acid is a central substance of which on the one hand hydroxy cinnamic acid derivatives (HCAs) are synthesized and on the other hand flavonoids are produced. P-coumaric acid is activated with Coenzyme A (CoA) and the second benzene ring is formed by the second important key enzyme chalcone synthase (CHS). This product is called naringenin chalcone and is an important key substance in flavonoid biosynthesis. The first (dihydro-) flavonol is dihydrokaempferol. The enzyme flavonol-3'-hydroxylase (F3'H) converts dihydrokaempferol to dihydroquercetin. Kaempferol and quercetin are the most important flavonoids in *A. thaliana*. Both are glycosidized to 3-O-glycosides and deposited in the vacuoles and both are precursors for anthocyanidin biosynthesis. Accumulation of flavonoids and HCAs occurs in the same cell in which they are synthesized (Dixon & Paiva 1995).

Besides the post-transcriptional regulation of enzyme activity, one general principle of regulation of gene expression involves binding of transcription factors (TFs) to the promotor region of a specific gene. At the end of a signal transduction pathway, TFs will be activated by phosphorylation, so that they can bind to promotor regions of specific target genes which will in turn then be transcribed. Promotor regions are called *cis*-regulatory elements. As a consequence specific genes will be switched on or off by this mechanism. Due to the fact that promotor regions frequently constitute many



binding domains for several TFs, the amount of bindings will regulate transcription rates of specific genes.

In detail, regulation of flavonoid biosynthetic genes happens presumably by the action of three different types of TFs. They are designated as bZIP, bHLH and MYB (Weisshaar & Jenkins 1998, Hartmann et al. 2005, Mehrrens et al. 2005) and are often light-regulated. Activation of these light-dependent TFs involves frequently a phosphorylation step through cytosolic kinases. It is assumed that the status of phosphorylation determines the transport of for example bZIP-TFs in and out of the nucleus. However, the regulation of gene expression of these three groups of TFs is not yet completely resolved. Nuclear localized *COP*-, *DET*- and *FUS*-genes in *A. thaliana* were identified as negative regulators of flavonoid biosynthetic genes. Deletion mutants of these genes displayed increased flavonoid gene expression and increased flavonoid accumulation in darkness (Hardtke & Deng 2000). In addition, the gene *ICX1*, also a negative regulator of flavonoid biosynthesis appears to be epidermally localized and affects *CHS* gene expression at low temperature, UV-B, UV-A (via cryptochrome 1), phytochrome A and cytokinin (Wade et al. 2003). However, mutants of *ICX1* did not have any increased expression of *CHS* and accumulation of flavonoids in darkness. In addition to a regulation at the gene expression level, a post-translational phosphorylation of the biosynthetic enzyme PAL via a protein kinase with a calmodulin-similar domain is also discussed (Cheng et al. 2001). In addition and to make the whole story even more complex, indications exist that transcription factor activity itself is also regulated by various external and internal signals (vom Endt et al. 2002).

#### *Flavonoid accumulation is induced by various environmental stimuli*

It was mentioned earlier, that in addition to their function as epidermal UV-B shield (Li et al. 1993, Lois 1994), flavonoids and HCAs fulfil many more ecological and physiological functions in plants. A role in growth, development and reproduction (attraction of pollinating insects) was mentioned before as well as a role in defense of pathogen attack due to their antifungal, antimicrobial, insecticidal or oestrogenic activity (Dixon & Paiva 1995, Harborne & Baxter 1999).

An antioxidative function in plants in situations of oxidative stress is also frequently discussed (Landry et al. 1995), but is not proven yet *in vivo* (see reviews of Grace & Logan 2000 and Blokhina et al. 2003). Also, several other enzymatic and non-enzymatic systems exist to scavenge reactive oxygen species (ROS) within the cell. Examples are superoxid dismutases (SODs) involved in the Mehler reaction, ascorbate peroxidases (APXs), dehydroascorbate reductase (DHAR) and glutathion reductase (GR) as components of the ascorbate-glutathione cycle (see review of Foyer & Noctor 2000) and catalase (CAT) or ascorbate and  $\alpha$ -tocopherole as non-enzymatic

antioxidants. Thus, flavonoids and HCAs might just constitute one possibility among others which can protect the plant in case of oxidative stress.

The induction of flavonoid and HCA biosynthesis occurs by diverse environmental factors which is in turn often closely linked to the function of accumulated flavonoid and HCAs in the plant. Flavonoid biosynthesis is induced by various biotic and abiotic factors and nutritional stress (Dixon & Paiva 1995). However, best investigated is the gene expression of the key enzyme of flavonoid biosynthesis, CHS, at high light intensities and through UV-A and UV-B radiation (Feinbaum, Storz & Ausbel 1991, Christie & Jenkins 1996, Kolb et al. 2001).

Likewise, a role of blue and/ or red light and their perception in the induction of *CHS* gene expression via different light receptors like cryptochrome or phytochrome was also frequently reported (Lange et al. 1970, Jackson & Jenkins 1995). An induction of *CHS* by UV-A and blue light via cryptochrome 1 and an independent induction via UV-B involving calmodulin was also described. An induction by UV-B was intensified through blue light and UV-A (Fuglevand et al. 1996) and was diminished by the action of red light via phytochrome B (Wade et al. 2001). In contrast, a cryptochrome 1-mediated induction of *CHS* gene expression can be intensified by phytochrome A and B (Wade et al. 2001). In addition to the induction by different light qualities it has been reported, that the expression of flavonoid biosynthetic genes like *PAL* and *CHS* increases in a light-dependent manner at low temperature (Leyva et al. 1995).

*Which signals could be involved in the induction of flavonoid accumulation at low temperature?*

The observed accumulation of flavonoids at low temperature and moderate illumination (Leyva et al. 1995) is surprising and the question arises which signal might be responsible for an induction of flavonoid biosynthesis at low temperature in comparison to an induction of flavonoid accumulation at high light or UV-B.

Might also photoreceptors be involved in a flavonoid accumulation at low temperature? The temperature sensor in plants is not known yet. On the other hand, high light, UV-B radiation and low temperature may lead to oxidative stress associated with a production of ROS from different sources (Levine et al. 1994). At increased irradiance enzymatic processes for CO<sub>2</sub> fixation become rate limiting which leads to an overreduction of the photosynthetic electron transport chain. Consequently, the ROS species superoxide radical and hydrogen peroxide accumulate at photosystem I (PS I) (Karpinsky et al. 2001). At low temperature conditions the enzymes of the Calvin cycle slow down which results again in an overreduction of the photosynthetic electron transport chain with ROS production at PS I (Feierabend et al. 1992, Dat et al. 2000). Finally, UV-B radiation destroys D1 protein in photosystem II (PS II), resulting in formation of singlet oxygen at PS II (Hideg et al. 2000). However, several other cellular sources for ROS

production might also exist under the three conditions. The role of ROS in signal transduction processes involved in pathogen attack and hypersensitive response (HR) could already be proven (Levine et al. 1994). Also, some indications exist that ROS might be involved in flavonoid accumulation (Nagata et al. 2003), so that a ROS induced flavonoid biosynthesis is also conceivable. However, Mackerness et al. (2000) showed already that UV-B induced CHS gene expression was not related to ROS formation.

Furthermore, low temperature and high light lead to an overreduction of the photosynthetic electron transport chain as described above. This overreduction can be measured as an increased reduction state of the first electron acceptor  $Q_A$  in the photosynthetic electron transport chain by chlorophyll fluorescence (Bilger & Schreiber 1986, Gray et al. 1997, Huner et al. 1998, Kramer et al. 2004). The reduction state of  $Q_A$  functions as a sensor for several signal transduction pathways regulating gene expression of chloroplast-encoded and nuclear-encoded genes (Escoubas et al. 1995, Gray et al. 1997, Karpinski et al. 1997, Pfannschmidt et al. 1999). Thus, the reduction state of  $Q_A$  could also be involved in an induction of flavonoid biosynthesis. In conclusion, photoreceptors, ROS and the reduction state of  $Q_A$  are all hypothetical signals which might be involved in flavonoid accumulation at low temperature.

*Arabidopsis thaliana is an ideal model plant for studying the induction of flavonoid accumulation at low temperature*

In order to investigate the above questions properly, *Arabidopsis (A.) thaliana* and its photoreceptor mutants were chosen as plants for investigation. *A. thaliana* is a model plant for species of the mustard family which comprises food plants like cabbage (*Brassica oleracea*), mustard (*Sinapis sp.*), rocket (*Eruca sp.*) and others. Since the *Arabidopsis* genome is fully sequenced (*Arabidopsis* Genome Initiative 2000) and a large assortment of mutants and transgenic plants is available, this species is ideal to investigate flavonoid accumulation at different abiotic conditions. In addition, *A. thaliana* plants have several practical advantages as plants are easy to grow and to reproduce, they have small space requirements due to their small size and last but not least, the rosette leaves display horizontal growth, guaranteeing a homogeneous illumination of the whole leaf lamina.

*Key methods for investigating the induction and pattern of flavonoid accumulation at low temperature*

As mentioned before, flavonoids are mainly UV-A absorbing and accumulate primarily in the vacuole of the upper epidermis of leaves. Bilger et al. (1997, 2001) have developed a new fluorescence method which determines the epidermal UV-A transmittance (absorbance) in leaves non-invasively and quickly. This allows repeated

measurements of the epidermal accumulation of UV-A absorbing compounds like flavonoids in leaves of *A. thaliana* at different environmental conditions and at different timepoints. HPLC analyses were also performed to validate UV-A transmittance measurements and obtain detailed information on flavonoid and HCA pigment composition.

Flavonoid accumulation at high light, supplemental UV-B radiation or low temperature could be the result of equal or distinct signal transduction pathways. In order to discover common components of relevant signal transduction pathways microarray gene expression analysis was performed. This method is a relatively new tool to investigate the gene expression level of many, often several thousand, genes after one treatment in relation to a second, often control treatment in one single microarray experiment. Thus, a snapshot of the tissues' whole gene expression is obtained. However, individual gene expression results have to be validated by northern blotting techniques. Microarray analysis is also useful when investigating the expression level of genes belonging to a whole biosynthetic pathways like flavonoid biosynthesis. Differences in the pattern of activation of the flavonoid biosynthetic pathway also allows conclusions on whether equal or distinct signal transduction pathways were used.

## II. Aims and scope of this thesis

This PhD project aimed at the investigation of the influence of natural environmental conditions on the induction of flavonoid biosynthesis in *A. thaliana*. Besides high irradiances and UV-B radiation also low temperature induces flavonoid biosynthesis. From these observations the question arises how flavonoid biosynthesis is induced at low temperature and which signal carriers are responsible for the induction at low temperature. One common signal carrier or overlapping signal transduction pathways might be involved in the induction. Three different potential „candidates“ were chosen as potential signal carriers: reactive oxygen species (ROS), the reduction state of the photosynthetic electron transport chain (psETC) and blue light photoreceptors.

As flavonoids predominantly accumulate in epidermal vacuoles, where they screen the mesophyll of leaves against harmful UV-B and UV-A radiation, this characteristic allows to estimate the flavonoid content very rapidly and non-invasively by measuring the epidermal UV-A transmission in green leaves using a newly developed technique based on chlorophyll fluorescence (Bilger et al. 2001). HPLC analysis was applied to investigate the specific pigment composition at the different experimental conditions. In addition to physiological aspects of flavonoid biosynthesis, gene expression studies for key enzymes of flavonoid biosynthesis like chalcone synthase (CHS) and phenylalanine ammonia-lyase (PAL) as well as general comparative gene expression studies at different environmental conditions using microarrays and northern blotting techniques were also performed.

The comparative analysis of flavonoid accumulation aimed to get information on whether similar or distinct signal transduction pathways were used by the different conditions. The effect of potential signal carriers like the reduction state of psETC, ROS and light receptors was investigated aiming to find potential common signal carriers for the induction of flavonoid biosynthesis. The search for additional components of relevant signal transduction pathways leading to the induction of flavonoid biosynthesis was supported by microarray analysis.

Experiments were primarily performed with *A. thaliana* plants and their photoreceptor mutants and were carried out in climate controlled growth chambers and cabinets.

### III. Material and methods

#### III.1 Plant material and growth conditions

##### III.1.1 Control growth conditions

*A. thaliana* L. (Heynh.) wildtype (WT, ecotypes Columbia, Col and Wassilewskija, Ws) or mutants of *Arabidopsis* were sown on nutrient enriched compost (TKS-2, Floragard, Oldenburg, Germany), stratified for three days in a refrigerator at 4 °C and then grown in a climate controlled growth chamber (BBC York) at 22 °C air temperature and a photosynthetically active radiation (PAR) of 120-130  $\mu\text{mol}/\text{m}^2\text{s}$  white light from fluorescent tubes (TLD 58W, 830 New Generation, Philips, Holland) at long-day conditions, which was 16 hours light per day. Seedlings and plants were irrigated using tap water. After 14 to 19 days young plants were singled into plastic pots (TekuTerra TO7, volume = 0,13 l, Pöppelmann GmbH & Co KG, Lohne, Germany) containing TKS-2 and kept at the described conditions for another week until experiments started. Some experiments had to be performed in light controlled growth cabinets (Grobank, CLF Plant Climatics, Emersacker, Germany), which were placed within the climate controlled growth chambers. Different lamps (F32T8, TL741 Universal/ Hi-Vision, Alto collection, Philips, USA) and an additional ventilation system than in the growth chambers were installed in the growth cabinets, so that, if experiments were to be generated in such a growth cabinet plants were allowed to acclimate to the new conditions for one week after they were singled. After a period of 21 to 26 days of growing, plants routinely exhibited five to eight fully developed rosette leaves. These conditions described so far refer to as “control conditions”. If not stated otherwise only young and fully developed rosette leaves were used for experiments. Also, if not stated otherwise, all experiments generally lasted for seven days.

PAR and temperature were measured using a Li-COR Quantum/ Radiometer/ Photometer (LI-185B, Li-COR, USA) and a digital Minimum-Maximum Thermometer (Oregon Scientific, USA), respectively. Additionally, irradiance, air temperature and humidity in the climate chamber were followed 24 hours per day using a data logger based data acquisition system.

As mentioned earlier white light in the growth chamber and in the growth cabinet was supplied by different lamps. Whereas in the chamber TLD 58W, 830 New Generation (Philips, Holland) lamps were installed, in the growth cabinet Grobank lamps of type F32T8, TL741 Universal/ Hi-Vision (Alto collection, Philips, USA) were placed. Figure III.1 shows spectra of these lamps. A higher irradiance in the blue light (BL) region (400-500 nm) of the total PAR (400-700 nm) was detected for the cabinet. This is important as control conditions were set up at both places.

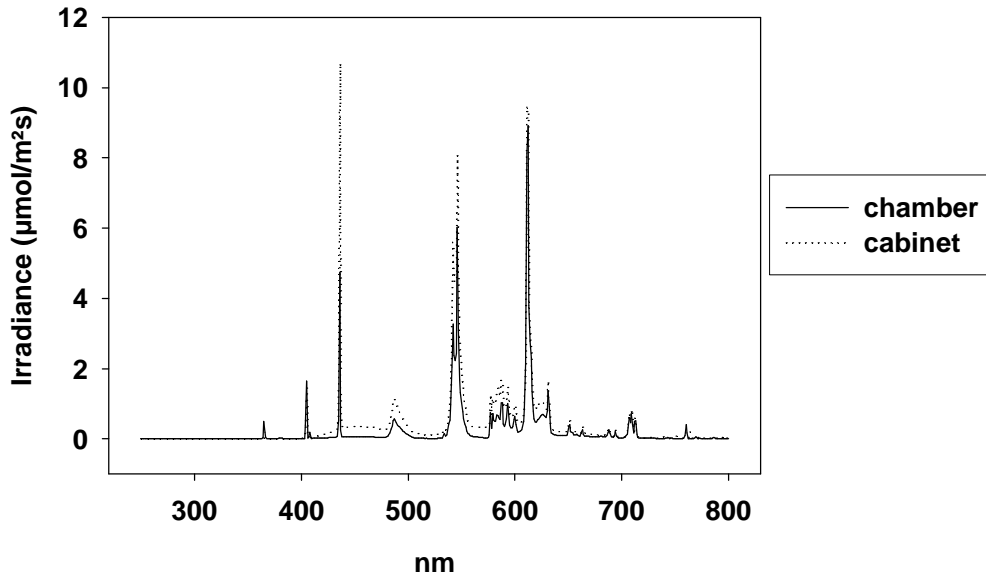


Figure III.1: Spectra of lamps installed in the growth chamber and the growth cabinet which were of different types recorded at a PAR of 120  $\mu\text{mol}/\text{m}^2\text{s}$ . A higher irradiance was detected in the blue light region of the PAR spectrum (400-500 nm) in the cabinet (dotted line) in comparison to the growth chamber (solid line).

Irradiance was summarized over PAR (400-700 nm) and over the BL region of the spectrum (400-500 nm) and the fraction of BL on total PAR was calculated (table III.1). In the growth cabinets this fraction was higher than in the growth chamber and counted for about 23 % of the total irradiance of PAR.

Table III.1: Calculated BL-fraction (400-500 nm) of total PAR (400-700 nm) at four different control growth places. Fraction was one third higher in growth cabinets than in growth chambers.

Growth place	Chamber		Cabinet	
Air temperature	9 °C	22 °C	9 °C	22 °C
<b>BL fraction (%)</b>	<b>14,7</b>	<b>14,7</b>	<b>22,5</b>	<b>22,8</b>

### III.1.2 Plant material

Besides the two ecotypes Col and Ws of *A. thaliana* the following blue light photoreceptor mutants were investigated: *phot1phot2* (background: Ws), *cry1cry2* (background: Col), which lacked intact phototropin1 and 2 and cryptochrome 1 and 2, respectively. The *phot1phot2* and *cry1cry2* mutants were kindly donated by Dr. Tatsuja

Sakai (RIKEN Plant Science Center, Japan) and Dr. Chentao Lin (University of California, USA), respectively. Also single mutants of cryptochrome were analysed in this study. *cry1* was devoid of cryptochrome 1 and *cry2* lacked cryptochrome 2. Both were ordered from NASC (Nottingham Arabidopsis Stock Center, University of Nottingham, Loughborough, UK ) and were in background of Col.



Image III.1: Three weeks old *A. thaliana* Col plants grown at control conditions in a state as used for the experiments.

In addition to *A. thaliana*, also fully developed plants of *Solanum (S.) tuberosum*, WT Desirée and antisense individuals with reduced leaf ferredoxin (Fd) contents were used for low temperature experiments. The Fd content was reduced by either 40 % (*FdA6*) and 50 % (*FdA7*) in comparison to WT individuals. They were kindly donated from Dr. Jan Backhausen (University of Osnabrück, Germany).

### III.2 Treatments

In this work leaves and whole plants of *A. thaliana* and *S. tuberosum* were treated with several different experimental conditions in order to investigate the role of flavonoid accumulation at low temperature. These treatments will be described and defined in detail in the following paragraphs.



### III.2.1 Low temperature

The “low temperature” condition for *A. thaliana* was as the control conditions but with a lower air temperature of 9 °C. It was set up in the growth chamber.

The “low temperature” condition for *S. tuberosum* was as the control conditions but with a lower air temperature of 9 °C and increased irradiance of 200  $\mu\text{mol}/\text{m}^2\text{s}$ .

Some experiments were conducted in low temperature and in darkness. For this purpose plants were put in a light-tight cardboard box.

Another experiment was performed in which the influence of a single period of time of low temperature conditions on epidermal UV-A transmittance was investigated. In this experiment *A. thaliana* Col plants were exposed to low temperature conditions for 0, 2, 4, 8 and 24 hours before they were placed back to control conditions. Transmittance was followed over a period of four days.

### III.2.2 High irradiances

“High light conditions” were as the control conditions but with a higher irradiance of generally 350 to 360  $\mu\text{mol}/\text{m}^2\text{s}$  and were also performed in the growth chamber. Light dependency experiments were set up in the growth chamber and different irradiances between 20 and 350  $\mu\text{mol}/\text{m}^2\text{s}$  were achieved by placing the plants at different distances to the light sources.

### III.2.3 Supplemental UV-B radiation

The “UV-B conditions” were always build up in the growth cabinet (Grobank, CLF Plant Climatics, Emersacker, Germany). In addition to a PAR of 120-130  $\mu\text{mol}/\text{m}^2\text{s}$  for 16 hours per day, UV-B radiation of about 145  $\text{mW}/\text{m}^2$  was supplemented for 14 hours per day, starting one hour after PAR started. Figure III.2 presents the white light spectrum supplementd by UV-B. The UV-B irradiance fraction (280-319 nm) accounted for 1,1 % of the total spectrum (280- 700 nm). In order to exclude UV radiation below 290 nm properly, UV fluorescent tubes (TL 40W/12R, Philips) were covered with cellulose-acetate foil (Rachow, Hamburg, Germany), which was pre-burned with the same lamp type for 24-hours in a seperate room.

One experiment was performed in which the influence of the UV-B dosage on epidermal UV-A transmittance was investigated. UV-B dosage was set to 0, 30, 55, 82, 109, 139, 143, 231, 279, 286 and 405  $\text{mW}/\text{m}^2$  UV-B.

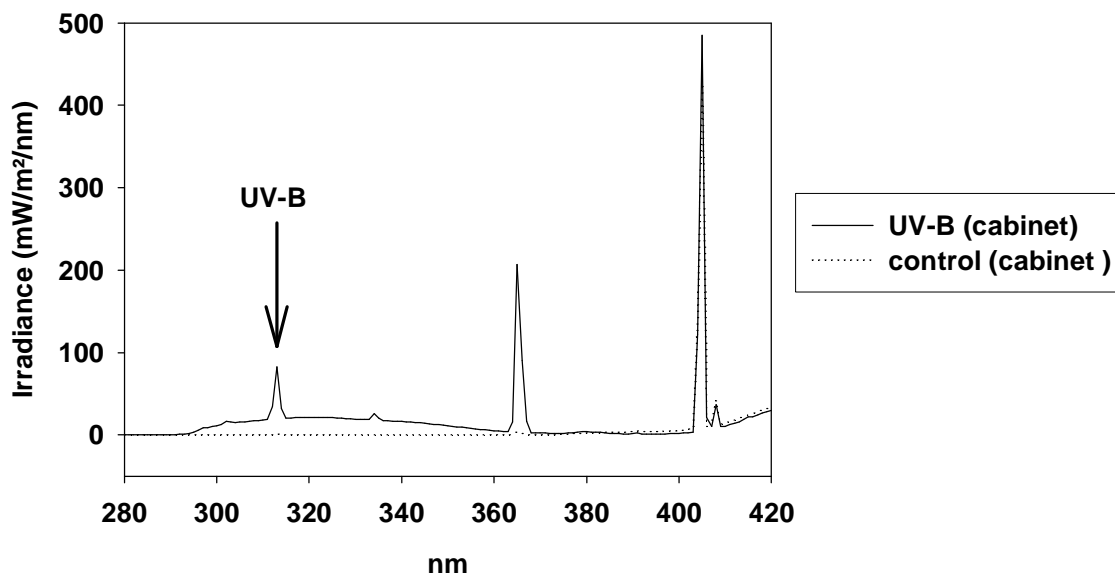


Figure III.2: Light spectrum between 280 and 420 nm recorded in the growth cabinet at control conditions (dotted line) and at supplemental UV-B conditions (solid line). The UV-B peak was visible at 280 nm (arrow). UV radiation below 290 nm was excluded by acetate foil.

#### III.2.4 Short photoperiod

“Short-day conditions” were as control conditions but with a photoperiod of only eight hours per day. Irradiance was set to 120 or 300  $\mu\text{mol}/\text{m}^2\text{s}$ . Experiments were performed in the growth cabinet.

#### III.2.5 Oxidative stress/ ROS-induction experiments

The role of ROS in the induction of flavonoid biosynthesis was investigated by applying ROS-generating solutions like riboflavin, hydrogen peroxide, SNAP (S-nitroso-N-penicillamin, MoBiTec, Göttingen, Germany) or SIN-1 (3-morpholinosydnonimine-hydrochloride, MoBiTec, Göttingen, Germany). Solutions were applied over a period of several days by brushing leaves carefully with the solutions twice a day. Control leaves were treated the same way but with tap water instead of the solution.

The positive release of ROS into leaf tissues by these solutions was investigated by loading cut leaves with NBT prior to treatment with solutions of riboflavin (1 mM) or SIN-1 (40  $\mu\text{M}$ ). Table III.2 shows the timetable for this experiment.

Table III.2: Time schedule for visualizing ROS in rosette leaves of *A. thaliana* Col by NBT after applying SIN-1 or riboflavin by a brush over a period of 24 hours (\* 8 h light and 8 h darkness (night)). Light treatment was 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light.

Total hours	Duration (h)	Condition	Description
0 – 1	1	22 °C, darkness, tap water	dark adaption
1 – 3.5	2.5	22 °C, darkness, NBT	NBT-loading
		22 °C, darkness, tap water, <b>1. brushing</b>	start of treatment
3.5 – 11.5	8	22 °C, light, tap water	incubation
		22 °C, darkness, tap water, <b>2. brushing</b>	re-treatment
11.5 – 27.5	16	22 °C, light*, tap water	incubation

### III.2.6 Illumination of abaxial leaf sides at low temperature conditions

In experiments, in which the abaxial side of the leaves was to be illuminated, young and fully developed rosette leaves were turned upside down and fixed carefully with pins without damaging them.

### III.2.7 Exclusion of blue light – illumination with yellow light

An influence of blue light perception was investigated by excluding blue light from the white light source by covering the white fluorescent tubes with a yellow foil (Lee filters, deep straw No.015, ZILZ direct, Pulheim, Germany). The irradiance was measured using the same PAR sensor as above and additionally by scanning the light spectra with a spectroradiometer (Bantham) (figure III.3).

### III.3 Parameters

Several parameters were measured and recorded in the experiments. Not all parameters were detected in all experiments with the exception of epidermal UV-A transmission measurements, which were always performed.

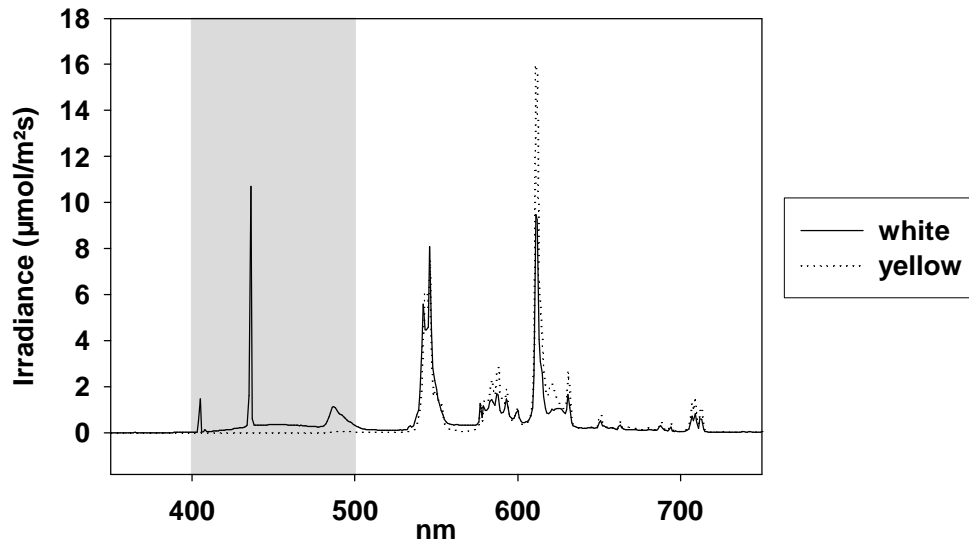


Figure III.3: Irradiance spectra between 350 and 750 nm recorded in the growth cabinet at white light conditions (solid line) and under excluded blue light, i. e. yellow light (dotted line) at 270  $\mu\text{mol}/\text{m}^2\text{s}$  PAR. No blue light was visible between 400 and 500 nm (grey field).

### III.3.1 Measurements of epidermal UV-A transmission in leaves

Flavonoids are UV-A absorbing pigments which primarily accumulate in the vacuoles of epidermal cells. Bilger et al. (1997) developed a method based on chlorophyll fluorescence, which allows to measure the relative epidermal UV-A transmittance in green tissues non-invasively and very rapidly (Bilger et al. 2001). The so-called UV-A-PAM instrument (Gademann Instruments, Würzburg) uses blue (B,  $\lambda_{\text{max}} = 470$  nm) or UV-A light ( $\lambda_{\text{max}} = 375$  nm) as excitation beam (figure III.4 b). If a leaf is irradiated with UV-A or blue light, it will emit chlorophyll fluorescence. UV-A absorbing compounds in the epidermis will lower the chlorophyll absorption of the UV-A beam but not of the blue light. As a result the fluorescence signal derived from the UV-A beam will be lowered whereas the signal derived from the blue light beam will stay constant (figure III.4 a).

The ratio of the emitted fluorescence from UV-A versus fluorescence from blue excitation estimates the relative epidermal UV-A transmission. As the emitted fluorescence is dependent on the incident irradiance, a blue foil (fluorescence standard, Walz, Effeltrich, Germany) was used to standardize individual measurements. A green non-fluorescing plastic foil (Gademann Instruments) served to correct against a small error due to reflected measuring light.

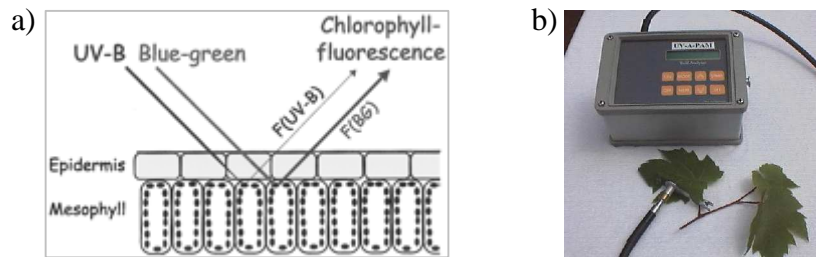


Figure III.4: Measurements of epidermal UV-A transmittance with the UV-A-PAM. a) Scheme showing the working principle of the measurement of epidermal UV-B-transmission. UV-B-absorbing compounds present in the epidermal vacuoles will lower the fluorescence signal from the mesophyll derived by UV-B excitation in comparison to blue-green excitation, which is not absorbed by these compounds. Figure from Bilger et al. (2001). b) Image of UV-A-PAM instrument (Gademann Instruments, Würzburg).

Determination of the value for 100 % epidermal UV-A transmittance for leaves was achieved by removing the epidermis before measurements (Bilger et al. 1997). Epidermis-free *A. thaliana* leaves yielded an  $F(\text{UV-A})/F(\text{B})$  value of  $1,30 \pm 0,24$  (SEM) after normalization of the signals to the blue standard foil. This value represents the average of ten different measurements on representative leaves and was used to calculate epidermal transmittance in all experiments. Measurements on epidermis-free leaves of *A. thaliana* Ws ecotype and of mutants *cry1cry2* and *phot1phot2* were also performed and showed similar transmission values (data not shown).

In order to calculate epidermal UV-A absorption from the transmission values the following equation was applied:

$$\text{Absorption} = - \log \text{ transmittance}$$

### III.3.2 Analysis of flavonoid and hydroxy cinnamic acid (HCA) composition in leaves

The composition of flavonoids and HCAs in leaves was analyzed using HPLC. The method was adopted from Kolb and co-workers (2001) and changed according to requirements. Only young and fully developed leaves were sampled.

For pigment extraction, the deep frozen leaf sample was homogenized for five minutes together with four glass beads of diameters between 1,7 and 2 mm (Carl Roth, Karlsruhe, Germany) in a mixer mill (MM2, RETSCH, Haan, Germany). Extraction buffer and internal standard were added to the homogenized sample, incubated on ice for five minutes and centrifuged for five minutes at 4 °C and 10.000 rpm. The supernatant was collected and the pellet was extracted again twice. Finally, the unified supernatant was centrifuged for 10 minutes at 10.000 rpm and a fraction of it was filtered

through syringe filters with 0,2  $\mu\text{m}$  pore width into HPLC vessels. The extraction buffer consisted of 50 % (v/v) HPLC grade methanol and 1 % (v/v) concentrated HCl.

The HPLC system used was an Agilent 1100 Serie HPLC system (Agilent Technologies, Waldbronn, Germany) with degasser, binaric pump, temperature controlled auto-sampler, column oven, a DAD detector and the Agilent ChemStation software, which was the system for instrument control, data acquisition and data evaluation. The DAD detector measured the individual peaks between 220 and 550 nm, giving an absorption spectrum for every eluted pigment. Chromatograms were recorded at 314 and 360 nm, but only the signal at 314 nm was used for quantification of compounds.

The column was an Agilent 5 $\mu\text{m}$  LiChrospher-100 RP-18 with a diameter of 4 mm and a length of 250 mm (Agilent Technologies, Waghäusel-Wiesental, Germany). Oven temperature was set to 20°C and the volume injected was generally 20  $\mu\text{l}$ . Flow rate was 0.75 ml/min.

The mobile phase consisted of a gradient of 0,01 % (v/v) phosphoric acid (solvent A) and 90 % (v/v) methanol-0,01 % (v/v) phosphoric acid (solvent B). Separation started with 20 % of the methanolic phase and increased in several steps up to 100 % according to table III.3.

Table III.3: Time schedule of separation gradient for HPLC of flavonoids and HCAs.

Time (min.)	Solvent A (%)	Solvent B (%)
0 - 9	80 - 55	20 - 45
9 - 19	55	45
19 - 30	55 - 30	45 - 70
30 - 32	30-0	70 - 100
32 - 38	0	100

Peak identification was achieved by comparing retention times and absorption spectra and also by molecular masses and fragmentation patterns derived from HPLC-ESI-MS analysis (see next paragraph). Chromatograms were analyzed using the software ChemStation for LC 3D (Rev. A 09.01[1206], Agilent Technologies). Peak areas (in AU\*s) were corrected for volumetric errors using Naringenin (Carl Roth, Karlsruhe, Germany) as internal standard, which is a flavanone absent in *A. thaliana* leaves, and calculated per leaf area (in  $\text{cm}^2$ ).

Generally, sampled leaves were scanned prior to freezing into liquid nitrogen and leaf area was calculated from scanned leaves using the free image processing and analysis program UTHSCSA Image Tool for Windows, Version 3.00

(<http://ddsdx.uthscsa.edu/dig/itdesc.html>). Sometimes only leaf discs were sampled instead of whole leaves, whose area was calculated from scanned leaf disc as described above. Leaf samples were quickly deep-frozen in liquid nitrogen prior to storage at  $-80^{\circ}\text{C}$  and until further use.

### III.3.3 Identification of flavonoids and HCAs found in leaves by HPLC-MS

Peaks found in HPLC were identified not only by retention time and absorption spectra but also by their molecular masses and specific fragmentation patterns derived from ESI-MS-HPLC analysis. Analytes were ionized in the negative mode by electrospray ionisation (ESI). This analysis was performed in cooperation with Dr. Eva-Maria Hubbermann at the institute for food technology (University of Kiel, Germany).

### III.3.4 Visualising polyphenols in leaf cross-sections and epidermal strips of *Arabidopsis*

Polyphenols were visualized in a confocal laser scanning microscope (CLSM, Leica TCS SP, Leica, Bensheim, Germany) with apochromatic objectives (20 X, 40 X, 63 X / 1,32 oil immersion) using the dye Naturfarbstoffreagenz A ( $\beta$ -aminodiethylester of diphenylboric acid, Nat. A) (Hutzler et al. 1998). Nat. A reacts with flavonoids to form yellow fluorescent substances. Excitation was at 364 nm with an ArUV (Argon/UV) laser and at 488 nm with an ArKr (Argon/krypton) laser, detection of the emitted fluorescence of bound Nat. A was at 515 - 550 nm and 560 – 600 nm. Chlorophyll fluorescence was detected at 660 – 720 nm. In addition images of transmission through the cross-sections were also recorded. Images were analysed with the software Leica TCS-Image.

Nat. A contained 0,25 %  $\beta$ -aminodiethylester of diphenylboric acid (w/v), 0,2 % Triton-X (v/v) in 1X PBS buffer. The reagent was prepared from aqueous 10 X concentrates of Triton-X (2 % (v/v) in  $\text{H}_2\text{O}$ ) and PBS (10 X in  $\text{H}_2\text{O}$ , i.e. 100 mM potassic phosphate buffer with pH of 6,8) and from an ethanolic 10 X concentrate of  $\beta$ -aminodiethylester of diphenylboric acid (2,5 % (w/v) in 100 % ethanol). One droplet of dye solution was added to the sample. After incubation for five minutes, Nat. A was removed by excessive washing with buffer.

### III.3.5 Visualising oxidative stress products: ROS

The abbreviation ROS summarizes several reactive oxygen species like hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), being the most stable ROS and superoxide radical ( $\text{O}_2^{\cdot-}$ ), being a

precursor of  $\text{H}_2\text{O}_2$ . Visualising of these two ROS was done using the dyes diaminobenzidin (DAB, BIOMOL GmbH, Hamburg, Germany) and nitrotetrazolium blue chloride (NBT, BIOMOL GmbH, Hamburg, Germany), which form with  $\text{H}_2\text{O}_2$  and  $\text{O}_2^{\cdot -}$  a brown and blue coloured precipitate, respectively.

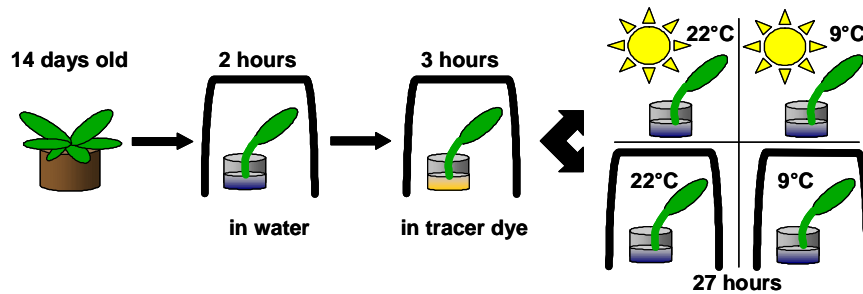


Figure III.5: Diagram presenting the procedure for visualizing ROS in rosette leaves of *A. thaliana* Col with the dyes DAB and NBT. After incubation leaves were fixed in bleaching solution.

Mature rosette leaves of *A. thaliana* plants were cut off and adapted for two hours to darkness at 22 °C air temperature (figure III.5). Then the leaves were allowed to take up dye solution via the transpiration stream for about three hours at the same conditions. After having loaded the leaves with dye they were placed to experimental conditions for 27 hours and finally fixed in bleaching solution to wash out chlorophyll and other pigments. Areas of ROS formation were visible under the microscope as brown or blue spots. Control leaves were kept at control conditions. During the process of lignin formation ROS are usually produced, so that colored petiole and veins were a good positive control for the successful uptake of dye solution.

The DAB dye solution was prepared as follows: 0,5 mg DAB was first dissolved in 200  $\mu\text{l}$  HCl (0,1 M) and then 800  $\mu\text{l}$   $\text{H}_2\text{O}$  was added to 1 ml total solution. The NBT dye solution contained 4 mg NBT per ml PBS (with pH = 6,9). Dye solutions were kept in darkness. Bleaching solution was a 1 : 5-mixture of pure ethanol and chloroform, to which 0,15 % trichloric acid was added. Bleached plant material could be stored in a 1:1-mixture of PBS and glycerin for several weeks in the refrigerator.

### III.3.6 Measurements of the reduction state of the photosynthetic electron transport chain

Analysis of the reduction state of the pool of the first electron acceptor in the psETC,  $Q_A$ , (as  $1-q_L$  according to Kramer et al. 2004) were performed using a pulse amplitude modulated fluorometer (PAM 101). Measurements were based on chlorophyll fluorescence as described by Schreiber et al. (1986).



A pulsed PAR light source (SP) of very high intensity was used to induce maximum fluorescence ( $F'_m$ ) and a continuous PAR light source (AL) with variable light intensities was used as actinic light source to induce photosynthetic activity. The reduction state of the  $Q_A$ -pool was estimated by measuring the photochemical quenching of fluorescence with a saturating light pulse (SP).  $1-q_L$  was calculated as indicated in Kramer et al. 2004 according to the following equation:

$$1-q_L = 1 - ((F'_m - F_t) / (F'_m - F'_o)) * (F'_o / F_t)$$

with  $F'_m$  = maximum F during illumination with AL  
 $F_t$  = terminal F during illumination with AL  
 $F'_o$  = F in darkness after illumination with AL

Leaves were dark adapted for one hour and also analysed at ambient temperatures using a thermostatically controlled chamber. During measurements leaves were kept turgid by watering them via the petiole and  $CO_2$  starvation was overcome by airing the chamber with an aquarium pump.  $F'_o$  was determined after rapidly darkening the leaf with a black cloth. The lowest level of the fluorescence signal after AL was switched off was taken for calculation.

Measurements of  $1-q_L$  on leaves of *S. tuberosum* were performed at experimental conditions using a Mini-PAM by the same procedure as described before for *Arabidopsis* leaves but with the actinic light source replacing the ambient light in the experiment.

#### III.4 Analysis of differential gene expression

Plants were treated with either low temperature, high light or UV-B for four hours before three to four rosette leaves of each plant and of control plants were cut, weighed and frozen into liquid nitrogen very quickly. Samples stayed deep frozen until further examination. Total RNA was extracted from treated and non-treated leaves using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the protocol therein. Residual DNA was digested using two Kunitz units DNase I (RNase-Free DNase Set, Qiagen, Hilden, Germany). Total RNA was precipitated with 4 M NaCl, diluted in RNase-free water and quantified photometrically against water in a NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, USA). Integrity of extracted RNA was confirmed by agarose gel electrophoresis. A loading buffer (Blue/orange loading dye, 6 X, Promega, Mannheim, Germany) and SYBR Green (SYBR Green I nucleic acid gel stain, 10.000 X concentrate in DMSO, Invitrogen, Karlsruhe, Germany) were added to each RNA sample and were used for control of run distance and visualizing separated RNA with an UV-illuminator (GelDoc2000, BioRad, Munich, Germany), respectively. 25 and 7,5  $\mu$ g total RNA were used for microarray analysis and northern blotting, respectively.

### III.4.1 Microarray experiments

Whereas treatment of plants, sampling, RNA extraction and RNA purification was done by myself, gene expression studies with this RNA were carried out by Dr. Sabine Milde and Dr. Anne Hoffmann (both centre for biochemistry and molecular biology, ZBM, University of Kiel, Germany) in Turku, Finland. For detailed information on the procedure please refer to Dr. Anne Hoffmann's dissertation and the material & methods section therein ([http://e-diss.uni-kiel.de/diss\\_1583/](http://e-diss.uni-kiel.de/diss_1583/)). Gene expression studies were performed in cooperation with the research group of Prof. Eva-Mari Aro (Plant Physiology and Molecular Biology, University of Turku, Finland) on Incyte microarray chips using the Cy3/Cy5 dUTP labelling system with green and red fluorescence emission (GE Healthcare, Freiburg, Germany). Microarrays were based on the GEM1 (gene expression microarray) cDNA collection of 7,942 ESTs of *A. thaliana* from Incyte Genomics (Palo, USA). These 7,942 ESTs represented approximately 6,500 genes of *A. thaliana*, which is about 25% of the genome. Arrays were manufactured by Prof. Aro's group and the Centre of Biotechnology (Biocity, Turku, Finland). Clones originated from sequence-verified libraries of InCyte® Genomics and the Ohio State University (USA). All ESTs were spotted in three technical replicates and were divided on two glass slides.

Labelled mRNA of control plants and treated plants was hybridized simultaneously (figure III.6). A sampling time of four hours after transfer to new conditions (9 °C; 360  $\mu\text{mol}/\text{m}^2\text{s}$  white light; 143  $\text{mW}/\text{m}^2$  UV-B radiation) was chosen. For detailed information on treatments please refer to table III.4 below.

Table III.4: Characteristics of different treatments of *A. thaliana* Col plants, which were used for microarray analysis and northern blotting. Per treatment several leaves were sampled in order to obtain enough total RNA for microarray analysis and northern blotting.

<b>Treatment</b>	<b>Temperature (°C)</b>	<b>Duration (h)</b>	<b>Light quality</b>	<b>Irradiance (<math>\mu\text{mol}/\text{m}^2\text{s}</math>)</b>
Control	22	4	White light	120
Low Temperature	9	4	White light	120
High Light	22	4	White light	360
UV-B	22	4	White light + UV-B	120 + 143 $\text{mW}/\text{m}^2$ UV-B

Three chips were performed per treatment and each chip contained RNA from one treated and one control plant. Slides were scanned using a ScanArray Express 5000 device (GSI Lumonics, USA). Fluorescence images of slides were saved as .tif-files. Assignment of spots to rows and columns, a process called gridding, and quantification

of spot intensity was done using ScanArray ExpressMicroarray Analysis System 2.0 (Perkin-Elmer, Boston, USA). Subsequently, the software GeneSpring 7.2 (Agilent, Böblingen, Germany) calculated normalized mean values, i.e. expression values, for each EST from the three technical replicates. In a second step, those ESTs were selected, which were up- or down-regulated by a factor of more than 1.5 or by a factor of less than 0.75, respectively in comparison to expression level of control plants in all three technical and biological replicates. As each EST had a specific and systematic annotation clone number, it was possible to assign a gene name and an At-code (AGI code) to most of the ESTs. The At-code describes the specific gene number of the *A. thaliana* genome. *CHS* for example has the following At-code: At5g13930. Information on specific genes were obtained by searching several databanks on the internet like the TAIR homepage (The *Arabidopsis* Information Resource, <http://www.arabidopsis.org>) and scientific articles.

#### III.4.2 Validation of microarray results by sequencing

Several genes of the flavonoid biosynthetic pathway and some of the genes, which were up- or down-regulated in all three treatments by a factor of more than 1.5 or less than 0.75 were investigated further. From all of these selected genes, a small amount of DNA-material from the array was amplified and sequenced with the capillary sequencer MegaBACE 1000 (GE Healthcare, Freiburg, Germany). The PCR products used for spotting had been prepared from cloned cDNA fragments using SP6 and T7 primers. Samples of these PCR products were obtained from Prof. Aro's group. Firstly, DNA was amplified in a PCR reaction using also SP6 and T7 primers and the quality of the PCR products was checked by an agarose gel. A loading buffer (Blue/Orange loading dye, 6 X, Promega, Mannheim, Germany) and SYBR Green (SYBR Green I, Invitrogen, Karlsruhe, Germany), added to each sample, were used for control of run distance and visualization of bands with an UV-illuminator (GelDoc2000, BioRad, Munich, Germany), respectively. Size of PCR products was estimated by adding 1 kb ladder or  $\lambda$  DNA/Hind III marker (Promega, Mannheim, Germany) to the same gel. Products were purified with the QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). The sequencing reaction was set up using the DYEnamic ET Dye Terminator Cyler Sequencing Kit (Amersham, GE Healthcare, Freiburg, Germany) and 50 ng of the raised PCR product. The purified sequencing reaction was subsequently sequenced in the MegaBACE according to manufacturer's instructions. Calculation of sequences from the chromatograms, a process called "base-calling", was performed with the MegaBACE software "Sequence Analyzer". Sequences were evaluated using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>), which compares the entered sequence with other sequences from many different organisms in the database.

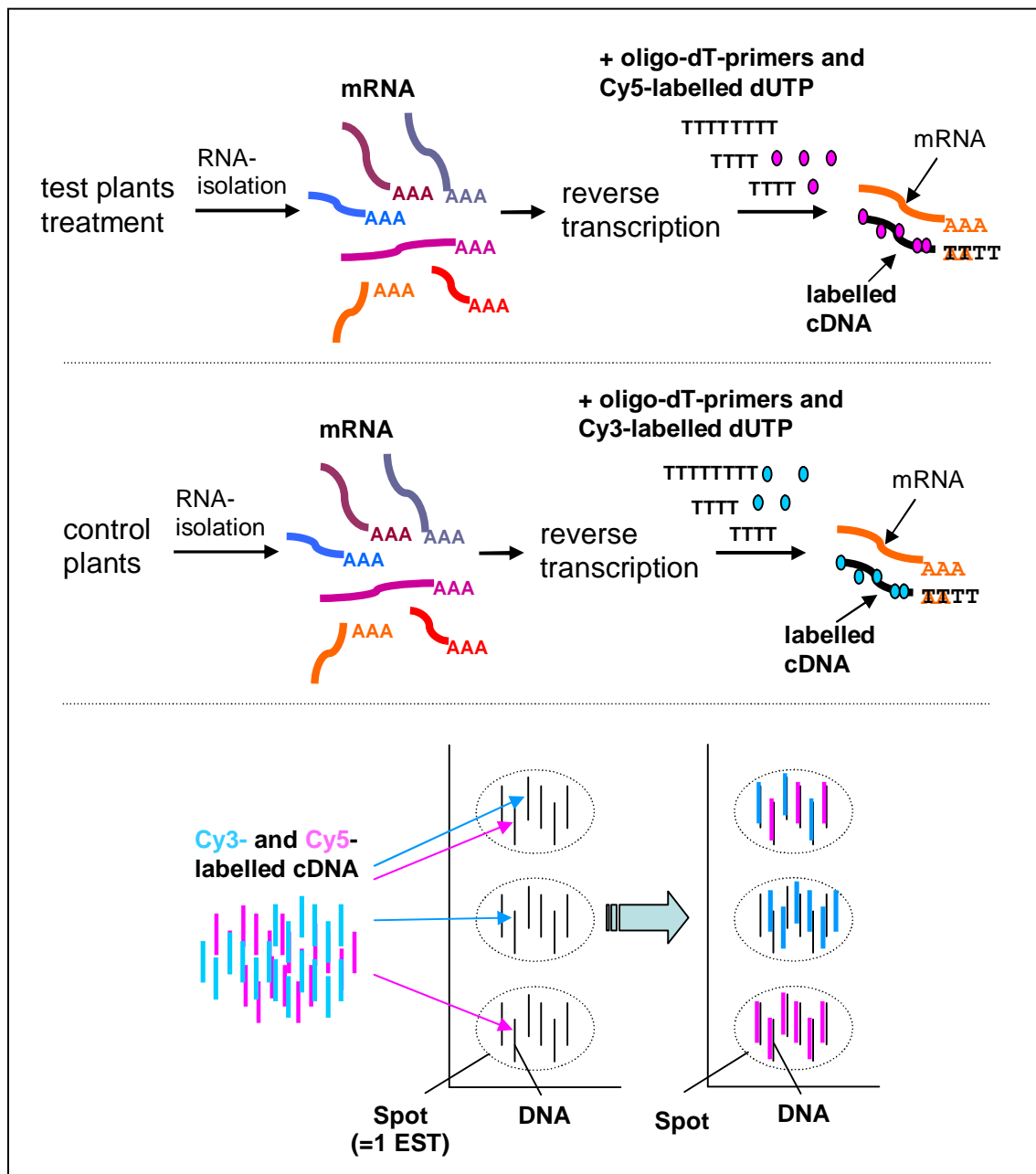


Figure III.6: Scheme demonstrating the labelling and hybridization step in microarray experiments (scheme adopted from Dr. Sabine Milde, ZBM, University of Kiel, Germany). mRNA in isolated total RNA (25  $\mu$ g) was labelled by direct incorporation of Cy3- or Cy5-labelled dUTPs (GE Healthcare, Freiburg, Germany) using oligo-dT-primers and Superscript II reverse transcriptase (both from Invitrogen, Karlsruhe, Germany). After purification and quantification of labelled cDNA, equal amounts (around 20 pmol) of control cDNA, labelled with Cy3 and treated cDNA, labelled with Cy5, were combined and pipetted on BSA-blocked microarray slides. Slides were covered and hybridized overnight at 65  $^{\circ}$ C. Depending on the presence of homologous sequences in the control and treated samples, labelled cDNA could bind to cDNA on the slides.

### III.4.3 Validation of microarray results by northern blotting

In addition, for some of these genes, microarray results were validated by performing northern blotting using the DIG-labelling system (Digoxigenin, Roche, Mannheim, Germany). Specific primer pairs for northern blotting were designed in order to produce fragments of 150 – 250 bp in length. For specificity reasons at least one primer was chosen to partly cover the 3'- untranslated region of cDNA. Primer pairs were designed using the software “Primer3” ([http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)) and applying the parameters in table III.5. Primers were checked for high specificity using the BLAST algorithm (<http://www.ncbi.nlm.nih.gov/BLAST/>) and were ordered from MWG (MWG-Biotech AG, Ebersberg, Germany).

Table III.5: Criteria for the design of primer pairs for specific *A. thaliana* genes with the software “Primer3” on [http://frodo.wi.mit.edu/cgi-bin/primer3/primer3\\_www.cgi](http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi).

<b>Parameter</b>		<b>Size</b>
Product size range		150 – 250 bp
Primer size	Minimum	15 bp
	Optimum	18 bp
	Maximum	24 bp
Melting temperature T <sub>m</sub>	Minimum	50 °C
	Optimum	60 °C
	Maximum	65 °C
	maximum difference	2 °C
GC-content	Minimum	40 %
	Maximum	60 %
Maximum complementary		3.0
Maximum 3' complementary		1.0

Sampling time points for northern blots were after 0, 4, 8, 24 and 48 hours of induction. Only whole, young and fully developed leaves were sampled by cutting, weighing and immediate deep-freezing in liquid nitrogen. All the following preparation steps were carried out in an RNase-free environment. Total RNA was extracted with the same procedure as described earlier (chapter III.4). 7.5 µg of total RNA was mixed with self-mixed loading buffer containing formamid, formaldehyd (37 %), MOPS buffer (3-(N-morpholine) propanesulfonic acid) and SYBR Green (SYBR Green I, Invitrogen, Karlsruhe, Germany), the RNA in the mixture was denaturated at 60 °C for ten minutes in a water bath and then separated in a denaturing formaldehyde-containing agarose

gel. One slot was loaded with loading buffer (Blue/Orange loading dye, 6 X, Promega, Mannheim, Germany) for control of run distance. The gel was photographed by a gel documentation system (GelDoc2000, BIO-RAD, Munich, Germany) and the positions of both start line and RNA bands were marked by a fluorescent ruler. The separated RNA was blotted, i.e. transferred to a nylon membrane (Hybond N<sup>+</sup>, Amersham, GE Healthcare, Freiburg, Germany) over night at room temperature by a capillary blot. RNA on the blot was fixed by cross-linking with 120 mJ UV-light for one minute in the UV1800 Stratalinker (Stratagene, Heidelberg, Germany). Until further use the membrane was stored shrink-wrapped in a freezer bag in the refrigerator. Membranes were hybridized with DIG-labelled probes in a formamide-containing buffer. This step was performed at 50 °C overnight (12 to 48 hours) in a rotating oven (GFL, Wunstorf, Germany). After hybridization, blots were washed thoroughly in order to remove non-bound probes before hybridization with the antibody against the bound DIG-labelled probes (Anti-DIG-AP, Fab fragments, Roche, Mannheim, Germany). Blots were visualized by chemiluminescence using CDP-Star substrate (Roche, Mannheim, Germany), which contains 1,2-dioxetane as substrate for the alkaline phosphatase to which the anti-DIG antibody was conjugated. 1,2-dioxetane is dephosphorylated by alkaline phosphatases to a meta-stable dioxetane phenolate anion which decomposes within few minutes and emits light at 466 nm. Finally blots were stored shrink-wrapped in freezer bags. For documentation and visualization of the northern blots, a chemiluminescence-sensitive film (Hyperfilm ECL, Amersham Biosciences, Freiburg, Germany) was laid on the membrane in a film cassette and exposed for five or ten minutes in a dark room. Film was developed for five minutes with developer (Roentoroll HC, Tentenol, Norderstedt, Germany), thereafter rinsed with water for one minute followed by fixation for ten minutes (Superfix MRP, Tentenol, Norderstedt, Germany).

Hybridization probes were prepared in two different ways. First, by DNA material from the array was amplified by PCR with SP6 and T7 primers using the PCR Master mix (Promega, Mannheim, Germany), then purified, eluted and quantified before a small fraction of it was labelled in another PCR reaction using DIG-labelled dNTPs (deoxynucleotide triphosphates; PCR Dig Labelling mix from Roche Diagnostics GmbH, Mannheim, Germany) and specific or universal SP6 and T7 primers.

A second way was to use RNA from treated leaf material. Total RNA was extracted and quantified as described before and subsequently reversly transcribed into cDNA by Omniscript reverse transcriptase (Omniscript RT kit, Qiagen, Hilden, Germany) and oligodT primers (Promega, Mannheim, Germany). This cDNA was quantified and gene specific cDNA fragments were raised by RT-PCR with specific primers and 40 ng RT-cDNA. Raised PCR-products were purified, eluted and quantified again before in a second PCR-reaction with DIG-labelled nucleotides (dNTPS), gene-specific primers and 500 pg cDNA, a gene-specific probe was prepared.

Quality of the probes was assessed in an agarose gel electrophoresis and quantified by comparison with 1  $\mu\text{g}$  DNA of HindIII-digested- $\lambda$ -phage (Promega GmbH, Mannheim, Germany) which was loaded to the same gel. Before use, hybridization probes had to be denaturated (first use: ten minutes at 100 °C, next use(s): ten minutes at 68 °C).

### III.5 Statistical analysis

Means and standard errors of the means (SEM) were calculated with Excel 2002. Datasets with only two means were tested for significant differences using a t-test (Microsoft Excel 2002 SP3). More complex datasets with either more than two means or more than one factor of influence were tested by one-way, two-way or three-way ANOVAs which were followed by an appropriate post-hoc test. This post-hoc test was in most cases a tukey-test (SigmaStat for Windows 3.10 Systat Software, Inc.)

## IV. Results & Discussions

### 1 First part: Characterisation of epidermal flavonoid formation at non-inducing conditions with respect to different ecotypes, developmental stages and daylength

#### 1.1 Results

This first part deals with aspects of flavonoid accumulation which circle around the main topic of this work, namely the regulation of flavonoid accumulation at low temperature conditions. Therefore it is possible to skip this part but it is anyhow worth reading it as it gives some essential background information on natural variation in epidermal flavonoid accumulation with respect to different ecotypes (chapter 1.1.1 to 1.1.4), to innerplant leafage (chapter 1.1.5) or to the choice of the photoperiod (chapter 1.1.6). This part also contains the identification of UV-A absorbing pigments by HPLC-MS (high performance liquid chromatography–mass spectrometry) in *A. thaliana* (chapter 1.1.7) and demonstrates that a certain variation in pigment content and composition existed between the ecotypes Columbia (Col) and Wassilewskija (Ws) (chapter 1.1.8 to 1.1.11). These last chapters and also chapters 1.1.1 to 1.1.4 anticipate partially results of part two, because few results to treatments with high irradiance, supplemental UV-B radiation and low temperature are included. However, this is necessary and they are presented very briefly only for the purpose to point out important differences between the two ecotypes.

##### 1.1.1 Epidermal UV-A transmittance at control conditions in the ecotypes Columbia and Wassilewskija of *A. thaliana*

Both *Arabidopsis* ecotypes, Columbia (Col) and Wassilewskija (Ws), are genetical backgrounds for mutants used in this work. They differed in growth pattern and in their reaction to changing environmental conditions. Whereas Col plants had about 12 to 14 rosette leaves until they started flowering after three to four weeks growing at control conditions, Ws plants only had about eight rosette leaves when starting flowering after two to three weeks growing at control conditions (image 1.1). Leaves of Ws appeared generally brighter green, were thinner and more even than those of Col plants.

Epidermal UV-A transmission measurements with the UV-A-PAM were performed throughout this work. The high level of UV-A transmittance before an experiment started was always checked and was essential for induction experiments. As mentioned earlier in the material section (III.1.1), control conditions, which were always applied during the time before an experiment started, were set up at two different locations. Treatment with supplemental UV-B radiation was not possible in the growth chamber, but had to be set up in a special growth cabinet, where UV-B tubes could be mounted. Therefore, two locations for “control conditions” were used. Different white light tubes





Image 1.1: Images of *A. thaliana* WT Ws (left plant) and Col (right plant) after growth at control conditions for three weeks.

resulted in slightly different spectral compositions in the chamber and in the cabinet (figure III.1). The blue light portion of the irradiance was increased in the growth cabinet in comparison to the growth chamber (table III.1). Thus, epidermal UV-A transmittance at control conditions had to be compared between the two locations.

Figure 1.1 presents epidermal UV-A transmittance values for Col and Ws plants grown in the chamber or in the cabinet at control conditions. Small, mostly non significant differences in epidermal UV-A transmittance were observed when comparing values after growth in chamber and growth in cabinet. In conclusion, despite this spectral difference, no obvious difference in transmittance between growth chamber and cabinet was observed in this experiment. However, Ws plants always displayed higher transmittance values in comparison to Col, independently of growth place. This indicates a characteristically lower content of epidermal UV-A absorbing compounds, which was also confirmed by HPLC analysis (see later in figure 1.11).

All experiments were performed by measuring the three youngest fully developed leaves at the beginning (which were routinely about the sixth leaf of the plants) and by measuring the identical leaves and the youngest, normally newly developed leaves at the end of the experiment. At 22 °C, usually about five new leaves developed on the plants, whereas at low temperature, only three new leaves developed. These youngest leaves were almost fully outgrown and were sampled for HPLC analysis after measurements of epidermal transmittance. In Col ecotypes they usually displayed decreased transmittance values in comparison to leaves which existed already at the start of the experiment (figure 1.1). This observation was surprising as experimental conditions were not changed. This was true for Col plants growing in the climate chamber as well as for those in the growth cabinet. *Arabidopsis* plants from the Ws ecotype did not show any difference in transmittance between the young leaves at the

start and young leaves at the end of the experiment. This age dependence was explored in more detail in additional experiments, where epidermal transmittance of all leaves of the investigated plants was determined. Results will be presented in chapter 1.1.5.

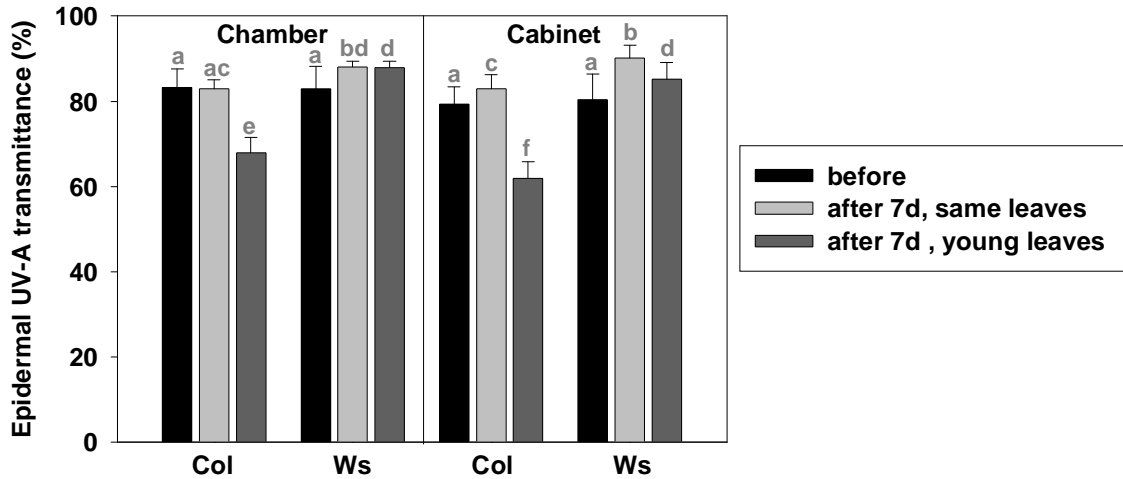


Figure 1.1: Epidermal UV-A transmittance in Col and Ws ecotypes of *A.thaliana* before and after a period of seven days at control conditions in the growth chamber (ch) and in the growth cabinet (ca). Every third column shows transmittance values of new leaves, which developed during this period ( $n = 9$  for all data points, except of ca/Col, where  $n = 18$ ). Epidermal transmittance values were tested separately for significant differences with a one-way ANOVA on ranks and Dunn's-method as post-hoc test. Significantly different values are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$  with the following exceptions: ch/Ws(day 0) vs. ch/Ws(day 7) with  $P = 0.011^*$ , ca/Col(day 0) vs. ca/Col(day 7) with  $P = 0.014^*$  and ca/Ws(day 7, same leaves) vs. ca/Ws(day 7, young leaves) with  $P = 0.002^{**}$ .

### 1.1.2 Epidermal UV-A transmittance at high light conditions in the ecotypes Columbia and Wassilewskija of *A. thaliana*

The low temperature-induced accumulation of epidermal flavonoids in comparison to the high light- and UV-B-induced accumulation was a central part of this work. Characteristics of this accumulation will be presented in detail in part two. In order to point out differences between the ecotypes Col and Ws, their response to high irradiances with respect to flavonoid accumulation will be presented now briefly. Col plants, treated with 360  $\mu\text{mol}/\text{m}^2\text{s}$  white light for seven days showed markedly and significantly decreased transmittance values, which were even lower for young and newly developed leaves ( $P < 0.001^{***}$ ). In Ws plants a similar pattern was observable, however absolute values were higher than for Col plants suggesting a weaker response (figure 1.2).

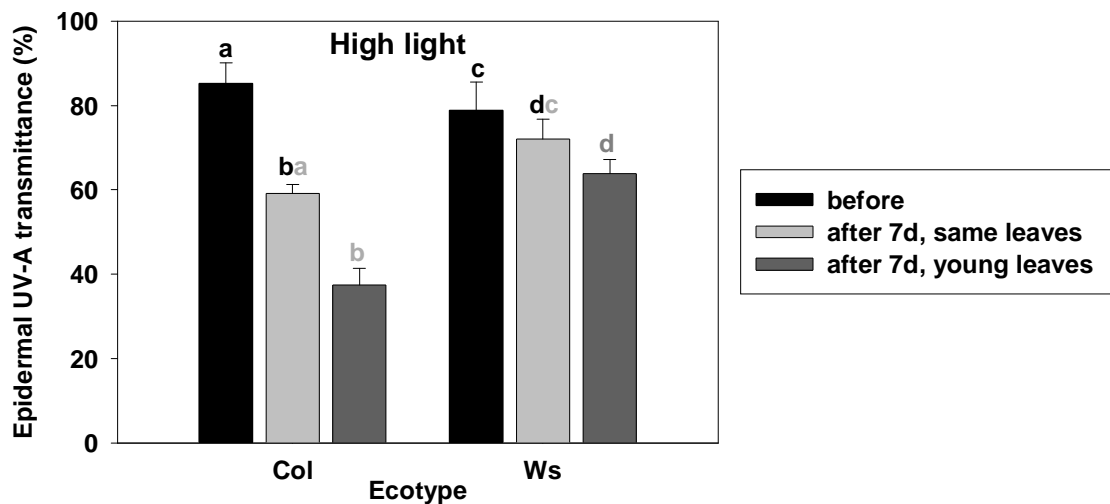


Figure 1.2: Epidermal UV-A transmittance of leaves of *A. thaliana* WT ecotypes Col and Ws after a seven day treatment with high light conditions (350  $\mu\text{mol}/\text{m}^2\text{s}$  irradiance;  $n = 9$  for all mean values). All means were tested by two-way ANOVAs and tukey-tests to be significantly different to each other. Level of significance was always high with  $P < 0.001^{***}$  with the exception of start values (“before”) for Col and Ws with  $P = 0.014^*$  and of “before” and “after 7d, same leaves” for Ws with  $P = 0.009^*$ . Significantly different values are indicated by different letters of same colour.

### 1.1.3 Epidermal UV-A transmittance at UV-B in the ecotypes Columbia and Wassilewskija of *A. thaliana*

Figure 1.3 shows the reaction of Col and Ws plants to UV-B radiation before and after seven days in leaves, which were present at start of the experiment and which developed during the experiment. Both ecotypes had strongly and highly significantly decreased transmittance values indicating a strong accumulation of flavonoids in the upper epidermis ( $P < 0.001^{***}$ ). Here, in contrast to the high light treatment, no obvious difference between the response of “young” and “old” leaves was detectable. However, a significant difference between old and young leaves at day 7 was detected for both ecotypes ( $P = 0.027^*$  for Col and  $P = 0.025^*$  for Ws). Again, significantly smaller transmittance values were observed for Col leaves in comparison to Ws leaves ( $P < 0.001^{***}$ ).

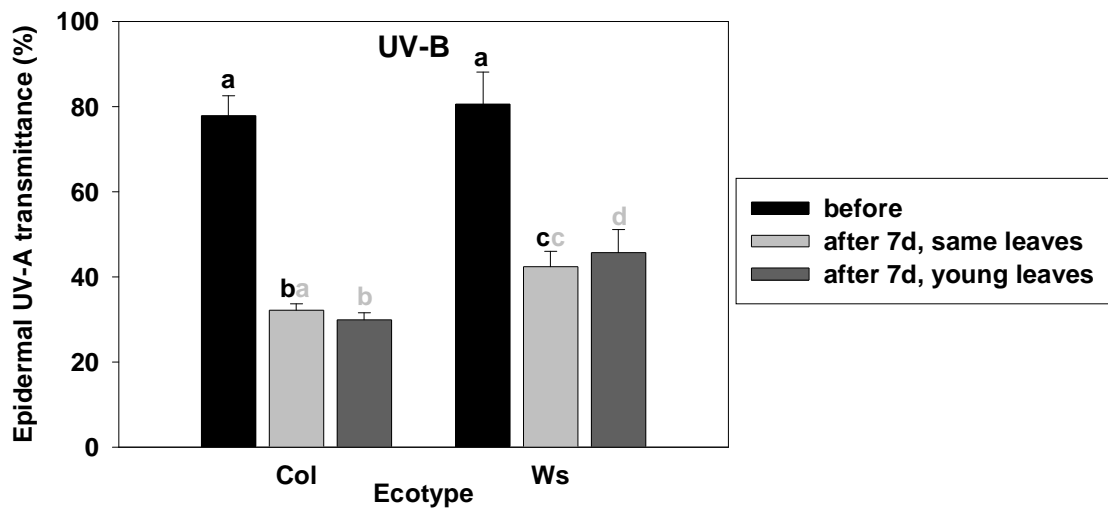


Figure 1.3: Epidermal UV-A transmittance in Col and Ws ecotypes of *A. thaliana* WT after supplemental UV-B radiation for seven days ( $n = 18$  and  $9$  for Col and Ws, respectively). Mean values were tested for significant differences using two-way ANOVAs and tukey-tests. Significantly different transmittance values are indicated by different letters of same colour and level of significance was always high with  $P < 0.001^{***}$  with two exceptions:  $P = 0.027^*$  and  $P = 0.025^*$  when comparing old and young leaves after seven days of ecotypes Col and Ws, respectively.

#### 1.1.4 Epidermal UV-A transmittance at low temperature conditions in the ecotypes Columbia and Wassilewskija of *A. thaliana*

The low temperature treatment is in focus of this work. Again, differences in the response to low temperature of Col and Ws will now be pointed out very briefly as it will be presented in detail in part two of this work. The figure 1.4 shows transmittance values for Col and Ws before and after a seven day low temperature treatment. Both ecotypes had markedly and significantly decreased transmittance values ( $P < 0.001^{***}$ ), pointing to a strong accumulation of UV-A absorbing compounds in the upper leaf epidermis. However, the reaction of Col plants was more intense, resulting in even lower transmittance values as for Ws plants ( $P < 0.001^{***}$ ). In both ecotypes youngest, newly developed leaves at the end of the experiment had significantly lower transmittance values than leaves, which were already present at the beginning of the experiment ( $P < 0.001^{***}$  for Col and  $P = 0.002^{**}$  for Ws). Results are very similar compared to the UV-B treatment (figure 1.4).

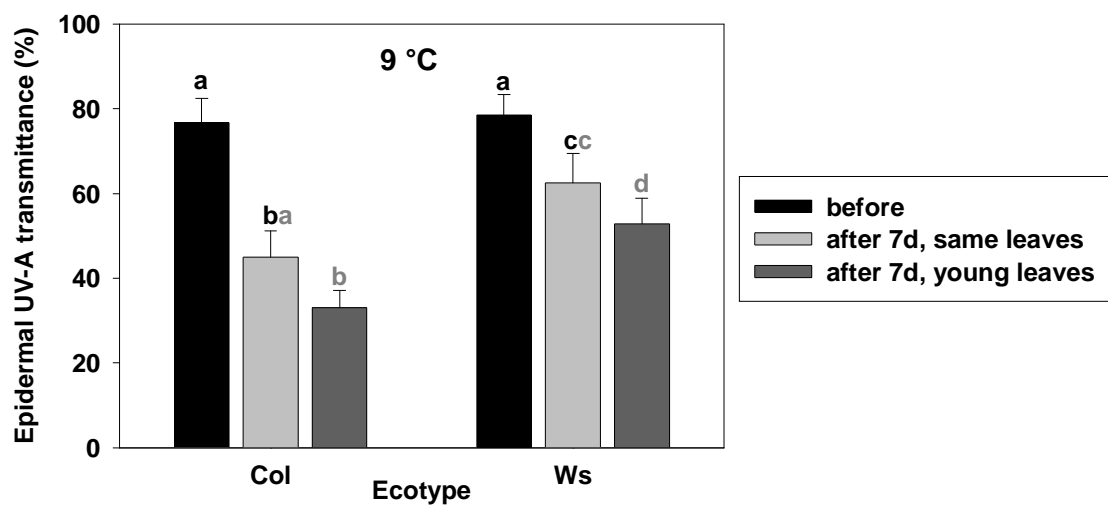


Figure 1.4: Low temperature induction of *A. thaliana* ecotypes, Col and Ws. Epidermal UV-A transmittance after seven days ( $n = 15$  and  $9$  for Col and Ws, respectively). With the exception of transmittance at start of the experiment, all values were tested to be statistically significantly different to each other, applying two-way ANOVAs and tukey-tests ( $P < 0.001^{***}$ , with the exception for Ws(day 7) vs. Ws(day 7, young leaves), where  $P = 0.002^{**}$ ).

### 1.1.5 The influence of developmental stage, i.e. leaf age on epidermal UV-A transmittance of *A. thaliana* Col

*A. thaliana* plants grow by developing rosette leaves until they start producing a flowering stem which will then be attached by small, short and lanceolate leaves. When starting experiments I had to decide which of all rosette leaves I would select for measuring and analysing epidermal UV-A transmittance. Thus, I started my work by measuring all leaves of each plant in order to get an idea about the natural and plant specific variation in epidermal UV-A transmittance. The figure 1.5 presents data from a low temperature induction experiment. *A. thaliana* plants were exposed to 9 °C or at control conditions (22 °C) for seven days and rosette leaves were measured clockwise starting at a randomly chosen rosette leaf. Transmittance values of non-induced plants ranged between 60 and 90 %, the values for low temperature-induced leaves varied extremely between 20 and 70 % (figure 1.5).

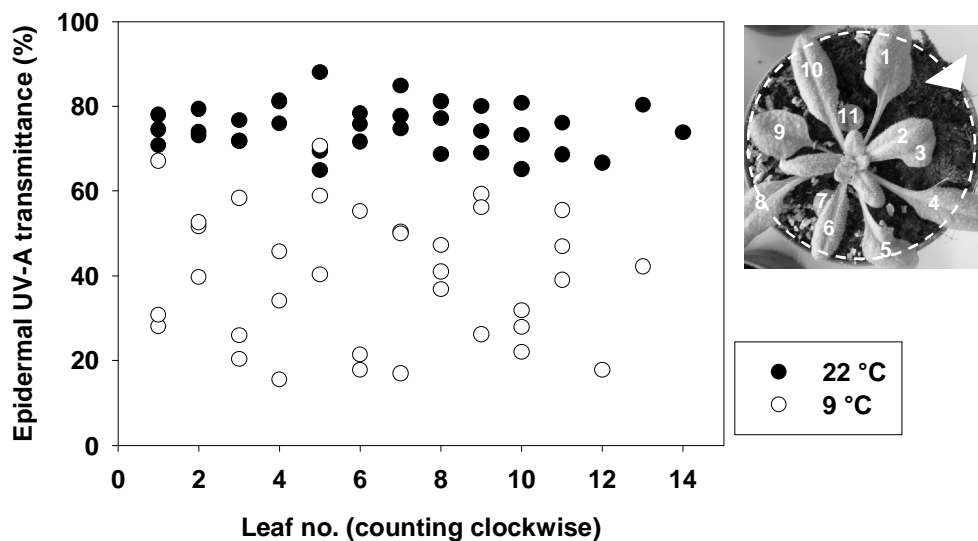


Figure 1.5: Epidermal UV-A transmittance of all rosette leaves of *A. thaliana* WT Col plants, exposed to low temperature (9 °C) and control (22 °C) conditions (n =35 for both data sets). Leaves were counted and measured clockwise starting at a randomly chosen rosette leaf. Both plants exhibited 13 and 14 rosette leaves. Datasets were statistically significant different to each other (t-test:  $P < 0.001^{***}$ ).

The figure 1.6 presents the same data set, with the difference that leaves were classified into three groups: those, which were old (class 1), those which were young (class 3), and those, which could not be assigned neither to class 1 nor to class 3 (class 2). The result was surprising: a clear relationship of epidermal transmittance with leaf age class was detected. Old leaves had under control conditions the highest transmittance and these values were only weakly but still highly significant ( $P < 0.001^{***}$ ) reduced after a low temperature treatment. Young leaves displayed lowest transmittance values under

control conditions and after low temperature treatment these leaves had the lowest transmittance values of all low temperature treated leaves.

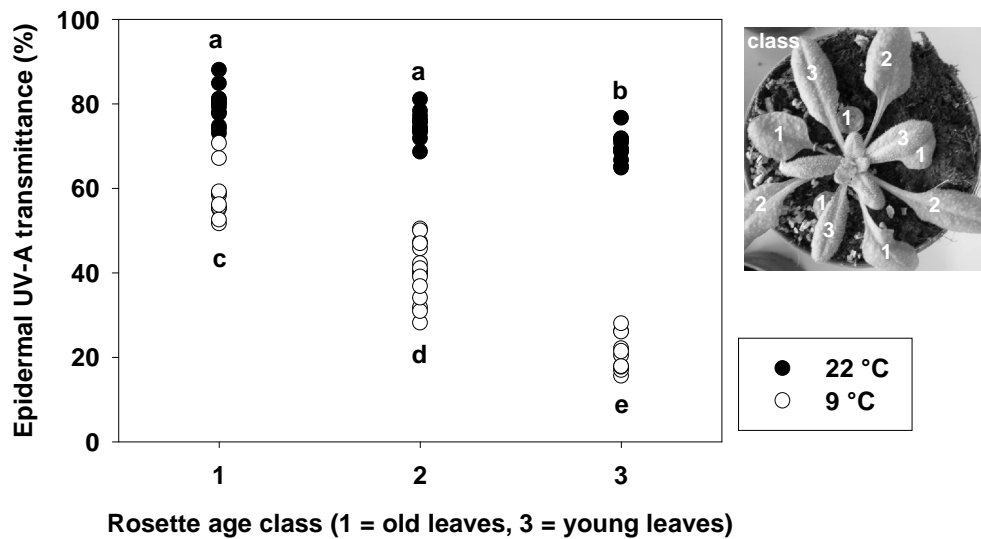


Figure 1.6: Epidermal UV-A transmittance of all rosette leaves of *A. thaliana* WT Col plants, exposed to low temperature (9 °C) and control (22 °C) conditions (n = 35 for both datasets). Leaves were classified into age classes as “old”, “young” and “middle” from their individual habitus. Data are the same as in the figure before. Statistical significant differences between individual leaf age classes and temperature were identified using a two-way ANOVA with a tukey-test as post-hoc test and are indicated by different letters. Level of significance was always high with  $P < 0.001$ \*\*\* with the following exception: class2(22 °) vs. class3(22 °C),  $P = 0.031$ \*.

A similar age dependency was found when high light was used to induce screening pigment synthesis (figure 2.1). In plants which were exposed to 360  $\mu\text{mol}/\text{m}^2\text{s}$  white light for five days a gradient in epidermal UV-A transmission was also observed. Whereas the oldest leaves had a transmission of 85 % the youngest leaves had a transmission of only 45 %. Again, with decreasing leaf age the transmission value decreased steadily.

These data show that leaf age plays a crucial role in the induction of flavonoid biosynthesis in *A. thaliana* Col. Old leaves accumulated only few UV-A absorbing compounds at inducing conditions, whereas young leaves were fully inducible. This was already observed earlier when UV-A transmittance in leaves of Col were compared with those of Ws after growth at control conditions (figure 1.1 for example). Additionally, frequently newly developed leaves started at control conditions with a low transmission value which increases with increasing age and development. Therefore new leaves are “born” with a certain UV-A-shield of about 70 % which is reduced to about 80 % if non inducing conditions continue. This is confirmed by sometimes higher transmittance

values for control plants measured after a period of seven days at the same conditions as visible in figure 1.1 and figure 1.7. During this period of time young leaves developed further and after seven days this increase in age was sometimes marked by an increase in UV-A transmittance. As a consequence, in all subsequent experiments only youngest, but fully developed leaves were sampled.

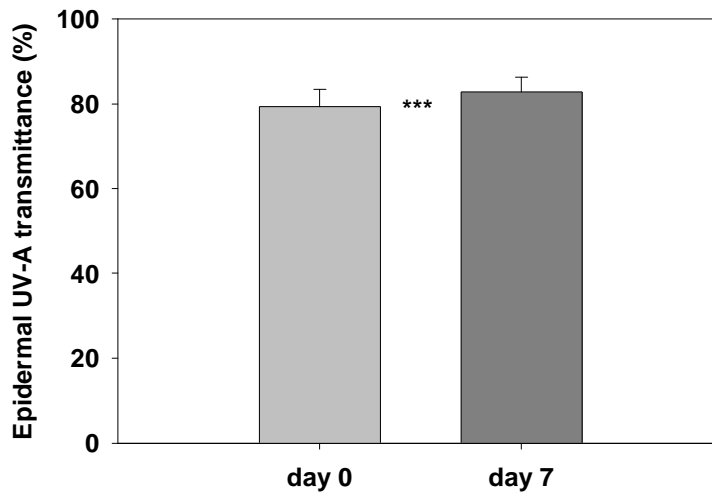


Figure 1.7: Small increase in epidermal UV-A transmittance after seven days at control conditions (n = 18, t-test:  $P < 0.001$ \*\*\*). Only same leaves were measured.

#### 1.1.6 The influence of daylength on the potential to decrease epidermal UV-A transmittance in *A. thaliana* Col

All experiments were generally done at long-day conditions with a photoperiod of 16 hours per day. In order to check any influence of a potential circadian clock on the accumulation of epidermal flavonoids at low temperature conditions, short-day experiments with a photoperiod of only 8 hours per day were performed using *A. thaliana* Col plants. These experiments were executed in the growth cabinet and lasted for seven days.

Image 1.2 presents images of *A. thaliana* Col which were treated with low temperature at short-day and a long-day conditions. Plants were equally developed but leaves of long-day plants were curled and appeared darker green.

Epidermal UV-A transmission measurements on these plants revealed that under short-day conditions nearly no low temperature-induced decrease in epidermal transmittance was observed (figure 1.8). Obviously, 8 h light per day was not enough to accumulate flavonoids at low temperature conditions. Only long-day conditions were able to induce an accumulation of these pigments.





Image 1.2: Image of an exemplary short-day-treated and long-day-treated *A. thaliana* Col at 9 °C air temperature. Treatment lasted seven days.

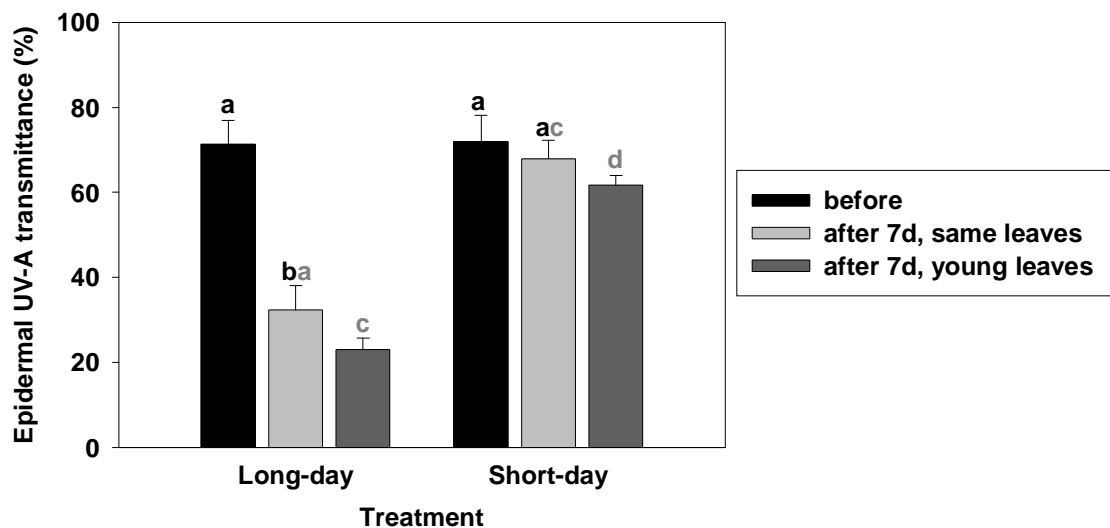


Figure 1.8: Low temperature induction of leaf epidermal UV-A transmittance in *A. thaliana* Col under long- and short-day conditions with photoperiods of 16 and 8 hours per day, respectively (n = 15 for day 0 and day 7, same leaves; n = 14 for day 7, young leaves). Mean values were tested for significant differences using two-way ANOVAs and tukey-tests as post-hoc tests. Significant different means are indicated by different letters of same colour. Level of significance was always high with  $P < 0.001^{***}$ .

An effect of the photoperiod at 9 °C air temperature could be proven. The question arose whether at 22 °C a similar effect was observable or whether a higher irradiance could compensate for a short photoperiod. The figure 1.9 combines data from three different experiments and shows the effect of short-day conditions on UV-A transmittance at low temperature and/or high PPFD in comparison to long-day conditions. Under long-day and 22 °C air temperature epidermal UV-A transmittance

was significantly decreased from about 70 % to 40 % when irradiance was raised from 120 to 300  $\mu\text{mol}/\text{m}^2\text{s}$  ( $P < 0,001^{***}$ ), which was a “normal” high light effect. At 9 °C air temperature transmittance was already 40 % at 120 $\mu\text{mol}/\text{m}^2\text{s}$  due to the temperature effect and was further significantly lowered to 30 % when irradiance was increased to 300  $\mu\text{mol}/\text{m}^2\text{s}$  (see left part of figure 1.9,  $P < 0,001^{***}$ ). Under short-day conditions and warm temperatures no significant decrease in transmittance was observed when irradiance was raised to 300  $\mu\text{mol}/\text{m}^2\text{s}$ . In contrast, low temperature alone had only a small but significant effect on transmittance ( $P < 0,001^{***}$ ). It was lowered from 70 to about 60 %. Transmittance decreased further when plants were exposed to 300  $\mu\text{mol}/\text{m}^2\text{s}$  at low temperature. The combined effect of low temperature and high irradiance on epidermal UV-A transmittance was similar for long-day- and short-day-treated plants; mean values were not statistically significant different. In summary, under short-day conditions the effect of light on flavonoid accumulation was only present at low temperature conditions and the effect of temperature was weakened when irradiance was low.

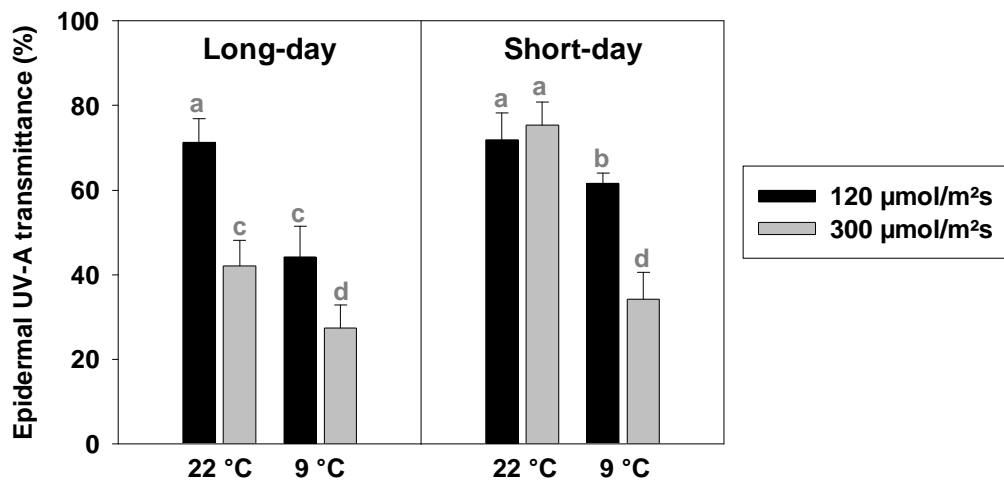


Figure 1.9: Influence of photoperiod on flavonoid induction in *A. thaliana* Col after low temperature and additional high light treatment. Data from three different experiments ( $n = 9$  for longday, 9 °C, 120 and 300  $\mu\text{mol}/\text{m}^2\text{s}$ ;  $n = 15$  for 22 °C, 120  $\mu\text{mol}/\text{m}^2\text{s}$ , long- and shortday;  $n = 27$  for shortday, 300  $\mu\text{mol}/\text{m}^2\text{s}$ , 22 and 9 °C;  $n = 14$  for shortday, 9 °C, 120  $\mu\text{mol}/\text{m}^2\text{s}$  and  $n = 18$  for longday, 22 °C, 300  $\mu\text{mol}/\text{m}^2\text{s}$ ). Differences among all values were identified using a one-way ANOVA and a tukey-test and are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$ .

### 1.1.7 Identification of flavonoids and HCAs in leaves of *A. thaliana* with HPLC-MS:

The previous chapters showed epidermal UV-A transmittance values for plants of the Ws and Col ecotype. In order to get information about the pigments which were responsible for a special value of epidermal UV-A transmittance, HPLC (high performance liquid chromatography) analysis in connection with HPLC-MS (mass spectroscopy) were performed. In Cooperation with Dr. Eva-Maria Hubbermann (Institute for Food Technology, University of Kiel, Germany) a large portion of the substances found in the HPLC chromatograms could be identified clearly. The figure 1.10 presents a typical HPLC chromatogram of a low temperature treated leaf sample. Altogether eleven different peaks were selected and analysed. In all following HPLC analyses these eleven substances were observed.

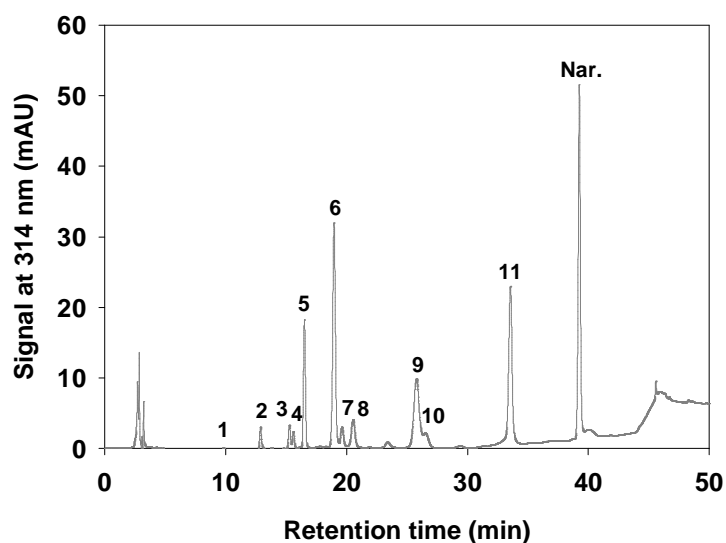


Figure 1.10: HPLC chromatogram of a low temperature treated leaf from *A. thaliana* Col, which was detected at 314 nm. The numbers indicate peaks, which were analysed further (Nar. = internal standard Naringenin).

Substances found in chromatograms were identified by HPLC-MS, absorption spectra and retention times. Identified substances were additionally compared with results from Veit and Pauli (1999) and finally, a satisfying identification of peaks was achieved (Table 1.1). Five different HCAs and each three different quercetin and kaempferol derivatives were identified. By comparing retention times of flavonols one can observe that a substance appeared earlier when more sugars were attached to the aglykon. In addition, a quercetin always appeared before a kaempferol derivative. One would expect that a higher glycosylation and hydroxylation lead to shorter retention times. This was confirmed. Table 1.1 shows a list of identified substances in the order as they appear in the chromatograms.

Table 1.1: Identification of substances via absorption spectra and HPLC analysis and molecular masses ( $m/z$ ) as well as fragmentation patterns from MS-analysis. Peak numbers refer to peaks in the chromatogram above (figure 1.10). The  $m/z$  originates from the MS-analysis. Masses of molecular fragments are placed in brackets. 5-Hydroxyferulate, sinapate, quercetin and kaempferol have masses of 210, 223, 301 and 285. Glucose, rhamnose, malate and HCl have masses of 162, 146, 116 and 37, respectively. Molecules attached to the aglykon are attached according to their pattern of fragmentation. A question mark indicates some uncertainties during identification with MS, which was partly caused through overlaying peaks (glc = glucopyranoside; rha = rhamnopyranoside, deriv. = derivative).

Peak no.	Substance (spectrum)	$m/z$	Deduced substance
1	HCA	372 (210 + 162)	5-Hydroxyferuloylglucose
2	HCA	385 (223 + 162)	Sinapoylglucose (1)
3	Quercetin glycoside	755 (301 + 2*146 + 162)	Quercetin-rha-glc-rha
4	HCA	422 (223 + 162 + 37)	Sinapoylglucose-HCl (?) (3)
5	Kaempferol glycoside	739 (285 + 2*146 + 162)	Kaempferol-rha-glc-rha
6	HCA	339 (223 + 116)	Sinapoylmalate
7	HCA	608 (2*223 + 162)	Disinapoylglucose (2)
8	Quercetin glycoside	none (301 + 162 + 146 = 609)	Quercetin-glc-rha (?)
9	Kaempferol glycoside	593 (285 + 162 + 146)	Kaempferol-glc-rha
10	Quercetin derivative	none (301 + 2*146 = 593)	Quercetin deriv. (-rha-rha ?) (3)
11	Kaempferol glycoside	577 (285 + 2*146)	Kaempferol-rha-rha

In total, eleven distinct substances were found regularly in the chromatograms, of which five were HCA derivatives, three were kaempferol derivatives and three were quercetin derivatives. Peak no. 6, sinapoylmalate was found as a main compound in all leaf extracts.

#### 1.1.8 Composition of UV-A absorbing pigments at control conditions in rosette leaves of ecotypes Col and Ws of *A. thaliana*

Returning to the growth of Col and Ws plants at control conditions in the chamber or in the cabinet, HPLC analysis was performed on young leaves of both ecotypes after growth for seven days at control conditions (figure 1.11). At control conditions leaf extracts from Col plants contained significantly more pigment per leaf area than those of Ws plants (t-test:  $P < 0.001^{***}$ ). In addition, the small decrease in transmittance visible for Col plants in the growth cabinet (figure 1.1) became more obvious in results from HPLC analysis. Statistically significantly more pigment was detected in Col leaves grown in the cabinet in comparison to those grown in the chamber (t-test:  $P = 0.020^*$ ). Leaves of Ws plants also displayed slightly reduced transmittance values and increased pigment content after growth in the cabinet, but these differences were found not to be statistically significant. The observed increase in Col plants was due to significantly enhanced amounts of both, HCAs and kaempferol glycosides (two-way ANOVAs and

tukey-tests with  $P = 0,043^*$  for HCAs and  $P = 0,008^{**}$  for kaempferol glycosides). Small amounts of quercetin glycosides were only detected in leaves of the Ws ecotype.

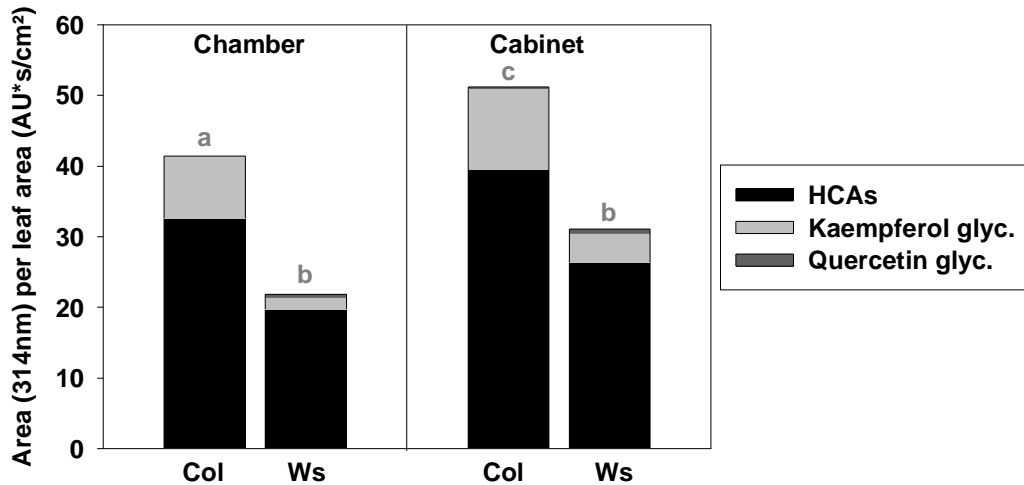


Figure 1.11: Composition of main UV-A absorbing compounds per leaf area as measured with HPLC in young leaves of *A. thaliana* ecotypes Col and Ws after seven days at control conditions in the growth chamber (ch) and in the growth cabinet (ca,  $n = 8$  and  $15$  for Col in chamber and cabinet, respectively;  $n = 7$  and  $4$  for Ws in chamber and cabinet, respectively). Differences between total pigments were tested for significance using a two-way ANOVA and a tukey-test as post-hoc test. Level of significance was high with  $P < 0.001^{***}$  with the exception of ch/Col vs. ca/Col with  $P = 0.020^*$  (glyc. = glycosides)

As mentioned before, statistically significantly increased amounts of HCAs were found in Col plants in comparison to Ws plants. The same observation was made when Col plants were grown in the growth cabinet in comparison to the climate chamber (figure 1.12). This difference in the total amount of HCAs between the ecotypes was due to an increase in sinapoylmalate (only significant for the chamber with  $P = 0.023^*$ ), sinapoylglucose (1) ( $P < 0.001^{***}$ , not shown) and 5-OH-feruloylglucose ( $P = 0.005^{**}$  for chamber and  $P < 0.001^{***}$  for cabinet, not shown) in Col plants. In Ws plants feruloylglucose was nearly missing. The difference between climate chamber and growth cabinet was primarily due to a significant increase in sinapoylglucose (1) in Col plants ( $P < 0.001^{***}$ ) and a non-significant increase in sinapoylmalate in Ws plants, when grown in the growth cabinet (figure 1.12).

An increased content of flavonoids was also observed in plants grown in the cabinet in comparison to the growth chamber (figure 1.13), which was significant only for Col plants ( $P = 0.007^{**}$ ). Col plants in the cabinet displayed increased values for all three kaempferol glycosides and an additional quercetin derivative (quercetin rhamnopyranosyl- glucopyranoside-rhamnopyranoside) appeared. In comparison, Ws plants contained only a small fraction of total flavonoids. Grown in the climate chamber, Ws leaves contained small amounts of all three kaempferol glycosides and

also of two quercetin glycosides. In the growth cabinet Ws leaves contained increased amounts kaempferol glycosides and the quercetin-rha-glc was increased as well. Surprisingly, the third quercetin derivative, was not present in Ws leaves grown in the growth cabinet.

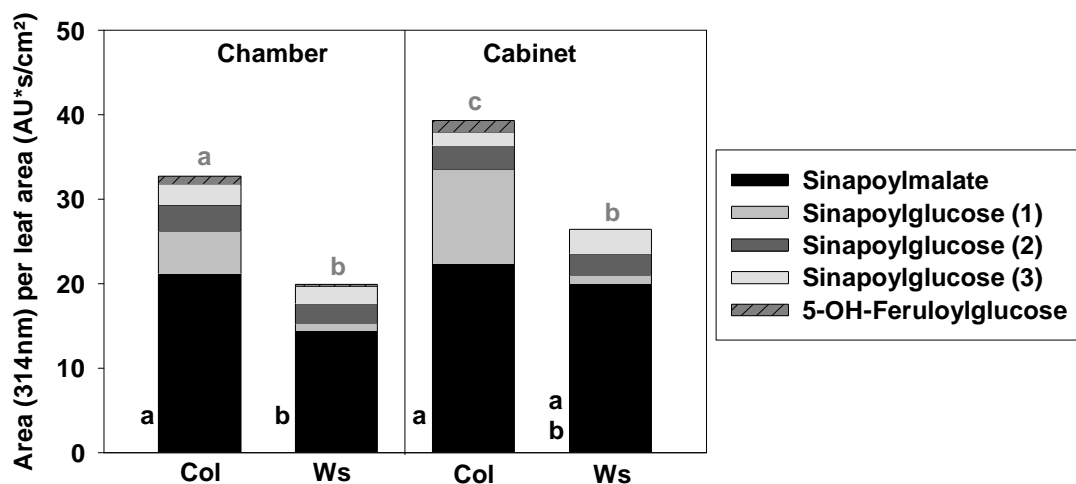


Figure 1.12: Composition of HCAs in rosette leaves of two different ecotypes of *A. thaliana* after seven days at control conditions in the growth chamber (ch) and in the growth cabinet (ca, n = 8 and 15 for Col in chamber and cabinet, respectively; n = 7 and 4 for Ws in chamber and cabinet, respectively). Total amounts of HCAs and individual amounts of several other HCAs were tested for significant differences using a two-way ANOVA and a tukey-test as post-hoc-test. Significant different values for total HCAs and sinapoylmalate are indicated by different letters of same colour. Levels of significance for total HCAs were as follows: ch/Col vs. ca/Col,  $P = 0.043^*$ , ch/Col vs. ch/Ws,  $P = 0.002^{**}$  and ca/Col vs. ca/Ws,  $P = 0.004^{**}$ . Level of significance for sinapoylmalate for ch/Col vs. ch/Ws was  $P = 0.023^*$ .

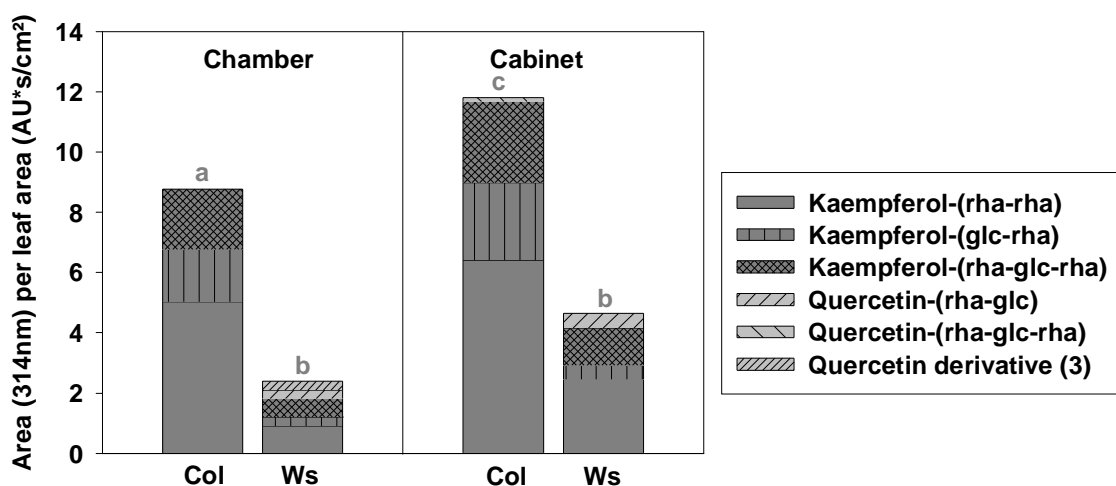


Figure 1.13: Composition of flavonol derivatives in rosette leaves of two different ecotypes of *A. thaliana* after seven days at control conditions in the growth chamber (ch) and in the growth cabinet (ca, n = 8 and 15 for Col in chamber and cabinet, respectively; n = 7 and 4 for Ws in

chamber and cabinet, respectively). Significant different values for total flavonoids were tested by a two-way ANOVA and a tukey test and are indicated by different letters. Level of significance was very high with  $P < 0.001^{***}$  for ch/Col vs. ch/Ws and was high with  $P = 0.007^{**}$  for ch/Col vs. ca/Col.

### 1.1.9 Composition of UV-A absorbing pigments after high light treatment in rosette leaves of ecotypes Col and Ws of *A. thaliana*

Rosette leaves of Col and Ws plants which were exposed to high light conditions for seven days were also analysed for pigment composition by HPLC. As transmittance measurements suggested (figure 1.2), the amount of UV-A absorbing compounds was significantly increased in Col plants in comparison to Ws plants (t-test:  $P = 0.003^{**}$ ). This increase could be explained by a higher amount of HCAs and kaempferol derivatives as shown in the figure 1.14.

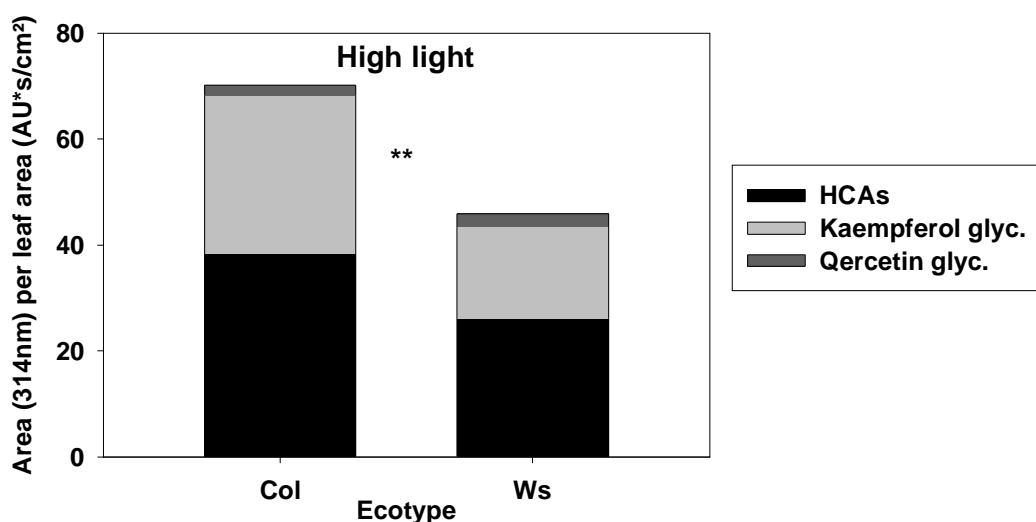


Figure 1.14: Main UV-A absorbing pigment composition after high light treatment in young rosette leaves of *A. thaliana* WT, ecotypes Col and Ws ( $n = 6$  and  $8$  for Col and Ws, respectively). The total pigment was significantly increased in Col plants in comparison to Ws plants (t-test:  $P = 0.003^{**}$ ). After high light treatment Col plants accumulated more HCAs and kaempferol glycosides (t-tests:  $P = 0.017^*$  for HCAs and  $P < 0.001^{***}$  for kaempferol glycosides), which resulted in a higher amount of total pigments.

The composition of HCAs after high light treatment varied among ecotypes (figure 1.15). The total fraction of HCAs was significantly smaller in Ws plants than in Col plants (t-test:  $P = 0.017^*$ ). A large fraction of sinapoylglucose (1) was observable in Col plants, whereas in Ws plants this HCA was only present in few amounts (t-test:  $P < 0.001^{***}$ ). The amount of sinapoylglucose (1) in Col was even higher than the amount of sinapoylmalate, which was generally the main compound in *A. thaliana*. However, the amounts of sinapoylglucose (1) and sinapoylmalate in Col were tested to be not

statistically significant different (t-test:  $P = 0.286$ , ns). The sinapic acid precursor 5-OH-ferulic acid, present in *A. thaliana* as 5-OH-feruloylglucose, was present in Col plants in respectable amounts, but in Ws plants this HCA was nearly completely missing.

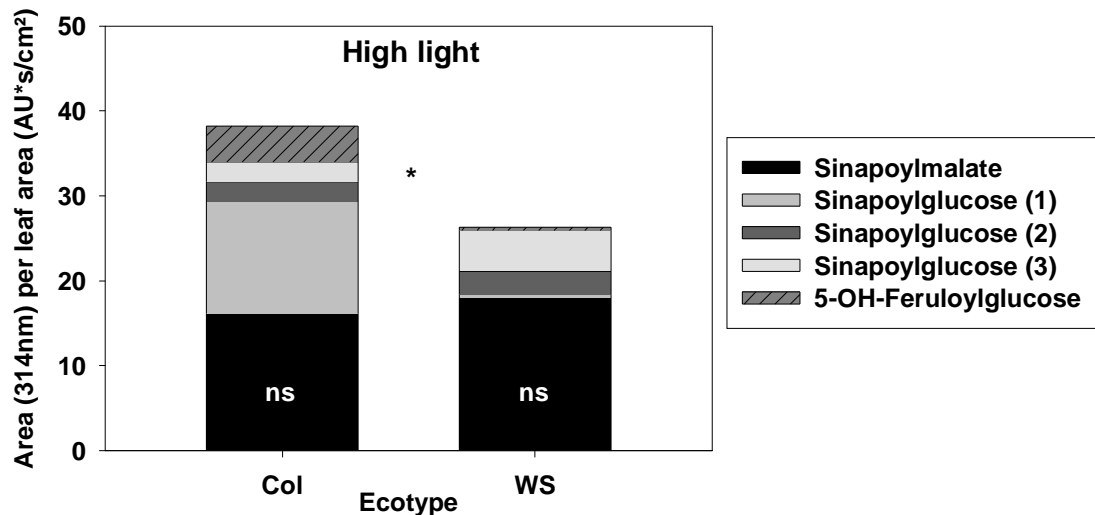


Figure 1.15: Composition of HCAs in young leaves of *A. thaliana* WT ecotypes Col and Ws after a seven day high light treatment ( $350 \mu\text{mol}/\text{m}^2\text{s}$  irradiance,  $n = 6$  and  $8$  for Col and Ws, respectively). Total amounts of HCAs and the amount of individual HCAs were tested for significant differences using t-tests. Col leaves contained significantly more HCAs than Ws leaves ( $P = 0.017^*$ ) and the composition varied between ecotypes, though all five derivatives were present in leaf extracts of both ecotypes. The amount of sinapoylmalate was not statistically significant different ( $P = 0.446$ , ns). Sinapoylglucose (1) was significantly increased ( $P < 0.001^{***}$ , not shown) and sinapoylglucose (3) was significantly reduced ( $P = 0.003^{**}$ , not shown) in Col in comparison to Ws.

Not only HCAs were increased in leaves of Col plants in comparison to Ws plants, but also flavonoid content was significantly increased (t-test:  $P = 0.001^{**}$ ). With exception of kaempferol rhamnopyranosyl- glucopyranoside-rhamnopyranoside, the amount of all kaempferol glycosides was largely increased (figure 1.16). The amount of total kaempferol derivatives was significantly higher in Col (t-test:  $P < 0.001^{***}$ ). Though total quercetin glycosides were found to be not significantly different (t-test:  $P = 0.588$ , ns), it is remarkable that all three quercetin glycosides were present in Col plants in small amounts, whereas in Ws plants only quercetin-rha-glc was accumulated to a higher amount.



### 1.1.10 Composition of UV-A absorbing pigments after UV-B treatment in rosette leaves of ecotypes Col and Ws of *A. thaliana*

Similar to results obtained after a high light treatment, the overall amount of UV-A absorbing pigments was significantly increased in Col plants in comparison to Ws plants (t-test:  $P = 0.009^{**}$ , figure 1.17). This effect was due to a higher fraction of quercetin glycosides in the Col ecotype (t-test:  $P < 0.001^{***}$ ). The amount of kaempferol glycosides and HCAs was nearly identical (t-tests:  $P = 0.702$ , ns for kaempferol glycosides and  $P = 0.055$ , ns for HCAs).

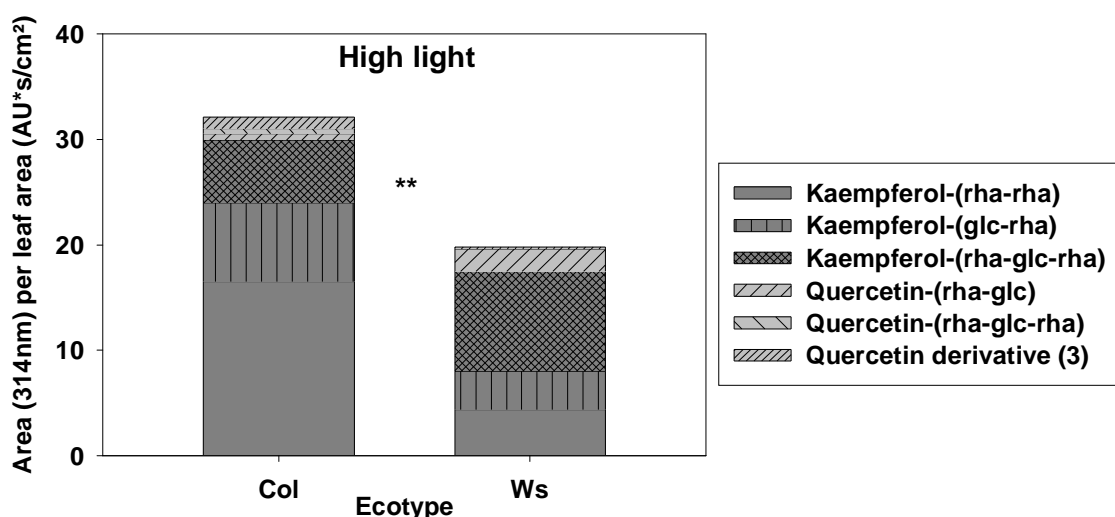


Figure 1.16: Flavonol composition after a seven day high light treatment ( $350 \mu\text{mol}/\text{m}^2\text{s}$  irradiance) in Col and Ws ecotypes of *A. thaliana* WT ( $n = 6$  and  $8$  for Col and Ws, respectively). The amount of total flavonoids and of individual flavonols were tested for significant differences using t-tests. A significantly higher flavonol content was detected in Col leaves than in Ws leaves ( $P = 0.001^{**}$ ), which was primarily due to a higher kaempferol content ( $P < 0.001^{***}$ , not shown). The total amount of quercetin derivatives was similar in both ecotypes ( $P = 0.588$ , ns, not shown).

As mentioned before, the amount of HCAs after UV-B treatment was nearly identical for both ecotypes. However, the composition of HCAs varied (figure 1.18). The amount of sinapoylglucose (1) was threefold higher in Col than in Ws (t-test:  $P < 0.001^{***}$ ). This was also detected at control conditions (figure 1.12) and after a high light treatment (figure 1.15). Sinapoylglucose (3) was significantly reduced in Col plants in comparison to Ws plants (t-test:  $P < 0.001^{***}$ ), an observation which was made also after a high light treatment. In both ecotypes 5-OH-feruloylglucose was present in similar amounts. This HCA was found earlier only in Col plants grown at control conditions or after treated with high irradiance.

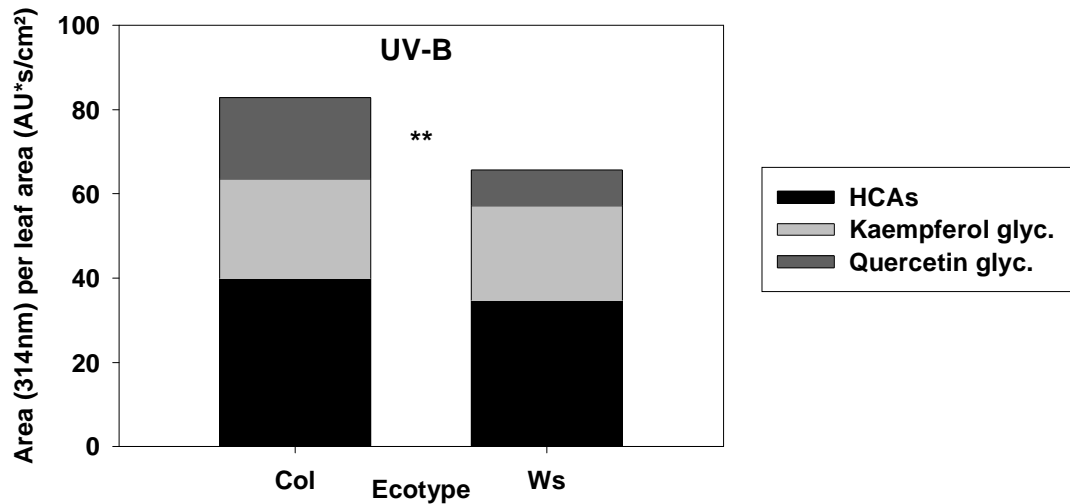


Figure 1.17: Main pigment composition in young rosette leaves of *A. thaliana* ecotypes Col and Ws after seven day treatment with supplemental UV-B radiation as analysed by HPLC (n = 18 and 6 for Col and Ws, respectively). Col plants contained significantly more UV-A absorbing pigments (t-test:  $P = 0.009^{**}$ ) primarily due to higher amounts of quercetin glycosides (t-test:  $P < 0.001^{***}$ ).

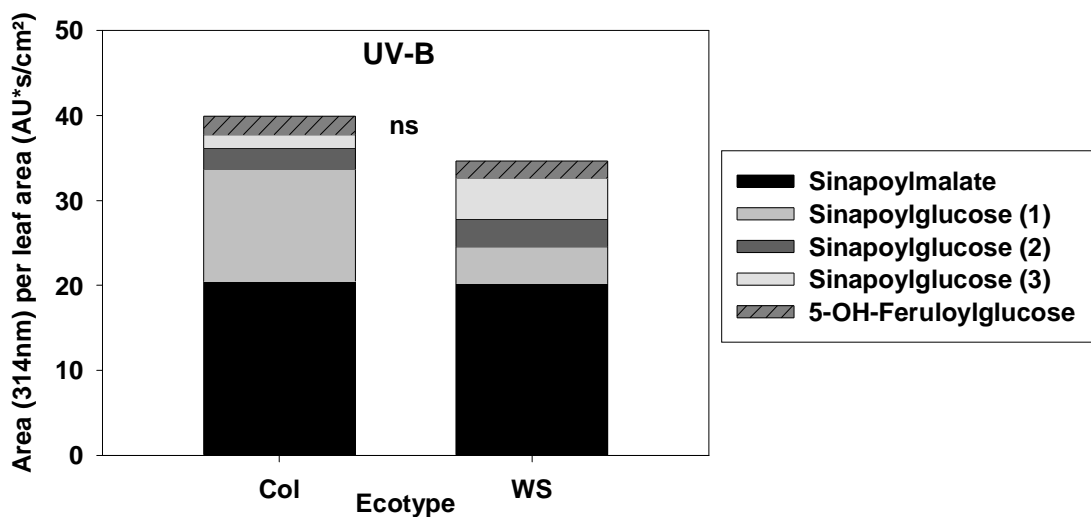


Figure 1.18: Composition of HCAs in leaves of *A. thaliana* WT, ecotypes Col and Ws, after a seven day UV-B treatment (n = 18 and 6 for Col and Ws, respectively). The total amount of HCAs was slightly but non-significantly (ns) higher in Col plants than in Ws plants (t-test:  $P = 0.055$ , ns). The composition varied among the ecotypes in a way that the amount of sinapoylglucose (1) was largely and significantly increased and sinapoylglucose (3) was significantly reduced in Col leaves in comparison to Ws leaves (t-test:  $P < 0.001^{***}$  for both).

In comparison to Ws plants, the amount of kaempferol glycosides was not significantly increased in Col plants (t-test:  $P = 0.702$ , ns, figure 1.19). However, the amount of quercetin glycosides was more than doubled in Col extracts due to a large amount of the quercetin derivative (3) (t-test:  $P < 0.001^{***}$  for both, total quercetin glycosides and quercetin derivative (3)). In both ecotypes all three kaempferol glycosides and quercetin glycosides were present. The fraction of quercetin glycosides was about 30 and 50 % of all flavonols in Ws and Col leaves, respectively.

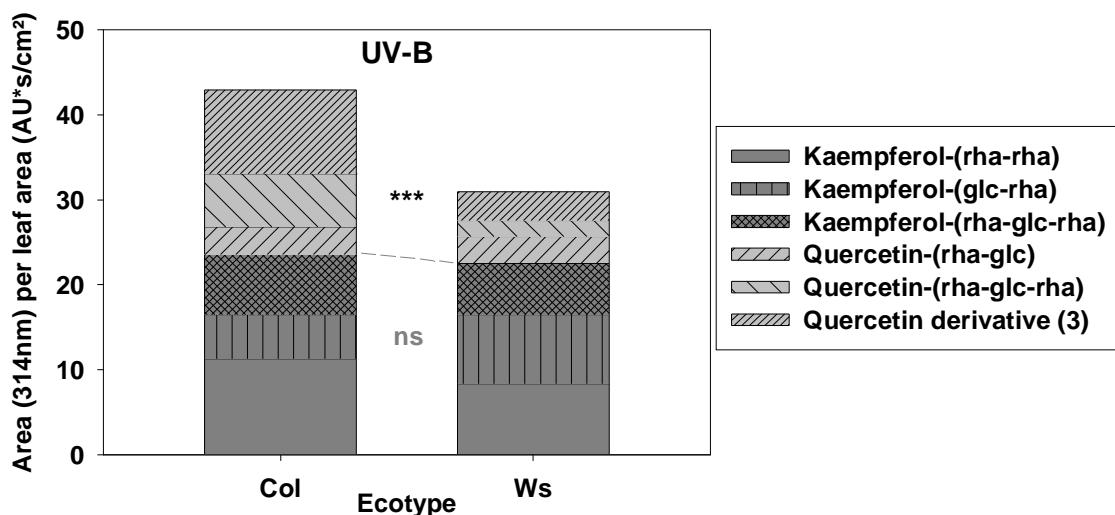


Figure 1.19: Flavonol composition in young rosette leaves of *A. thaliana* WT Col and Ws after UV-B treatment ( $n = 18$  and  $6$  for Col and Ws, respectively). Col leaves contained significantly more flavonol glycosides than Ws leaves (t-test:  $P = 0.003^{**}$ , not shown). The amount of total kaempferol derivatives was similar and not statistically significantly different for both ecotypes (t-test:  $P = 0.702$ , ns). Quercetin glycosides were present in high amounts in both ecotypes, and Col plants accumulated double the amount of quercetin glycosides than Ws plants after radiation with UV-B (t-test:  $P < 0.001^{***}$ ). The fraction of total quercetin glycosides of all flavonols was about 50 and 30 % in Col and Ws ecotypes, respectively.

#### 1.1.11 Composition of UV-A absorbing pigments after low temperature treatment in rosette leaves of ecotypes Col and Ws of *A. thaliana*

Plants which were exposed to low temperature conditions for seven days were also analysed by HPLC for pigment composition. HPLC analysis showed again a significantly higher amount of UV-A absorbing pigments in Col leaves than in Ws leaves (t-test:  $P < 0.001^{***}$ , figure 1.20). A statistically significant increase in all three fractions, HCAs, kaempferol glycosides and to a small amount quercetin glycosides explained this difference (t-tests:  $P = 0.003^{**}$  for HCAs,  $P < 0.001^{***}$  for kaempferol glycosides and  $P = 0.012^*$  for quercetin glycosides).

Differences in the composition of HCAs between both ecotypes were found primarily in a significantly increased amount of sinapoylmalate (t-test:  $P < 0.001^{***}$ , figure 1.21). Surprisingly, no or very few feruloylglucose was present in neither Col nor Ws plants. Until so far, this substance was present at least in Col leaves. Differences in peak areas per leaf area for the other three HCA derivatives were only small but still statistically significant (t-tests:  $P = 0.013^*$ ,  $P < 0.001^{***}$  and  $P = 0.003^{**}$  for sinapoylglucose (1), (2) and (3), respectively). Whereas sinapoylglucose (1) and (2) displayed increased amounts in comparison to Ws, sinapoylglucose (3) was reduced in Col. After treatment with high irradiances or UV-B radiation it was also observed that increased amounts were detected for sinapoylglucose (1) and reduced amounts for sinapoylglucose (3).

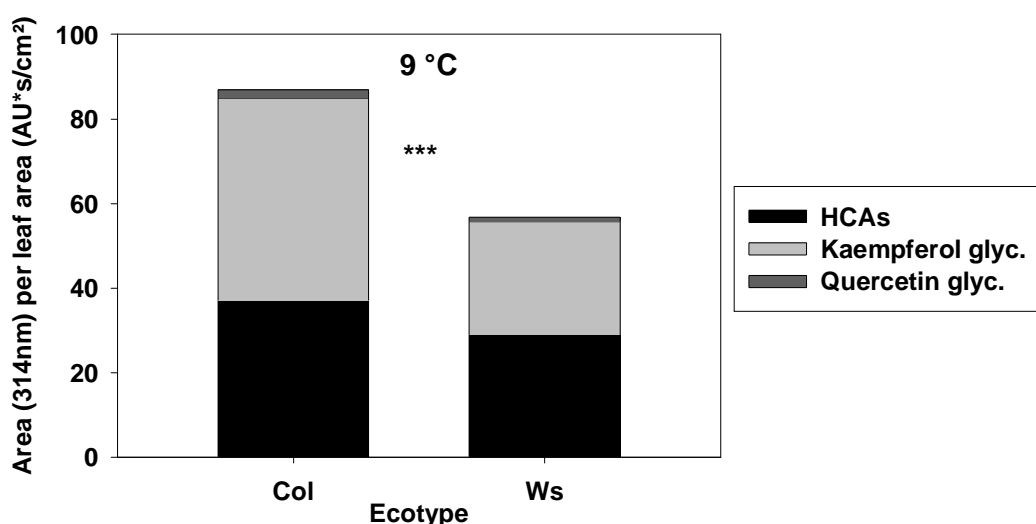


Figure 1.20: Low temperature induction of different ecotypes from *A. thaliana* WT. Main UV-A absorbing pigment composition after seven days at low temperature condition in young rosette leaves ( $n = 8$  for Col and Ws). Col leaves contained significantly more HCAs, kaempferol glycosides and quercetin glycosides in comparison to Ws leaves (t-tests:  $P = 0.003^{**}$  for HCAs,  $P < 0.001^{***}$  for kaempferol glycosides and  $P = 0.012^*$  for quercetin glycosides). Thus, the total amount of pigment in Col plants was also significantly increased in comparison to Ws plants (t-test:  $P < 0.001^{***}$ ).

Low temperature treated Col leaves contained significantly more kaempferol glycosides and quercetin glycosides than Ws leaves (t-tests:  $P < 0.001^{***}$  for kaempferol glycosides and  $P = 0.012^*$  for quercetin glycosides, figure 1.22). All three kaempferol glycosides were increased extremely and statistically significantly in Col leaf extracts in comparison to extracts from Ws leaves (t-tests:  $P < 0.001^{***}$  for kaemp-rha-rha and -glc-rha-,  $P = 0.004^{**}$  for kaemp-rha-glc-rha). The quercetin fraction was small for both ecotypes and differences in the composition were found. Whereas Ws plants only contained one quercetin derivative (quercetin-rha-glc), all three quercetin glycosides were present in small amounts in Col plants. The amount of quercetin glycosides and kaempferol glycosides in Col was nearly double the amount in Ws plants.

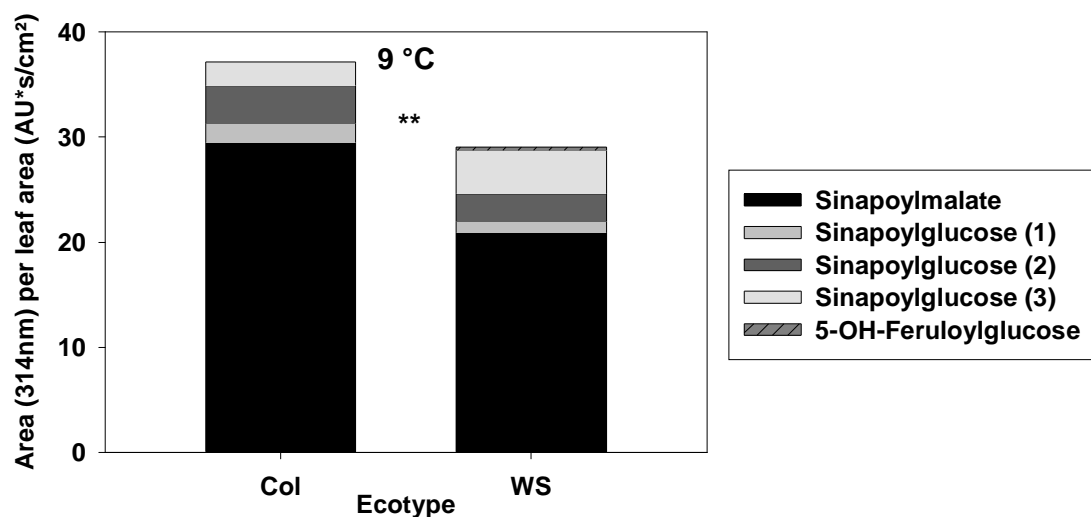


Figure 1.21: Composition of HCAs in young, low temperature treated leaves of *A. thaliana* WT Col and Ws plants ( $n = 8$  for Col and Ws). Total amount of HCAs was significantly increased in Col plants in comparison to Ws plants (t-test:  $P = 0.003^{**}$ ). This increase was mainly due to a significantly higher amount of sinapoylmalate (t-test:  $P < 0.001^{***}$ , not shown), but all three sinapoylglucoses were also significantly increased in Col plants (t-tests:  $P = 0.013^*$  for sinapoylglucose (1),  $P < 0.001^{***}$  for (2) and  $P = 0.003^{**}$  for (3), not shown).

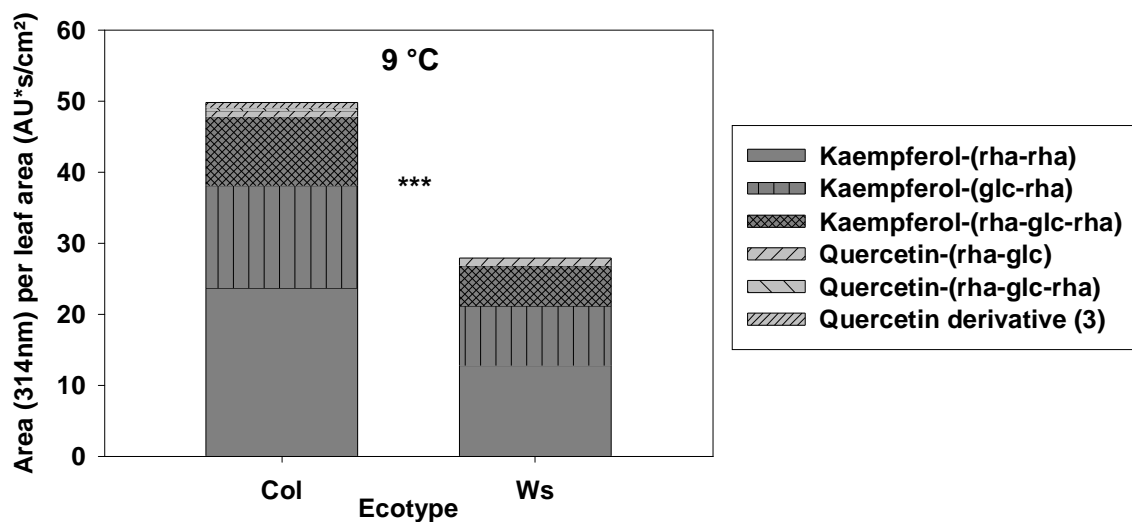


Figure 1.22: Composition of flavonol glycosides after a seven day low temperature treatment in young leaves of *A. thaliana* WT Col and Ws ( $n = 8$  for Col and Ws). Col plants contained significantly more flavonols than Ws plants (t-test:  $P < 0.001^{***}$ ) due to significantly higher amounts of kaempferol and quercetin derivatives (t-tests:  $P < 0.001^{***}$  for kaempferol glycosides and  $P = 0.012^*$  for quercetin glycosides).

## 1.2 Discussion (First part)

*The ecotype Ws of Arabidopsis exhibited intrinsically reduced amounts of UV-A absorbing compounds and fewer rosette leaves in comparison to the Col ecotype*

Most experiments in this work were performed with *A. thaliana* WT, ecotype Col. However, mutants were also used in this work which were in genetical background of the ecotype Ws, so that this ecotype had to be compared with Col with respect to epidermal flavonoid accumulation. In summary, results could demonstrate that under all circumstances epidermal UV-A absorbance and the amount of total pigments, total HCAs and total kaempferol glycosides were always and mostly significantly reduced in Ws plants in comparison to Col plants. Quercetin derivatives were reduced in the Ws ecotype after induction experiments with supplemental UV-B radiation or low temperature but not at control conditions or after treatment with high irradiances. Pigment composition was not changed markedly. In conclusion, it can be stated firstly, that the epidermal UV-A shield in rosette leaves of the Ws ecotype was always reduced in comparison to Col leaves independently of environmental condition and that secondly, Ws plants displayed a reduced sensitivity to changing environmental conditions in comparison to Col plants with respect to the accumulation of epidermal flavonoids. Epidermal UV-A transmittance was reduced by 56 to 62 % of control level in Col leaves treated with either high light, low temperature or supplemental UV-B radiation. In contrast, transmittance of Ws leaves was reduced by only 19 to 43 % of control levels (data from figures 1.3 to 1.5). This result is not surprising as natural variation in the reaction to an abiotic factor among different ecotypes of *Arabidopsis* is not unexpected and was detected before (see review of Tonsor et al. 2005). Torabinejad and Caldwell (2000) investigated seven different european ecotypes of *Arabidopsis* for their UV-B tolerance and concluded that different levels of UV-absorbing compounds might be the contributors to the observed ecotypic variation in UV-B tolerance.

An assessment of the molecular diversity of the 5' flanking region of of the key enzyme of flavonoid biosynthesis CHS, which contains the CHS promotor, revealed single nucleotide polymorphisms (SNP) among 28 different ecotypes from all over the world and also between Col (origin: USA) and Ws (origin: Russia). This polymorphism was found to be also linked to functional variation of the CHS promotor (de Meaux et al. 2005). Thus, the reduced level of flavonoids in the Ws ecotype could also be the result of a differential and less intensive gene expression of the CHS gene.

*Leaf and plant age dependency of epidermal UV-A transmittance at control, low temperature and high light conditions*

Some data were presented on the influence of leaf age on epidermal UV-A transmittance at control, low temperature and high light conditions. Two aspects became clear: firstly, at every timepoint, independently on the environmental condition,

a leaf age dependent gradient in transmittance existed, meaning that old leaves had higher transmittance values in comparison to young leaves. This gradient was even steeper according to the severeness of the environmental condition, in which the plant was growing. Secondly, at constant control conditions every single leaf displayed a slight increase in transmittance over time. “Born” with a certain UV-A shield, this shield decreased slowly from day to day, if certain conditions prolonged. A third aspect was observed, that on the other hand, young, non-induced *A. thaliana* Col leaves at the end of experiments exhibited a lower transmittance value than young leaves at the start of the experiment. Both were classified as “young” at timepoint of measurement, but a difference in transmittance was observed between “young, day 0” and “young, day 7”, suggesting that the initial UV-A shield of newly developed leaves increased with increasing plant age as well. Surprisingly, this phenomenon was only observed in the Col ecotype and did not appear in Ws plants. In conclusion, I can summarize, that at control conditions at every timepoint a leaf age dependent gradient in epidermal UV-A transmittance existed and that this gradient persisted during growth, resulting in even lower transmittance values for new leaves. The initial UV-A shield increased with every new leaf. During ageing every leaf “lost” a certain fraction of its initial UV-A shield down to a low level of epidermal UV-A absorbance, if control conditions prolonged, in a manner that the leaf age dependent gradient persisted. A leaf age dependency of flavonoid accumulation in *Arabidopsis* with or without UV-B irradiation was also investigated earlier (Lois 1994). A leaf age dependent gradient in flavonoid content, indicated as absorption values at 330 nm of ethanolic extracts per mg tissue, before and after irradiation with UV-B was also observed as a time dependent decrease in flavonoid content of every single leaf pair (first pair = leaf 1 + leaf 2, second pair = leaf 3 + leaf 4, etc.) independently of a UV-B treatment (Lois 1994). Thus, the first two illustrated aspects of my work can be confirmed. The third aspect, which is the concluded increase of the initial UV-A shield of newer developed leaves with plant age is also detectable in the work of Lois (1994) though the data presented there is not totally convincing: at non-inducing conditions the initial absorbance at 330 nm of each leaf pair increased with nearly every new leaf pair. In summary, it can be concluded that epidermal flavonoid accumulation is not only just induced by UV-B, low temperature or other factors but appears to be regulated by both individual leaf age and total plant age. Precisely, with increasing leaf age the amount of UV-A absorbing compounds decreases, and with increasing plant age every new leaf exhibits a higher UV-A shield than the next older leaf, and also factors which are capable in inducing flavonoid accumulation lead to a substantially higher level of the flavonoid content in all leaves at every timepoint. Thus, three aspects define the level of flavonoid content of a single leaf: 1. environmental condition, 2. leaf age and 3. plant age.

A similar leaf age dependency of epidermal UV-A transmittance after UV-B treatment was also observed by Bilger and co-workers in studies on *Vicia faba* (Bilger et al. 2001). However, only young and still developing leaves were capable in accumulating UV-A absorbing compounds, whereas old and mature leaves were not. A low temperature induced leaf age dependency of *Vicia faba* was also detected later (Bilger et

al. 2007). However, at warm temperature of 19 °C the opposite relationship was observed, with increased transmittance values of young leaves. It was also concluded that only those leaves which were still growing were capable in accumulating UV-A absorbing compounds and that old leaves which were completely full-grown could not regulate (up- or down) their UV-A transmittance. This last finding is in little contrast to my results because also old leaves retained at least a reduced capability to decrease their epidermal UV-A transmittance after treatment with low temperature or high light. The same was observed after UV-B treatment of *Arabidopsis* Col plants by Lois and Buchanan (1994).

#### *The interplay of photoperiod, temperature and irradiance on UV-A transmittance*

All experimental set-ups inclusive control conditions were performed under long-day (LD) conditions, i. e. a photoperiod of 16 hours per day. A short-day (SD) photoperiod of only eight hours per day was only effective in inducing epidermal UV-A screening when the temperature was lowered (to 9 °C). High irradiances of 300  $\mu\text{mol}/\text{m}^2\text{s}$  alone did not result in decreased transmittance values. When low temperature under SD was established, transmittance was slightly reduced and an additional light effect of 300  $\mu\text{mol}/\text{m}^2\text{s}$  was detected, which was similar to the light effect at 9 °C under LD conditions. This means that the influence of the photoperiod on epidermal screening was not present any more at 9 °C and 300  $\mu\text{mol}/\text{m}^2\text{s}$ .

#### *HCA and flavonoids in A. thaliana rosette leaves*

Altogether eleven UV absorbing compounds were identified by HPLC-MS in rosette leaves of *A. thaliana* ecotype Col, which were also detected in the ecotype Ws. In addition, all three kaempferol glycosides and five HCAs were also found in leaves which were grown at non-inducing conditions. Six different flavonol glycosides were identified of which each three were kaempferol glycosides and quercetin glycosides. The glycosylation pattern was the same for kaempferol glycosides and quercetin glycosides and a quercetin glycoside eluted always before the according kaempferol glycoside in the chromatograms (figure 1.10). One aglycone was glycosylated with two molecules of rhamnose and one molecule of glucose, the second flavonoid had each one molecule of rhamnose and glucose attached and the third compound had two molecules of rhamnose bound to the aglycone. However, the glycosylation pattern of the third quercetin could not be resolved unmistakably as its peak was overlaid partly with the second kaempferol glycoside.

Five different UV-A absorbing HCAs were also identified and four of them were sinapate esters and one was a hydroxy-feruloyl glycoside. Among the sinapate esters one compound was unmistakably identified as sinapoylmalate and was the main HCA in *A. thaliana*. The other three were all esterified with sinapate and a hexose (indicated as



sinapoylglucose (1)) but to two of them a further HCl molecule (sinapoylglucose (3)) or a second sinapate molecule (sinapoylglucose (2)) was attached. These two latter compounds seem to be artificially formed through the process of pigment extraction or during the separation process in the HPLC system. 1 % concentrated HCl was added to the ethanolic extraction buffer.

It is well established that leaf flavonoids in *Arabidopsis* are mainly kaempferol and quercetin glycosides. Four different kaempferol derivatives were initially detected in control and UV-B treated leaf samples of *A. thaliana* ecotype Landsberg *erecta* (Ler) by Li and co-workers (1993). Surprisingly, no quercetin derivatives were detected although HPLC analysis and electron impact-mass spectroscopy were performed. Not four, but only three different flavonoids, characterized as kaempferol glycosides and of which one was identified as a glycosylated kaempferol with two rhamnoses and one further sugar attached to the aglycone, were discovered in *A. thaliana* Col leaves before and after UV-B treatment later (Lois 1994, Lois & Buchanan 1994). However, in the image of the thin-layer chromatography of extracts from *Arabidopsis* leaves are six different spots visible (figure 4 in Lois & Buchanan (1994), indicating that there are altogether six different flavonoids in UV-B treated *Arabidopsis* leaves. Three quercetin derivatives were detected together with three kaempferol derivatives for the first time by Sheahan and Cheong (1998) in non-induced *A. thaliana* Ler by HPLC and diode array analysis. However, no quercetin derivatives were found in leaves but only in seedlings and flowers. Finally, Veit & Pauli (1999) could characterize the structure of leaf flavonoids of *A. thaliana* Ws in more detail by spectroscopic analysis (UV, NMR, MS) and chiral capillary zone electrophoresis. At non-inducing conditions three kaempferol glycosides were identified with the first having attached two molecules of glucose (glucopyranoside) and one molecule of rhamnose (rhamnopyranoside), the second having attached each one molecule of glucose and rhamnose and the third having attached two molecules of rhamnose to the aglycone. Thus, the glycosylated kaempferol discovered by Li and co-workers (1993) was confirmed by the work of Veit & Pauli (1999). After irradiation with supplemental UV-B radiation the corresponding quercetin glycosides of the identified kaempferol glycosides were detected. It is also mentioned that in addition to these six flavonoids different minor flavonoids also accumulate which contribute less than 10 % to the total flavonoid content. Hence, the flavonoid pattern detected in this work corresponds very well with the literature data.

Regarding HCA composition in *Arabidopsis*, major soluble phenolic esters of *Arabidopsis* were first identified by Chapple and co-workers in 1992 using thin-layer chromatography, HPLC and fast atom bombardment-mass spectroscopy. Besides 2-*O*-sinapoylmalate and 1-*O*-sinapoylglucose, also sinapoylcholine was detected, the two latter being found mainly in *Arabidopsis* seeds. In contrast to my findings, 5-hydroxy ferulic acid, the precursor for sinapic acid and all sinapic acid derivatives like sinapoylglucose and sinapoylmalate, was not detected in leaf samples. This could have been due to different detection limits. Sinapoylglucose is the precursor for sinapoylmalate, which is the main HCA in leaves of *A. thaliana*. Lorenzen et al. (1996)

found that about 10 % of sinapoylmalate (in pmol/ mg) was present as sinapoylglucose in leaves of *A. thaliana* Col after growth for three weeks under similar conditions as applied in this work (100  $\mu\text{mol}/\text{m}^2\text{s}$  irradiance, 23 °C air temperature and 16 hours light per day). The detected peak area at 314 nm per leaf area was the unit for pigment amount given in my work so that this 10 % fraction can not be compared directly with my results. So, the fraction of sinapoyl glycosides of sinapoylmalate was found to be frequently much higher than 10 %. However, the relation for peak heights and widths of HCAs to sinapoylmalate presented in the chromatogram of Lorenzen et al. 1996 (figure 2 B) appeared to be very similar to the relation of peak heights and widths as observed in my work (figure 1.10).

Finally it can be concluded, that until to date only one work exists that describes each three kaempferol and quercetin glycosides with equal glycosylation pattern in leaves of *A. thaliana* (Veit & Pauli, 1999) and that only one other work describes sinapate esters others than sinapoylmalate to be also present in leaves of *A. thaliana* grown at non-inducing conditions (Lorenzen et al. 1996). Hence, my results can confirm the two findings, that namely not more than six different flavonol glycosides were present in rosette leaf of *A. thaliana* and that quercetin glycosides were mostly missing in non-induced leaves. In contrast, the composition of HCAs was found to be comprised not only of sinapoylmalate but also of considerable amounts of sinapoylglucose in *A. thaliana* Col and Ws. In addition, one further precursor, namely 5-hydroxy feruloylglucose was also detectable albeit in very few amounts in leaf samples.

## 2      **Second part: Characterisation of epidermal flavonoid accumulation at low temperature in comparison to light- and UV-B-induced accumulation**

### 2.1      Results

The following paragraphs will present data which characterize and describe flavonoid accumulation in rosette leaves of *A. thaliana* Col after treatment with three different environmental stimuli: high irradiance, supplemental UV-B radiation and low air temperature. Before the effect of low temperature can be compared with the effect of high PPFD and UV-B, the induction of flavonoid accumulation by the latter two factors had to be characterized first. Therefore, I start with the characterization of a light-induced flavonoid accumulation (chapter 2.1.1), continue with the UV-B induced accumulation (chapter 2.1.2) and will then compare these results with a low temperature-induced flavonoid accumulation (chapter 2.1.3). The interplay of light and temperature will be elucidated in chapter 2.1.4. The potential for a reduction of epidermal UV-A screening will be shown in chapter 2.1.5, before transmittance measurements will be validated with HPLC results (chapter 2.1.6). Finally data on the cellular localisation of flavonoids (chapter 2.1.7) will conclude this second part, before I will discuss these results in chapter 2.2.

#### 2.1.1      Light-induced flavonoid accumulation

In the last part differences in responses to environmental stimuli of different ecotypes of *A. thaliana* WT were presented. It was also shown that individual leaf age influences the extent of the response to a certain stimulus. The following paragraphs will present some data on a light-induced decrease in epidermal UV-A transmittance in *A. thaliana* Col. But before this, I will again emphasise on the importance of leaf age when considering epidermal UV-A transmittance in *A. thaliana* Col leaves. Therefore, figure 2.1 shows a gradient in transmittance of a high light treated *Arabidopsis* plant according to leaf age. As it was shown for low temperature-induced plants, light treated old leaves had higher transmittance values than light-induced young leaves. Thus, the potential to accumulate UV-A absorbing flavonols in the upper leaf epidermis was greater in young leaves than in old leaves. In conclusion, only young leaves showed a strong response to increased irradiances and in all following experiments only young leaves were investigated and sampled.

##### 2.1.1.1      High light response of epidermal flavonoid accumulation in *A. thaliana* Col

In a timeplot of transmission measurements it becomes clear that 22°C air temperature and high irradiances of 350  $\mu\text{mol}/\text{m}^2\text{s}$  white light led to a significant decrease in epidermal UV-A transmittance from 85 % to about 60 % within two days (figure 2.2).

No further decrease was observed within the following five days. However, newly developed leaves had a transmission of only 40 %.

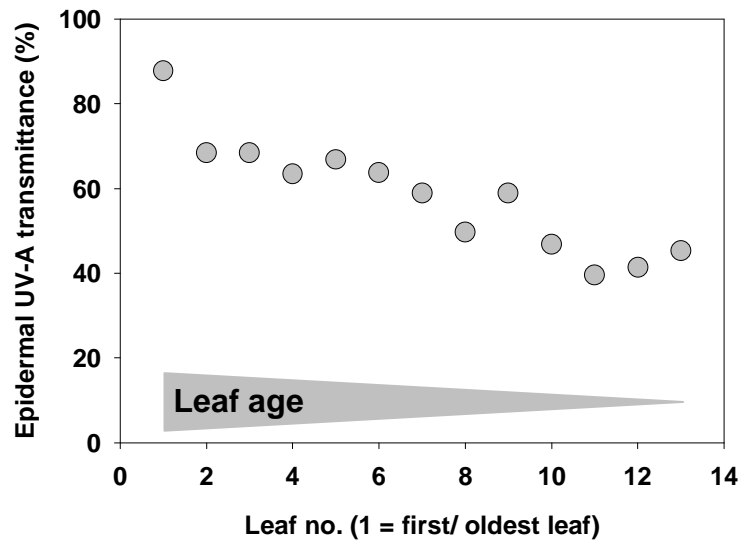


Figure 2.1: Epidermal UV-A transmittance in all rosette leaves of *A. thaliana* Col exposed to high light conditions ( $360 \mu\text{mol}/\text{m}^2\text{s}$ ) for five days in dependency of decreasing leaf age. Cotyledons were excluded. Eight leaves were present at start of the treatment.

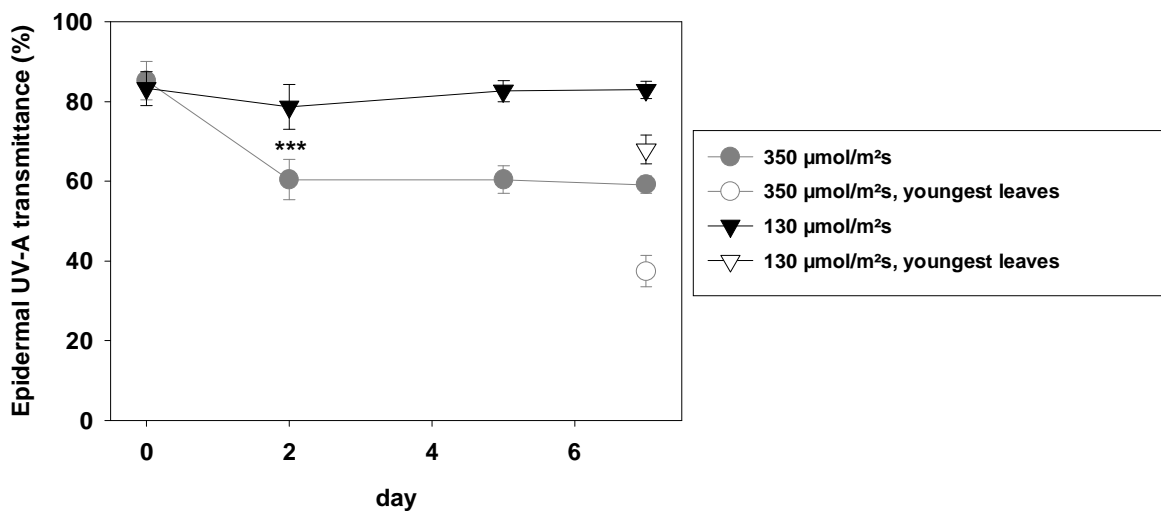


Figure 2.2: Timeplot of high light induction ( $350 \mu\text{mol}/\text{m}^2\text{s}$ ) of epidermal UV-A transmittance in *A. thaliana* Col plants in comparison to control conditions ( $n = 9$ ). In comparison to control conditions, after an induction period of two days epidermal transmittance was significantly lowered in plants which were exposed to  $350 \mu\text{mol}/\text{m}^2\text{s}$  (t-test:  $P < 0.001^{***}$ ). End values were tested to be highly significantly different to each other (t-test:  $P < 0.001^{***}$ ).

Figure 2.3 shows final transmittance values after a high light treatment as presented in figure 2.2 and demonstrates again the differences between “old” and “young leaves. Young leaves had transmittance values which were even lower than values of leaves which were present at the start of the experiment. This was true for both conditions, but the difference in transmittance between control conditions and high light treatment after seven days was about the same if considered “old” (grey columns) and young leaves (dark grey columns), namely 24 and 31 %, respectively.

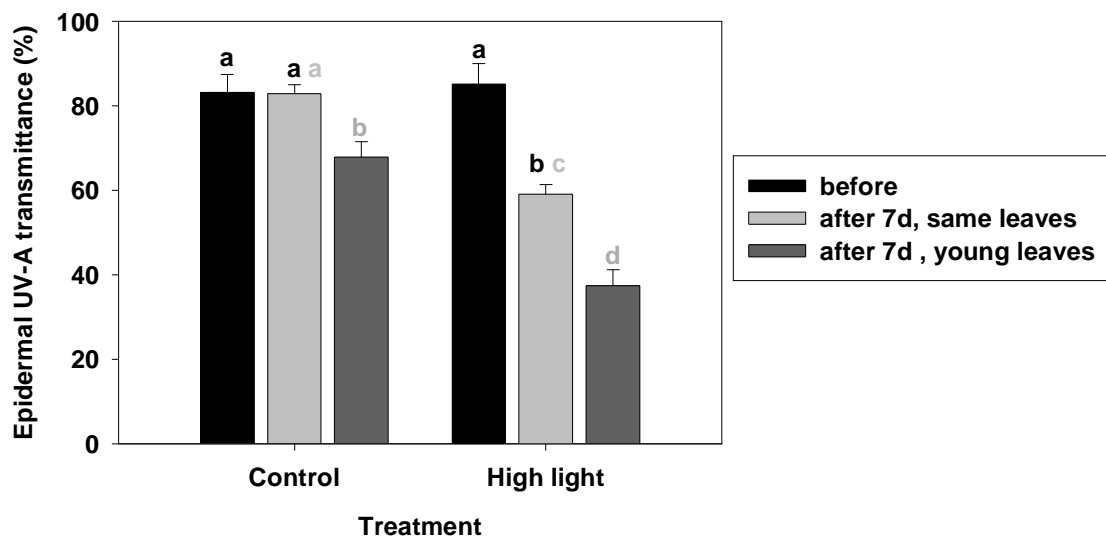


Figure 2.3: Epidermal transmittance values of leaves of *A. thaliana* Col before and after exposure to control and high light conditions for seven days (n = 9, end values of figure 2.2). Statistically significant differences were identified by two-way ANOVAs and tukey tests as post-hoc tests. They are indicated by different letters of same colour. P was always high with  $P < 0.001^{***}$ .

High light treated plants were also sampled and young rosette leaves were analysed for HCAs and flavonol glycosides with a HPLC system. Figure 2.4 shows that the overall amount of UV absorbing compounds was significantly increased after a high light treatment due to a strong increase in kaempferol glycosides and a weak accumulation of quercetin glycosides.

#### 2.1.1.2 Dose-response relationship for irradiance and flavonoid accumulation at 22 °C

As high light was shown to have a positive effect on flavonoid content in leaves of *A. thaliana*, the question arose whether lower irradiances were also capable in inducing flavonoid accumulation and whether this accumulation was correlated with the irradiance. As assumed, measurements of epidermal UV-A transmission could demonstrate, that the amount of UV-A absorbing compounds was indeed positively correlated with the irradiance. Leaves, grown at a photon flux density (PPFD) of 120

$\mu\text{mol}/\text{m}^2\text{s}$ , which were transferred to lower light intensities increased their epidermal transmittance and decreased it after transfer to higher irradiances (figure 2.5). After seven days at different PPFDs a gradient in transmission from 90 % at  $50 \mu\text{mol}/\text{m}^2\text{s}$  to 37 % at  $350 \mu\text{mol}/\text{m}^2\text{s}$  was observed.

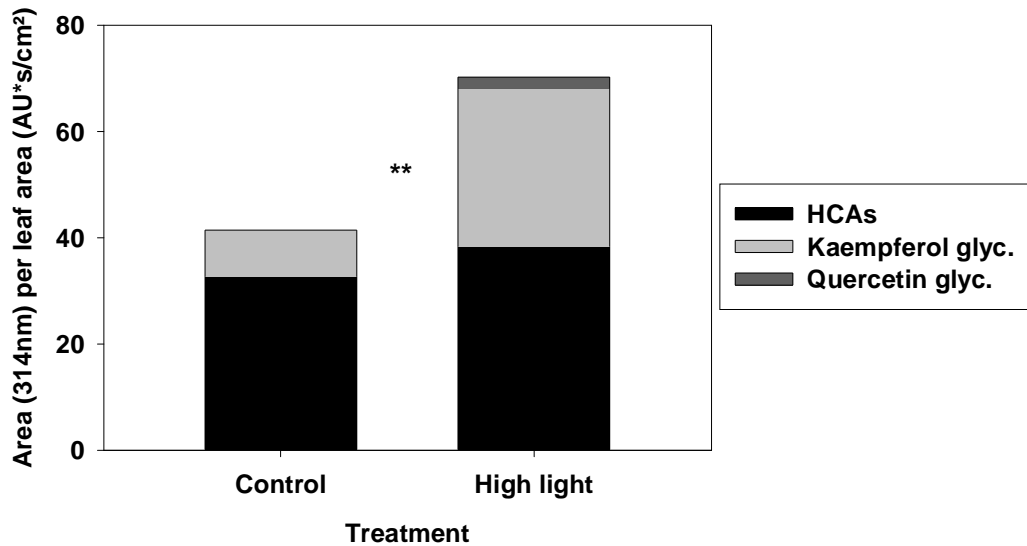


Figure 2.4: Main pigment composition as measured with HPLC in leaves of *A. thaliana*, which were exposed to control conditions and high light conditions for seven days ( $n = 8$  and  $6$  for control and high light, respectively). Total pigment content was tested to be significantly different (t-test:  $P = 0.004^{**}$ ).

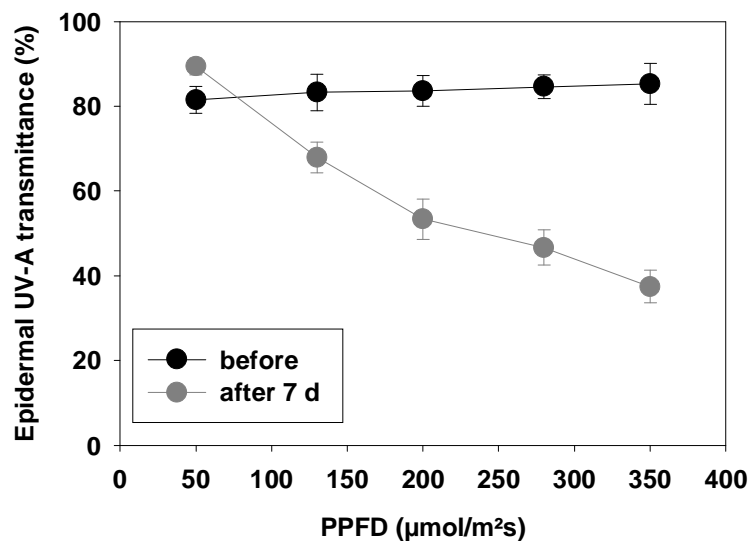


Figure 2.5: Dependency of epidermal UV-A transmittance in young leaves of *A. thaliana* Col on irradiance, which was varied from  $50$  to  $350 \mu\text{mol}/\text{m}^2\text{s}$  white light. Plants were exposed to different irradiances for seven days ( $n = 9$  for all PPFDs).

HPLC analysis of leaves of these treated plants were performed and the figure 2.6 demonstrates that HPLC analysis could confirm a strong and positive correlation between the PPFD and the total amount of flavonoids irrespective of HCAs. Up to a PPFD of 200  $\mu\text{mol}/\text{m}^2\text{s}$  this increase was significantly different to each lower PPFD, but with higher PPFDs the total pigment amount was not statistically different to the amount at 200  $\mu\text{mol}/\text{m}^2\text{s}$ . An increase in kaempferol glycosides was observed over the whole irradiance range. Quercetin glycosides appeared at an irradiance of 200  $\mu\text{mol}/\text{m}^2\text{s}$  upwards, but stayed small in their total amounts. The fraction of HCAs increased significantly from 50 to 130  $\mu\text{mol}/\text{m}^2\text{s}$  (one-way ANOVA and tukey,  $P < 0.001^{***}$ , figure 2.7), but at even higher PPFDs this fraction stayed more or less saturated.

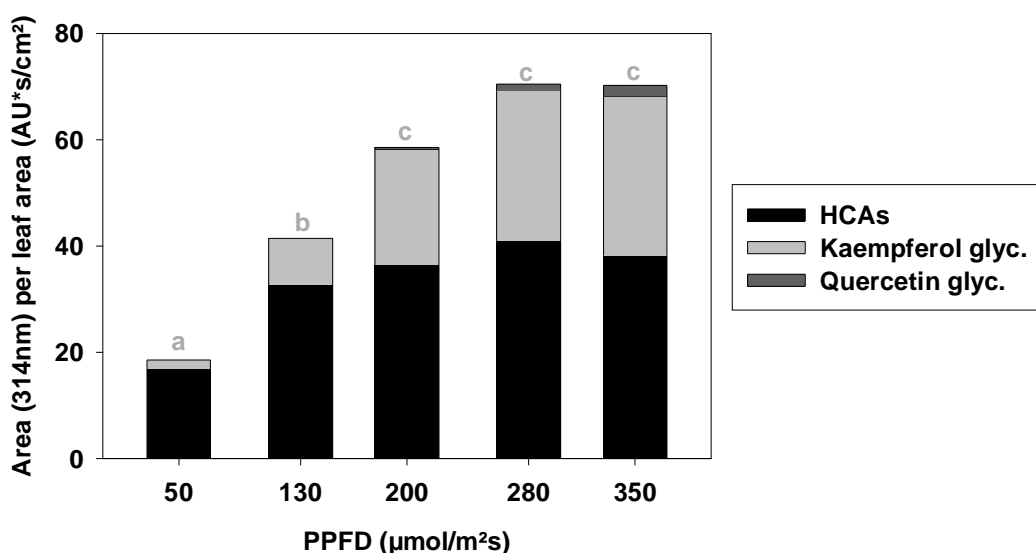


Figure 2.6: UV-A absorbing pigment composition in rosette leaves of *A. thaliana* Col, which were treated with increasing PPFDs for seven days as measured with HPLC ( $n = 9$  for 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ ,  $n = 8$  for 130 and 200 and  $n = 6$  for 350  $\mu\text{mol}/\text{m}^2\text{s}$ ). Values for total pigments were tested for significant differences with a one-way ANOVA and a following tukey-test. Significant differences are indicated by different letters and level of significance was always high with  $P < 0.001^{***}$  with the exception 130 vs. 200  $\mu\text{mol}/\text{m}^2\text{s}$ , where  $P = 0.019^*$ .

This increase was accompanied by an increase in all HCAs with exception of sinapoylglucose (2), whose amount stayed relatively constant. With a further increase in irradiance the amount of sinapoylglucose (1) and 5-OH-feruloylglucose increased steadily (figure 2.7).

The amount of kaempferol glycosides increased with increasing irradiances (figure 2.8). The amount of all three kaempferol derivatives accumulated in a light-dependent manner. Kaempferol glucopyranoside-rhamnopyranoside was not present at 50  $\mu\text{mol}/\text{m}^2\text{s}$ . The strongest increase was detected between the irradiance of 130  $\mu\text{mol}/\text{m}^2\text{s}$  and 200  $\mu\text{mol}/\text{m}^2\text{s}$ , whereas quercetin glycosides were found only at 200  $\mu\text{mol}/\text{m}^2\text{s}$

upward and their fraction of total flavonoids stayed small, i.e. 1.5 % at 200, 7.2 % at 350  $\mu\text{mol}/\text{m}^2\text{s}$ .

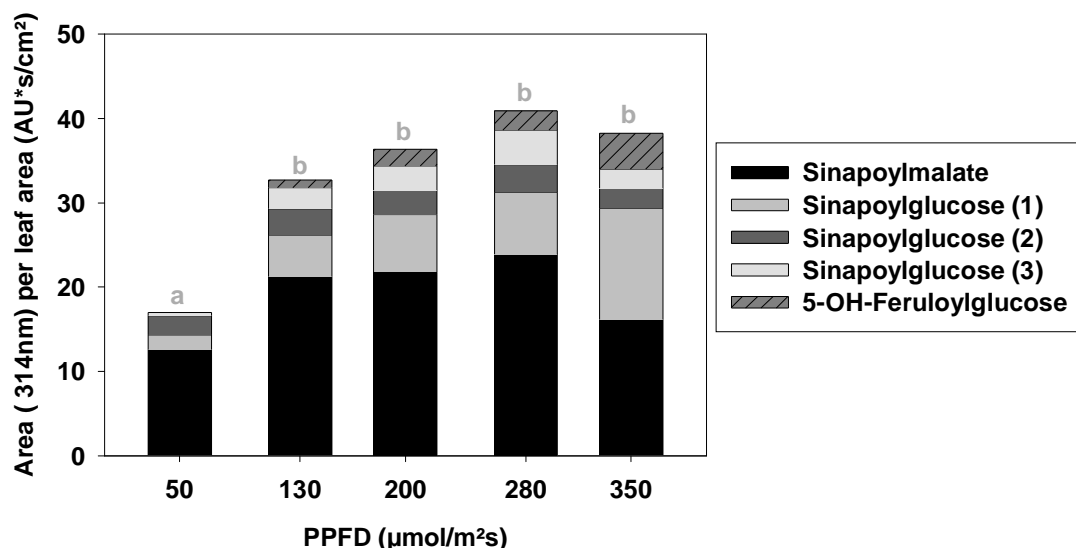


Figure 2.7: Detailed composition of HCAs in leaves of *A. thaliana* Col which were exposed to different irradiances for one week ( $n = 9$  for 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ ,  $n = 8$  for 130 and 200 and  $n = 6$  for 300  $\mu\text{mol}/\text{m}^2\text{s}$ ). A statistically significant increase was only detected between 50 and 130  $\mu\text{mol}/\text{m}^2\text{s}$  (one-way ANOVA with tukey-test:  $P < 0.001^{***}$ ).

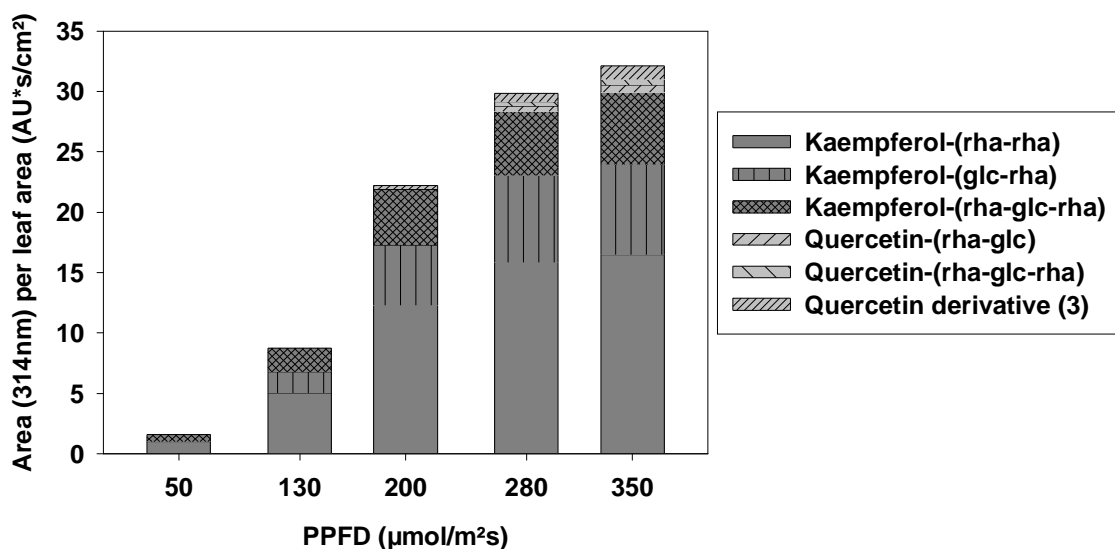


Figure 2.8: Flavonol glycoside composition in *A. thaliana* Col plants which were exposed to increasing PPFD ( $n = 9$  for 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ ,  $n = 8$  for 130 and 200 and  $n = 6$  for 300  $\mu\text{mol}/\text{m}^2\text{s}$ ). The contribution of quercetin glycosides to total flavonol glycoside content was always very small (not more than 7.2 %, which was found at 350  $\mu\text{mol}/\text{m}^2\text{s}$ ).



The figure 2.9 presents the data on kaempferol glycosides from the figure 2.8 as a line/ scatter plot. A light-dependent increase in amount pigment per leaf area could be observed for all kaempferol derivatives. The strongest increase was detected between an irradiance of 130 and 200  $\mu\text{mol}/\text{m}^2\text{s}$ . The according glycosides of kaempferol and quercetin derivatives were found to be in a similar quantitative relation to each other.

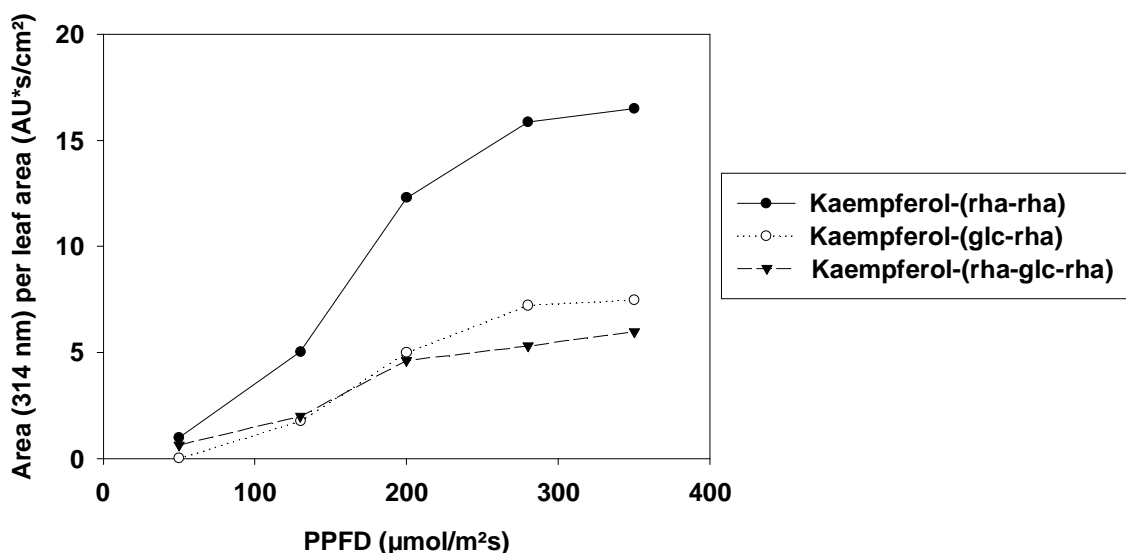


Figure 2.9: Kaempferol content in *A. thaliana* Col after exposure to increasing irradiances – same data set as figure 2.8 presented as a line/ scatter plot in order to demonstrate the correlation of all derivatives with the PPFD (n = 9 for 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ , n = 8 for 130 and 200 and n = 6 for 300  $\mu\text{mol}/\text{m}^2\text{s}$ ).

The amount of quercetin derivatives, presented as a line/ scatter plot in figure 2.10, was also correlated with the irradiance. However, quercetin glycosides were only present at light intensities of 200  $\mu\text{mol}/\text{m}^2\text{s}$  upwards, with the quercetin derivative (3) being the first quercetin detected in *A. thaliana* Col plants. The total amount of quercetin glycosides stayed small.

## 2.1.2 UV-B induced flavonoid accumulation

### 2.1.2.1 The effect of supplemental UV-B radiation on flavonoid accumulation

UV-B radiation is the “classical” environmental factor known to induce flavonoid accumulation in plants. In *A. thaliana* the epidermal UV-A transmittance decreased quickly from 80 % to 45 % after transfer from control conditions to UV-B conditions within two days and decreased a little further during the following three days down to

30 % upon exposure to UV-B (figure 2.11). Leaves which newly developed during the experiment displayed reduced transmittance values.

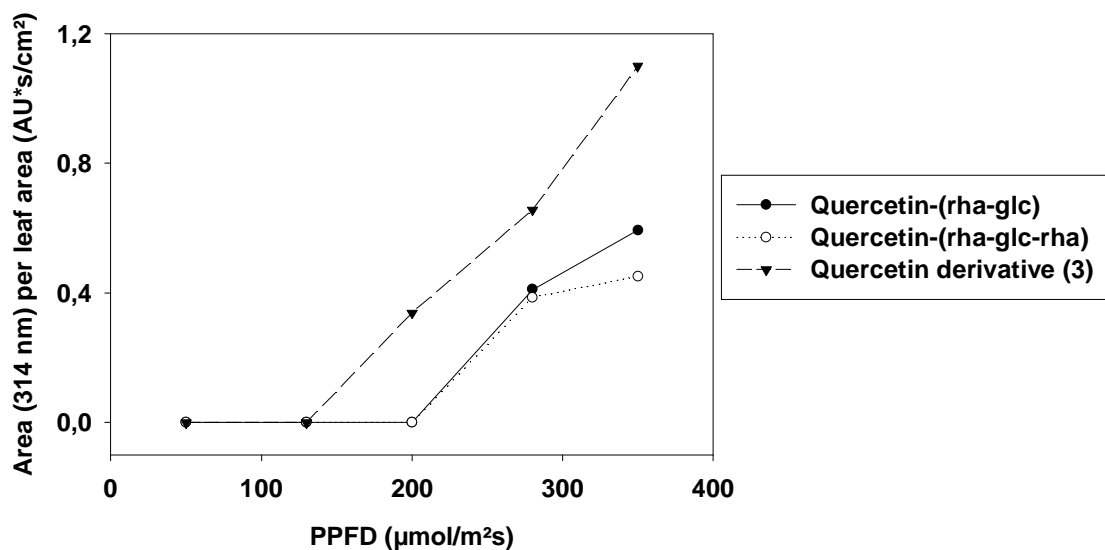


Figure 2.10: Quercetin content in *A. thaliana* Col after exposure to increasing irradiances (n = 9 for 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ , n = 8 for 130 and 200 and n = 6 for 300  $\mu\text{mol}/\text{m}^2\text{s}$ ).

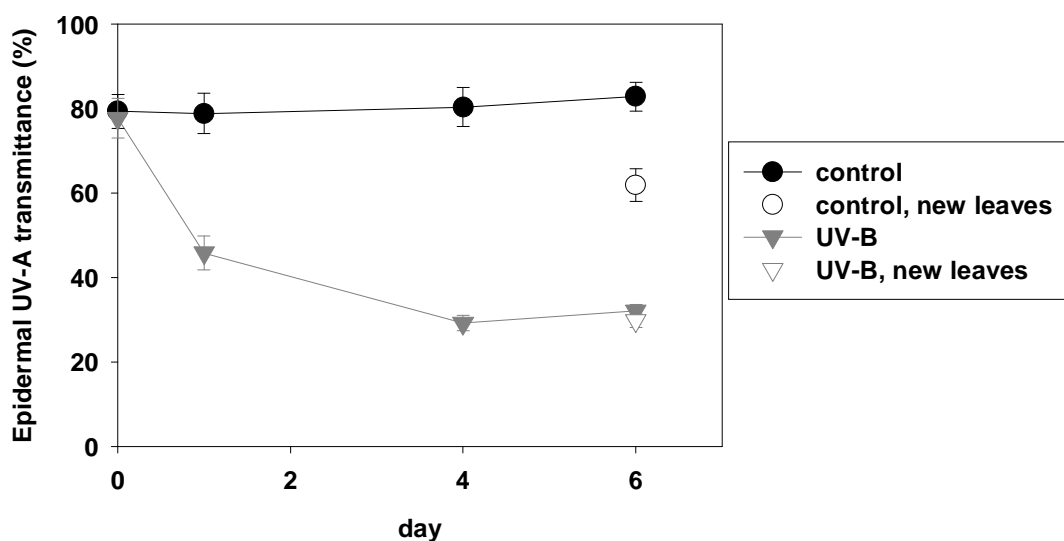


Figure 2.11: Timeplot of epidermal UV-A transmittance measured with the UV-A-PAM of rosette leaves of *A. thaliana* Col plants which were exposed to either control conditions (black symbols) or to supplemental UV-B radiation (grey symbols) as indicated in the method section III.2.3) (n = 18 for all data points).

Start and end values of transmittance of plants kept at control conditions and under UV-B as shown in figure 2.11 are presented below as a bar graph (figure 2.12). Epidermal UV-A transmittance in UV-B treated leaves was strongly reduced to only 30 %, independently of leaf age. Leaves of control plants which were also measured at start of the experiment displayed no reduction in transmittance, whereas young leaves of control plants had significantly lowered transmittance values of 60 %.

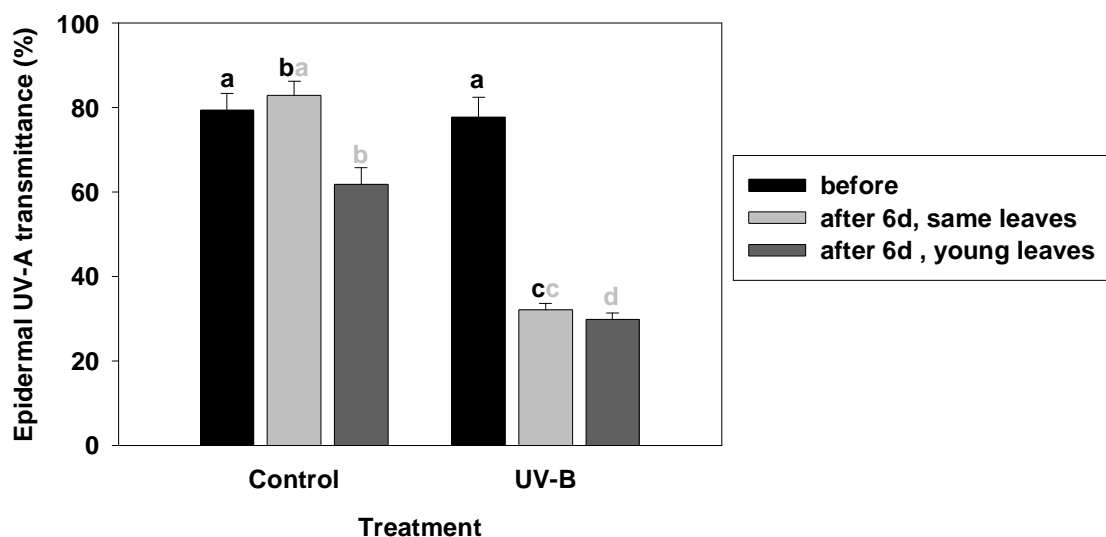


Figure 2.12: UV-B induction of epidermal UV-A transmittance in *A. thaliana* Col (right columns) in contrast to control conditions (left columns) (n = 18). Presented are start values of young leaves, final values of the same leaves and final values of new leaves, which had developed during the experiment. A statistically significant increase in transmittance was observed between day 0 and day 6 in leaves which were kept at control conditions (two-way ANOVA and tukey-test with  $P = 0.008^{**}$ , black letters). New, young control leaves displayed reduced values of transmittance in comparison to “old” control leaves (two-way ANOVA and tukey-test with  $P < 0.001^{***}$ , grey letters).

The composition of UV-A absorbing compounds as measured by HPLC analysis in newly developed leaves is shown in figure 2.13. In comparison to control plants the amount of kaempferol glycosides and quercetin glycosides was strongly increased in UV-B treated plants. Kaempferol glycosides were doubled. Both, kaempferol glycosides and quercetin glycosides were present in nearly equal amounts in UV-B treated leaves, which contrasts results from other induction experiments, in which the fraction of quercetin glycosides stayed always very small.

No difference in composition and amount of HCA derivatives per leaf area was observed between control and UV-B treated leaves (figure 2.14).

After UV-B treatment the amount of flavonoids was increased more than threefold (figure 2.15). Kaempferol glycosides were doubled in their amount and quercetin

glycosides were extremely accumulated from more or less zero (only about 0.1 AU\*s/cm<sup>2</sup>) to about 20 AU\*s/cm<sup>2</sup> after UV-B treatment. The main quercetin glycoside was the “quercetin derivative (3)”, which was also the main quercetin in other induction experiments.

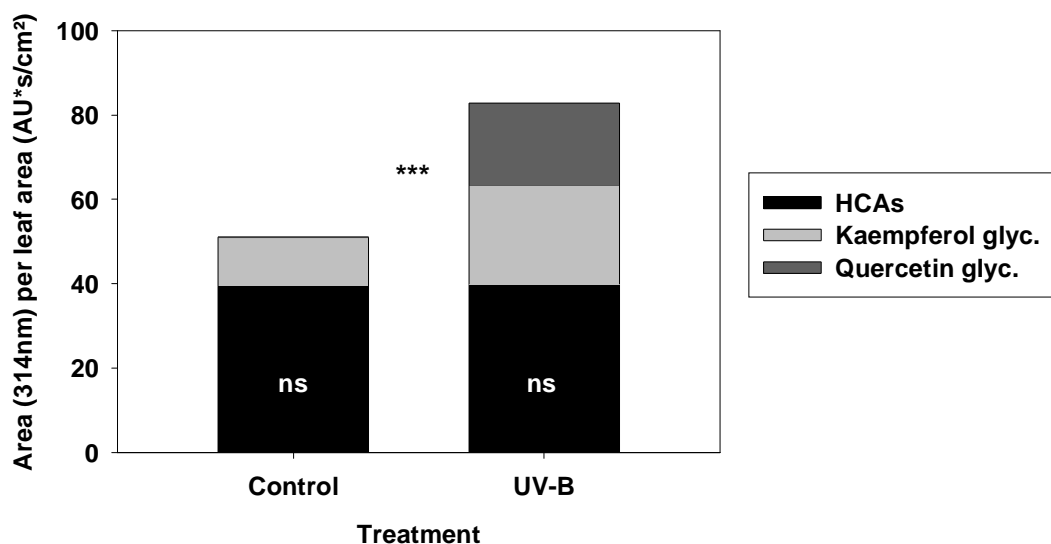


Figure 2.13: Composition of UV-A absorbing (314 nm) compounds in young rosette leaves of *A. thaliana* Col which were exposed to either control (n = 15) or UV-B conditions (n = 18) for six days. The total amount of pigment per leaf area was significantly higher in UV-B treated samples due to significantly higher amounts of kaempferol and quercetin glycosides (t-tests:  $P < 0.001$ \*\*\*). The amount of HCAs was tested to be not different (t-test:  $P = 0.844$ , ns).

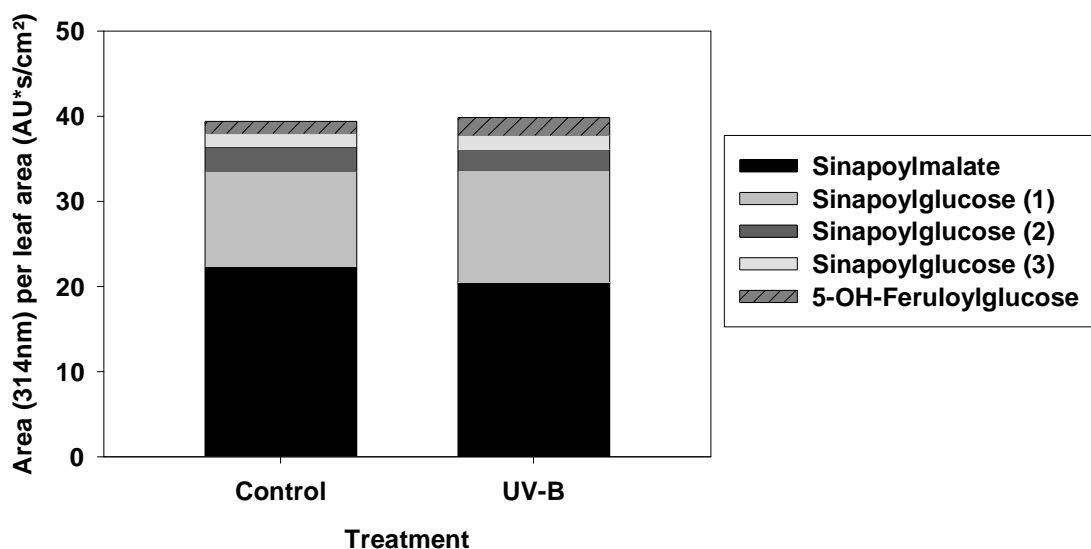


Figure 2.14: Composition of HCA components after UV-B treatment (right column, n = 18) in comparison to control conditions (left column, n = 15) in *A. thaliana* Col.

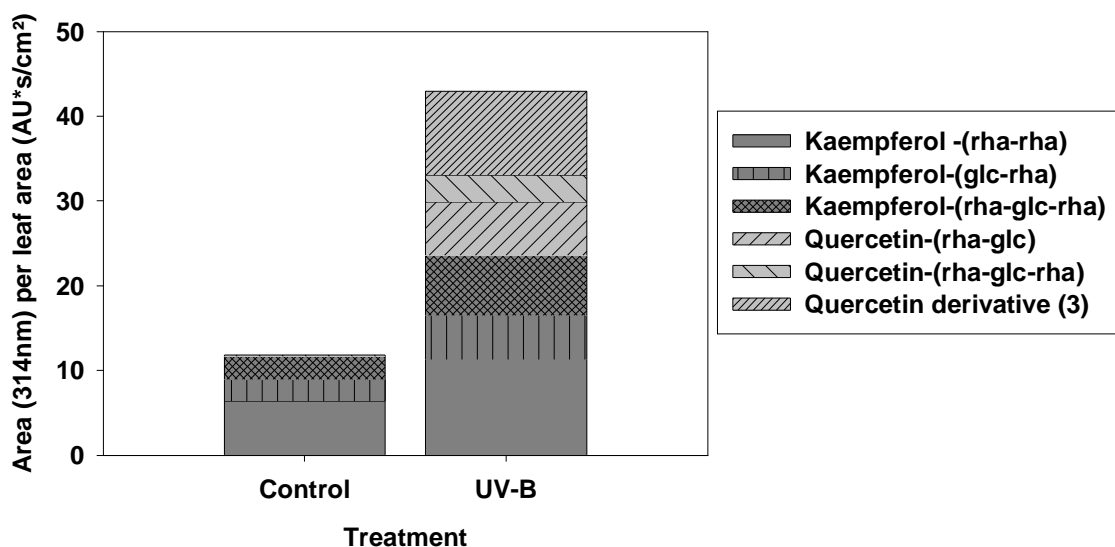


Figure 2.15: Composition of flavonol derivatives after a 6 day UV-B treatment in rosette leaves of *A. thaliana* Col plants (right column; n = 18) in comparison to leaves of control plants (left column; n = 15). Individual kaempferol glycosides were highly significant increased after UV-B exposure as tested with t-tests ( $P < 0.001$ \*\*\* for all kaempferol glycosides).

#### 2.1.2.2 Dose-response relationship for UV-B and epidermal UV-A transmittance

As the UV-B dosage chosen had a very intensive effect on flavonoid accumulation, the question arose, if, as it was true for the external factor irradiance, a direct correlation between the dosage and the amount of accumulated flavonoids would exist. The influence of different UV-B dosages on the epidermal flavonoid accumulation is presented in the figure 2.16 below. With increasing UV-B dosage the epidermal UV-A transmittance decreased steadily, indicating a strong and positive correlation between UV-B dosage and epidermal flavonoid accumulation.

#### 2.1.3 Low temperature-induced flavonoid accumulation

##### 2.1.3.1 The effect of low temperature on phenotype and epidermal accumulation of UV-A absorbing compounds

Low temperature experiments started generally with plants which had five fully developed rosette leaves. During a low temperature treatment of ten days plants grew and developed three new rosette leaves whereas control plants developed about seven new leaves during the same time period. Low temperature treated plants were markedly smaller in their habitus and their leaves were darker in color, smaller and thicker than control leaves (see image below). Often these plants had curled leaves. No difference was detected in leaf hairyness.

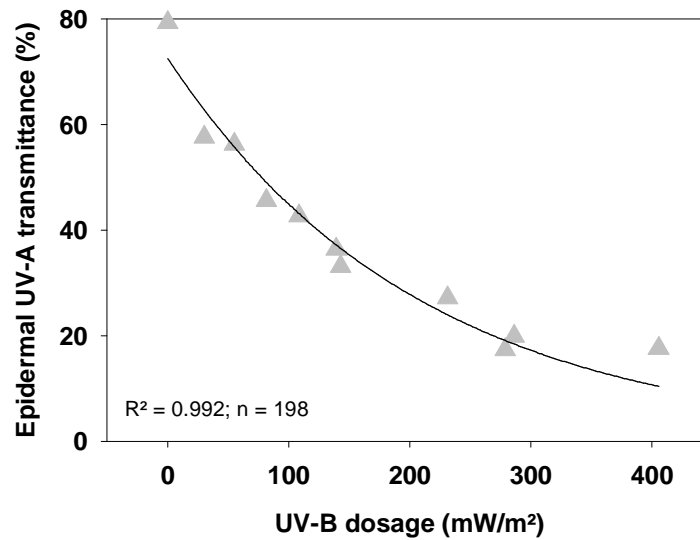


Figure 2.16: Dependency of epidermal UV-A transmittance as measured with the UV-A-PAM in rosette leaves of *A. thaliana* Col of incident UV-B dosage. Plants were illuminated with different supplemental UV-B radiation for four days (n = 18).



Image 2.1: Control (left plant) and low temperature (right plant) treated *A. thaliana* Col. Treatment lasted ten days.

Besides higher irradiance and UV-B, moderately lowered air temperature of 9 °C was also capable in inducing epidermal flavonoid accumulation. Figure 2.17 shows a

timeplot of transmittance values of plants kept at control conditions and of plants treated with low temperature. Epidermal transmittance of cold leaves decreased steadily throughout the experiment, whereas transmittance of control leaves did not change. High light and UV-B led to an abrupt decrease in transmittance after two days and no further reduction in transmittance was observed (compare with figures 2.2 and 2.14). The epidermal UV-A transmittance in leaves of *A. thaliana* Col was under control conditions at 80 % as estimated with the UV-A-PAM. After seven days at low temperature transmission was more than halved, which indicated a strong accumulation of UV-A absorbing compounds in the upper epidermis. A marked and significant decrease in transmission was observable only after three days of low temperature treatment (t-test,  $P = 0.004^{**}$ ). At the end of the experiment, even slightly lower transmittance values were detected for young and potentially newly developed leaves.

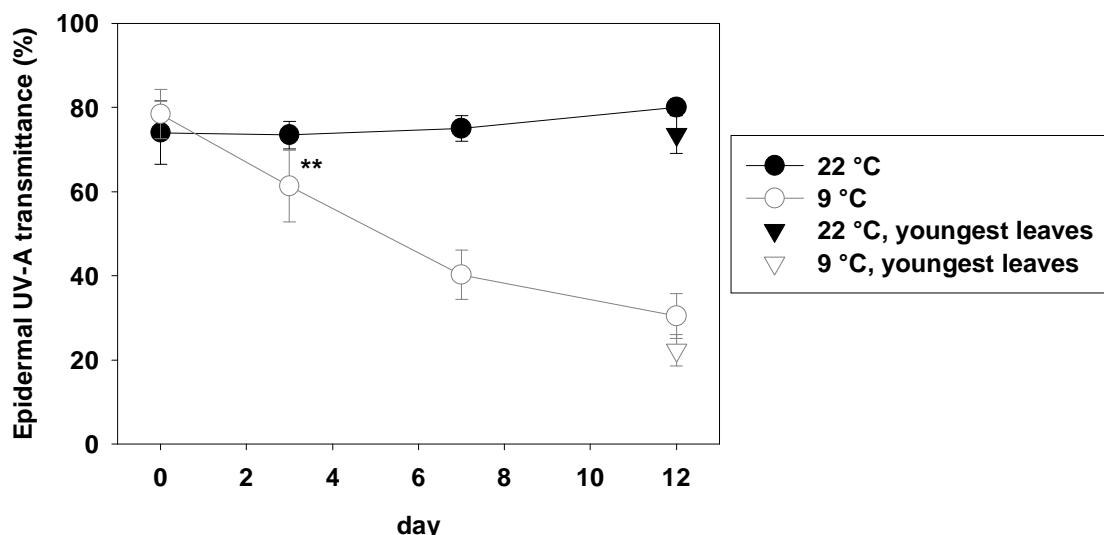


Figure 2.17: Timeplot of low temperature-induced reduction of epidermal UV-A transmittance as measured with the UV-A-PAM in rosette leaves of *A. thaliana* Col (grey line, n see table 2.1) in comparison to control conditions (black line). Measured leaves were neither young nor old but of medium age at each timepoint. Transmittance values at day 3 of control and treated leaves were statistically significant different with  $P = 0.004^{**}$  (t-test).

Table 2.1: Sample size n for low temperature experiment presented in figure 2.17 (y.l. = youngest leaves).

day \ °C	0	3	7	12	12, y.l.
22	11	7	12	16	9
9	12	9	15	14	10

The figure 2.18 presents the start and end values of figure 2.17 of a low temperature induction experiment as a bar graph. After seven days at 9 °C transmittance was lowered to 40 or 20 %, depending on the age of leaves investigated. Transmittance was significantly lowered in new leaves in comparison to old leaves after the low

temperature treatment. This was also observed after a high light exposure (figure 2.3). Only UV-B radiation had a similar effect on old and new leaves as transmittance values were reduced to about 30 % for all leaves (figure 2.12)

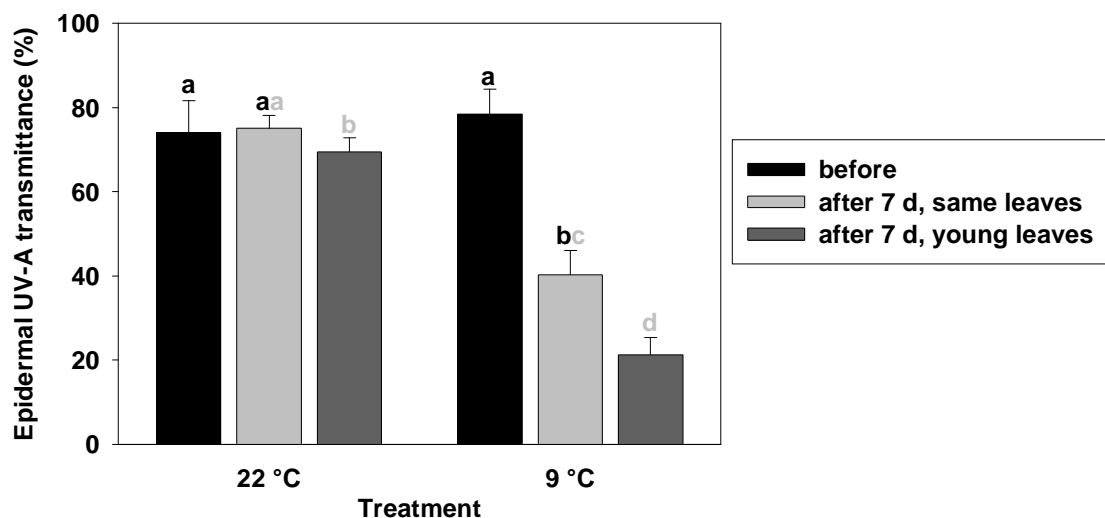


Figure 2.18: Low temperature (9 °C) induced decrease in epidermal UV-A transmittance in rosette leaves of *A. thaliana* Col plants (n = 15 and 10 for “same” and young leaves, respectively) in comparison to control conditions (22 °C, n = 12 and 10 for “same” and young leaves, respectively) after seven days. Significant differences were identified using two-way ANOVAs and tukey-tests as post-hoc tests. They are indicated by different letters of same colour and P was always high with  $P < 0.001^{***}$  with the following exception: same leaves(22 °C, day 7) vs. young leaves(22 °C, day 7), with  $P = 0.013^*$ .

HPLC analysis of low temperature treated leaves proved that, to a large extent, kaempferol glycosides and, to a small extent, quercetin glycosides were accumulated in the leaves in comparison to control leaves (figure 2.19), whereas the amount of HCAs stayed constant (t-test:  $P = 0.512$ , ns). The total amount of HCAs was comparable to amounts detected after a high light or UV-B treatment (figures 2.4 and 2.13). The total pigment amount was doubled in low temperature treated leaves.

The amount of HCA derivatives did not change upon lowering the temperature, but the composition of HCAs changed (next figure 2.20). An increase of the amount of sinapoylmalate was observed, while the amount of sinapoylglucose (1) was reduced and 5-OH-feruloylglucose was missing in the leaf extracts. Differences were found to be statistically significant. This result is in clear contrast to findings after high light exposure, where a decrease of sinapoylmalate was accompanied by an increase in sinapoylglucose (1) and 5-OH-feruloylglucose. Composition of HCAs after a UV-B treatment did not differ from control conditions (figure 2.14).



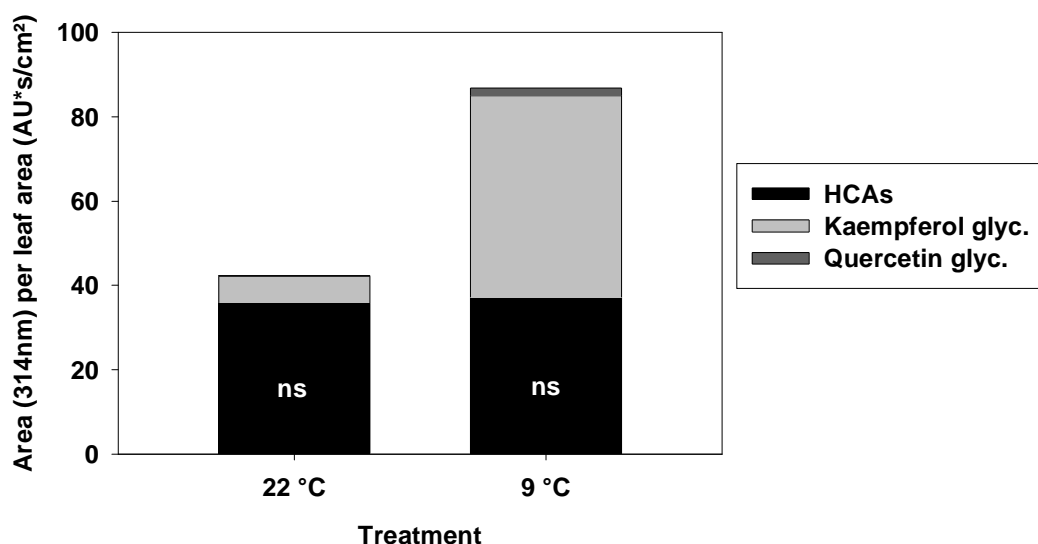


Figure 2.19: Main UV absorbing pigment composition in *A. thaliana* Col leaves after low temperature treatment for seven days in comparison to control leaves as measured with the HPLC (n = 8). While the amount of total HCAs did not change upon a low temperature treatment (t-test: P = 0.512, ns), total kaempferol glycosides and, to a lesser extent, quercetin glycosides accumulated in treated rosette leaves.

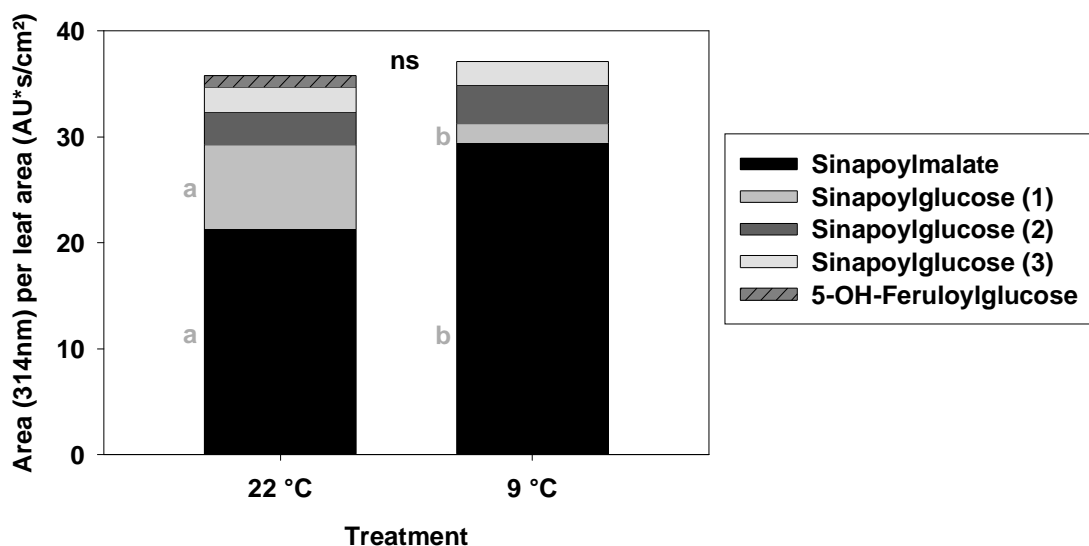


Figure 2.20: Detailed composition of HCAs in low temperature treated and control leaves of *A. thaliana* Col (n = 8). A marked and significant increase in sinapoylmalate was compensated by a significantly reduced level of sinapoylglucose (1) and the missing of 5-OH-feruloylglucose (t-tests with P < 0.001\*\*\* for sinapoylmalate and P = 0.013\* for sinapoylglucose(1); all others were not statistically different; differences are indicated by grey letters).

Kaempferol derivatives accumulated at low temperature more than sevenfold. The amount of all three kaempferol glycosides was increased strongly. Quercetin glycosides also accumulated and all three derivatives identified by HPLC-MS were present after low temperature treatment (figure 2.21). These findings are very similar to results obtained from a high light treatment. In contrast, supplemental UV-B radiation led to a rather weak accumulation of kaempferol glycosides but to a strong accumulation of quercetin glycosides (figure 2.15).

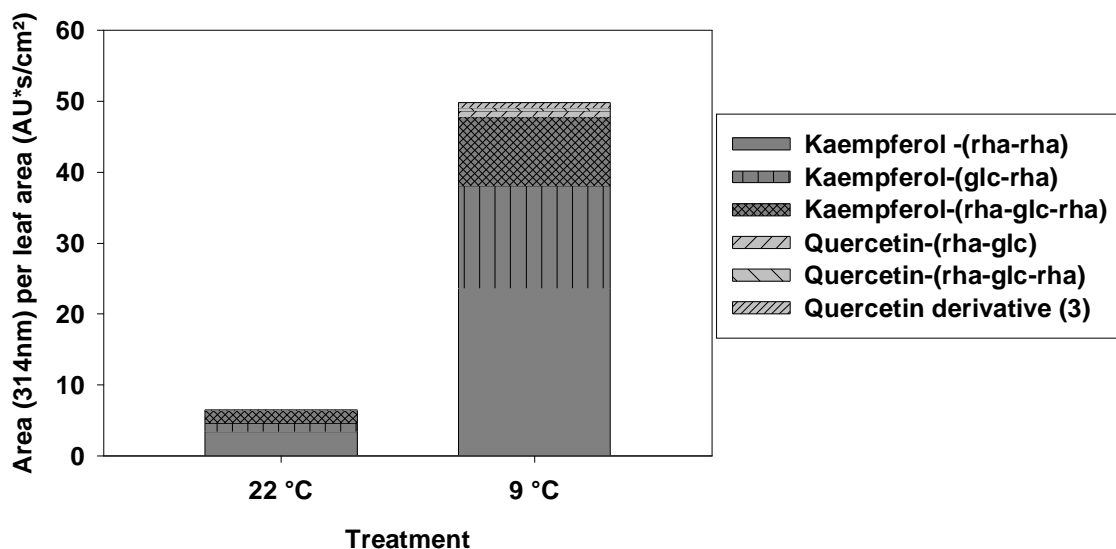


Figure 2.21: Composition of flavonoids after a seven day low temperature treatment in contrast to control conditions in rosette leaves of *A. thaliana* Col (n = 8). Quercetin derivatives counted for only 3.9 % of total flavonoid fraction after the low temperature treatment.

#### 2.1.3.2 Dose-response relationship for irradiance and flavonoid accumulation at 9 °C

In order to investigate a potential interaction between light and temperature, the following paragraphs will present data on a light dependency of flavonoid accumulation at low temperature which differs from a light dependency at warm temperature. After ten days at different PPFDs and 9 °C air temperature *A. thaliana* Col plants had decreased epidermal UV-A transmittance values which correlated nicely with the PPFD (figure 2.22). In contrast to 22 °C air temperature, plants which were exposed to very low PPFDs, namely only 20  $\mu\text{mol}/\text{m}^2\text{s}$ , also displayed reduced transmittance values. At warm temperatures this PPFD was not sufficient in accumulating any flavonol glycosides and only very few kaempferol glycosides were detected by HPLC (figure 2.5 and 2.8).

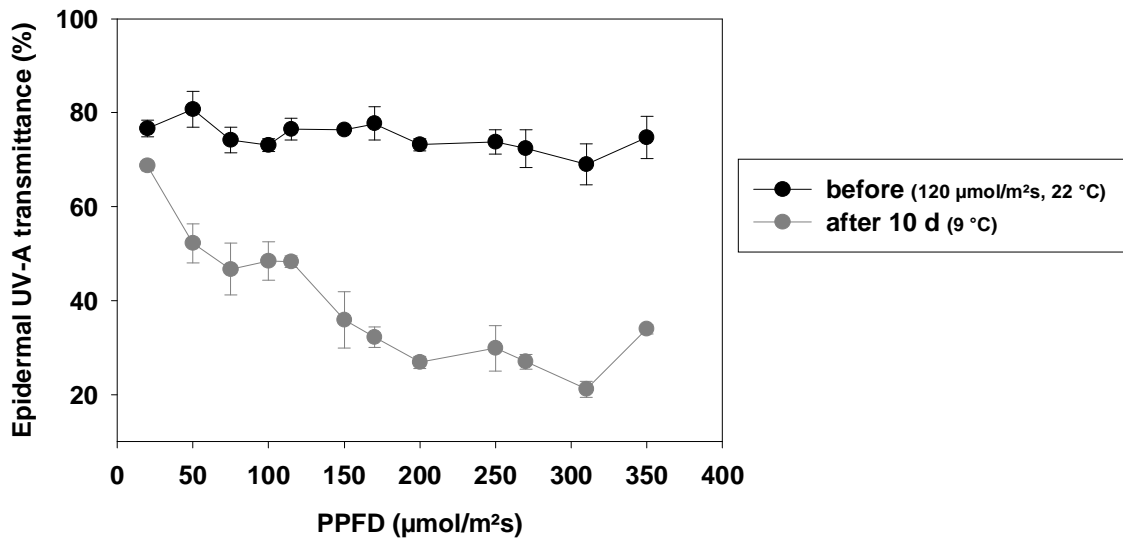


Figure 2.22. Light-dependency of epidermal UV-A transmittance at  $9 \text{ }^\circ\text{C}$  air temperature in *A. thaliana* Col (grey line). Plants were exposed to different PPFDs at low temperature for ten days. The black line present start values from the same plants taken before the treatment when plants were grown under control conditions (PPFD =  $120 \mu\text{mol}/\text{m}^2\text{s}$ ). Until start of the experiment plants were kept at control conditions, where usually  $120 \mu\text{mol}/\text{m}^2\text{s}$  were applied ( $n = 3$ ).

Plants which were kept at very low irradiances held their leaves straight upwards while plants exposed to high light intensity laid their leaves flat down to the substrate (see image 2.2).

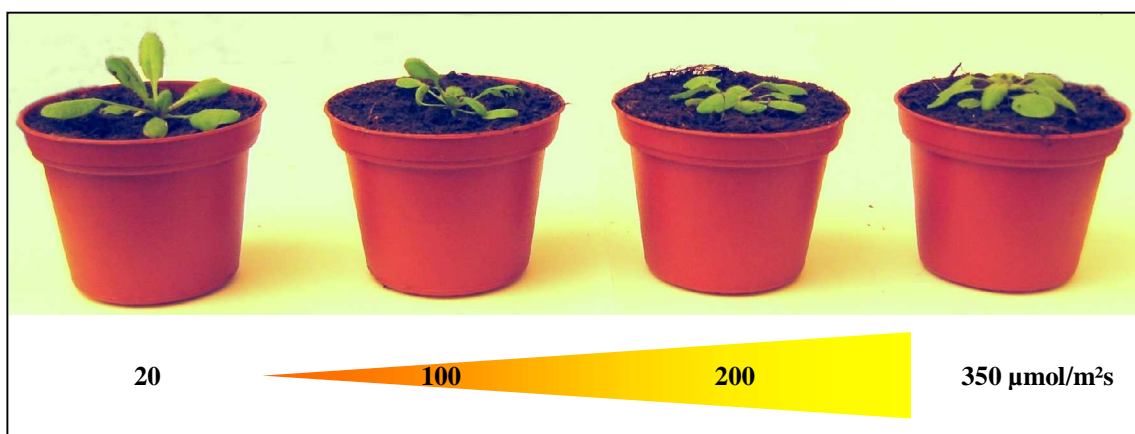


Image 2.2: *A. thaliana* Col plants kept at  $9 \text{ }^\circ\text{C}$  air temperature and different irradiances for several days.

As mentioned before, samples of these plants were analysed further by HPLC. The following diagrams present the results in detail. A relatively weak increase in total UV-

A absorbing compounds with increasing irradiances at low temperature conditions was detected. Mainly kaempferol glycosides and to a lesser extent quercetin glycosides were accumulated with increasing irradiance (figure 2.23). The amount of HCAs did not change upon increased irradiances, however at the lowest irradiance of 20  $\mu\text{mol}/\text{m}^2\text{s}$  white light the amount of HCAs was surprisingly high, namely about 55  $\text{AU}^*\text{s}/\text{cm}^2$ . At all other PPFDs and in all other experiments the amount of HCAs was always around 40  $\text{AU}^*\text{s}/\text{cm}^2$  (compare with figures 2.5, 2.10 or 2.17). In contrast, an increase in total HCAs at low PPFD was observed between 50 and 130  $\mu\text{mol}/\text{m}^2\text{s}$  in a light curve experiment at 22 °C (compare with figure 2.6).

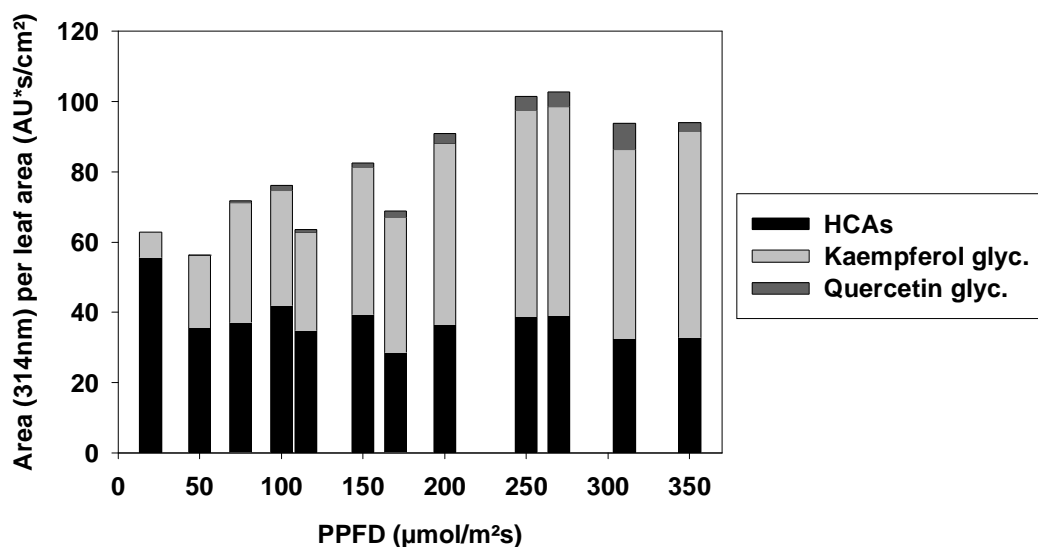


Figure 2.23: Main pigment composition in rosette leaves of *A. thaliana* Col treated with different PPFDs at low temperature (9 °C). Presented are the amount of HCAs, kaempferol and quercetin glycosides as a stacked bar graph against PPFD (n = 3).

Also, composition of HCAs in those low temperature treated leaves did not change markedly with increasing PPFD (figure 2.24). Sinapoylmalate was always the main HCA found and four additional glycosides were present in all leaves in small amounts. However, in leaves which were illuminated with only 20 and 50  $\mu\text{mol}/\text{m}^2\text{s}$  the pigment 5-OH-feruloyl glycoside was completely missing. In addition, leaves illuminated with only 20  $\mu\text{mol}/\text{m}^2\text{s}$  displayed strongly increased amounts of sinapoylmalate (t-test:  $P = 0.020^*$ ) and sinapoyl glycoside (1) (t-test:  $P = 0.063$ , ns) in comparison to 50  $\mu\text{mol}/\text{m}^2\text{s}$ . In summary it can be stated that sinapoylmalate was increased upon the low temperature treatment from about 20 at 22 °C to 30  $\text{AU}^*\text{s}/\text{cm}^2$  (compare with figure 2.7) and that sinapoyl glycoside (1) was reduced with increasing PPFD. Thus, the lowest amount of this compound was found at the highest PPFD. The HCA 5-OH-feruloyl glycoside was missing at low temperature only if the irradiance was low, meaning at higher PPFDs this substance was present in the leaf samples. In conclusion, the sinapoylmalate precursor sinapoylglucose (1) was slightly increased with increasing PPFD at 22 °C and was slightly reduced with increasing PPFD at 9 °C air temperature.

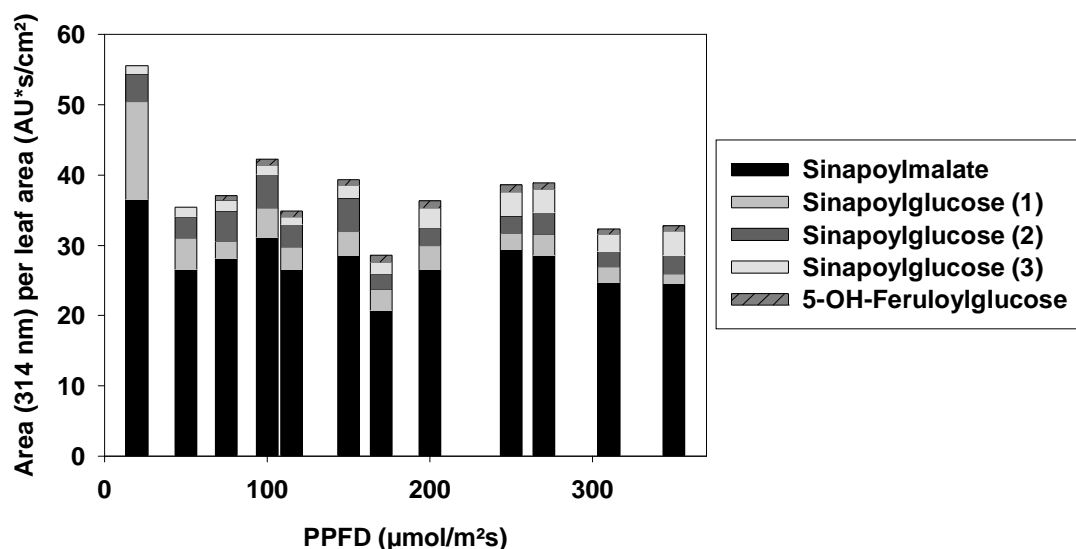


Figure 2.24: Composition of HCAs in leaves of *A. thaliana* Col exposed to increasing irradiances at 9 °C air temperature for ten days (n = 3).

Flavonol glycosides, detected as derivatives of kaempferol and quercetin, accumulated with increasing PPFD at low temperature (figure 2.25). The amount of quercetin glycosides was very small in comparison to kaempferol derivatives though their concentration was much higher at irradiances of 200  $\mu\text{mol}/\text{m}^2\text{s}$  upwards than in earlier single-factor experiments (compare with figure 2.8). In addition, at 22 °C quercetin glycosides were detected only at a PPFD of 200  $\mu\text{mol}/\text{m}^2\text{s}$  upwards, whereas at low temperature conditions these substances were accumulated in leaves already at 100  $\mu\text{mol}/\text{m}^2\text{s}$  upwards. Three different kaempferol glycosides were present in nearly equal parts. No further increase in total flavonol glycosides was detected from a PPFD of 200  $\mu\text{mol}/\text{m}^2\text{s}$  upwards indicating a maximum flavonol content of about 63  $\text{AU}\cdot\text{s}/\text{cm}^2$ . In comparison, at 22 °C the maximum flavonol content was only the half of it namely about 32  $\text{AU}\cdot\text{s}/\text{cm}^2$ . However, composition of kaempferol glycosides at 22 and 9 °C was very similar. Surprisingly, quercetin derivative (3) was not detected in any leaf sample of the light curve experiment at 9 °C.

The light dependency of kaempferol accumulation at low temperature becomes clearer if single derivatives were displayed as a line/scatter plot (figure 2.26). All three kaempferol glycosides were accumulated in a light-dependent manner with the steepest increases between 20 and 75  $\mu\text{mol}/\text{m}^2\text{s}$  white light. The strongest relationship between the amount of pigment and PPFD was detected for kaempferol rhamnopyranoside-rhamnopyranoside which was the main kaempferol glycoside found in leaf samples of *A. thaliana* Col.

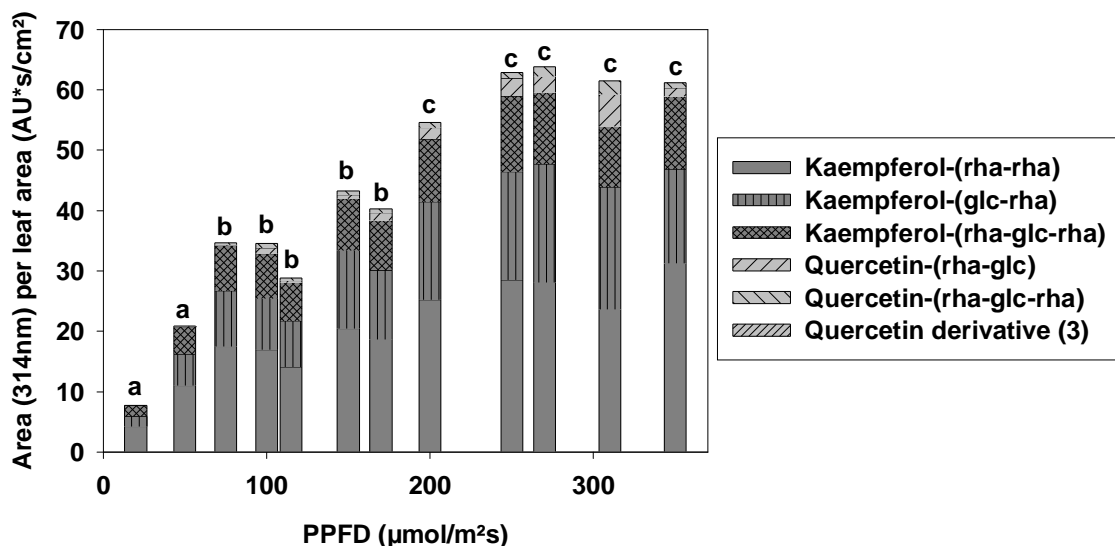


Figure 2.25: Flavonoid composition in leaves of *A. thaliana* Col exposed to increasing irradiances at 9 °C as detected by HPLC (n = 3). Significant differences among PPFDs for total flavonoids were discovered by a one-way ANOVA and a tukey-test as post-hoc-test and are indicated by different letters. For level of significance refer to table 2.2.

Table 2.2: Results of the tukey-test presented for individual PPFDs. Different letters indicate significant differences. In cases where level of significance was not  $P < 0.001$ \*\*\*, the actual P-value is given in brackets.

PPFD	20	50	75	100	115	150	170	200	250	270	310	350
350	a	a	a(0.003)	a(0.003)	a							<b>b</b>
310	a	a	a(0.009)	a(0.008)	a(0.001)						<b>b</b>	
270	a	a	a(0.001)	a	a	a(0.037)	a(0.011)			<b>b</b>		
250	a	a	a(0.002)	a(0.001)	a		a(0.016)		<b>b</b>			
200	a	a	a(0.046)	a(0.040)	a(0.004)			<b>b</b>				
170	a						<b>b</b>					
150	a	a(0.018)				<b>b</b>						
115	a(0.024)				<b>b</b>							
100	a(0.002)			<b>b</b>								
75	a(0.002)		<b>b</b>									

The preceding experiments showed a flavonol accumulation at low temperature conditions in a light-dependent manner, meaning that with increasing irradiance more and more flavonol glycosides accumulate up to a concentration of about 63 AU\*s/cm<sup>2</sup> (figure 2.25). As expected, both factors, high light and low temperature, intensified each other in the induction of flavonol biosynthesis. This was especially prominent in increased amounts of quercetin glycosides. In contrast, no inducing effect was observed on HCA biosynthesis.

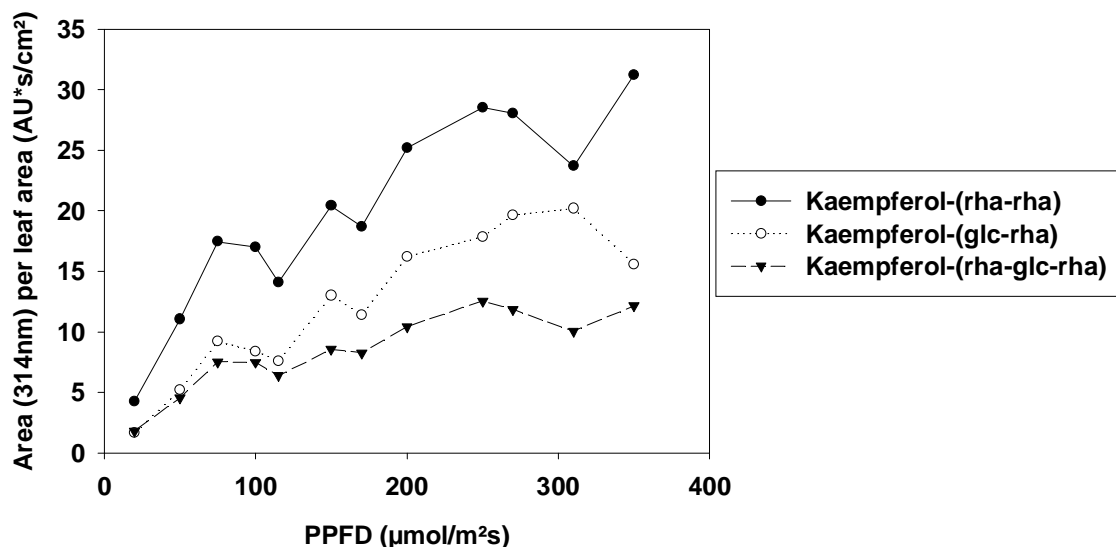


Figure 2.26: Flavonoid composition in leaves of *A. thaliana* Col exposed to increasing irradiances at 9 °C presented as a line/ scatter plot. This diagram shows the same data for kaempferol derivatives (ten-day low temperature and light treatment of rosette leaves of *A. thaliana* Col for ten days, n = 3) as in the figure 2.27.

#### 2.1.4 The role of temperature in epidermal flavonoid accumulation

##### 2.1.4.1 Irradiance-UV-A transmittance relationships modulated by different temperatures

In the previous paragraphs I could show a flavonoid accumulation as a result of moderately adverse environmental conditions like increased irradiance, lowered temperature and increased UV-B burden. I also showed dose-response relationships for the factors light and UV-B. In order to investigate the additional role of temperature, light curves at 22°C, 15 °C and 9°C air temperature were performed. The figure 2.27 presents transmittance data of these light curves. With increasing irradiance UV-A transmittance decreased for all temperatures. The steepness of the curves was very similar whilst the levels of transmittance were different. The low temperature curve ranged from 60 to 20 %, the warm temperature curve from 90 to 50 % and the 15 °C-curve was placed directly between these two. These results demonstrate that both factors, light and temperature had a positive effect on flavonoid accumulation and both factors could amplify themselves. Findings also indicate that *A. thaliana* would finely regulate epidermal flavonoid accumulation according to present temperature and irradiance.

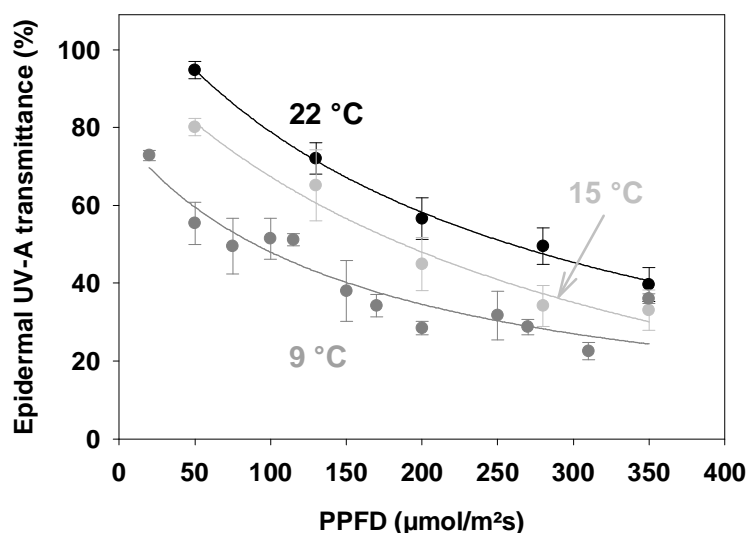


Figure 2.27: Epidermal UV-A transmittance in *A. thaliana* Col in dependence of irradiance after exposure to various irradiances at different temperatures for seven (22 °C and 15 °C,  $n = 9$  for all data points,  $R^2 = 0.951$  and  $0.954$ ) or 10 days (9 °C,  $n = 3$ ,  $R^2 = 0.881$ ).

#### 2.1.4.2 Darkness and low temperature

The light-dependent accumulation pointed to a special role of the factor PPFD in the accumulation of flavonoids at low temperature. Therefore, *A. thaliana* Col plants were exposed to low temperature conditions in complete darkness for several days and transmittance and pigments were determined after seven days. A timeplot of epidermal UV-A transmittance showed no change in transmittance throughout the experiment pointing to a lack of additional low temperature-induced flavonol accumulation in the upper epidermis when light was missing (figure 2.28).

A comparison of dark-treated plants with plants which were exposed to “normal” low temperature conditions under long-day conditions is presented in figure 2.29. It becomes obvious that without any light no epidermal UV-A shield could be established.

However, transmittance measurements can only give insight in the *epidermal* UV-A shield but not into deeper cell layers. Thus it could be possible that in darkness and low temperature flavonoids would accumulate in other tissues than the adaxial epidermis. As a consequence HPLC analysis was performed on these leaves in comparison to control leaves, which were monitored simultaneously and “normal” low temperature treated leaves. Dark-treated plants displayed significantly reduced amounts of HCAs and identical amounts of kaempferol glycosides in comparison to control plants (figure 2.30, grey letters).



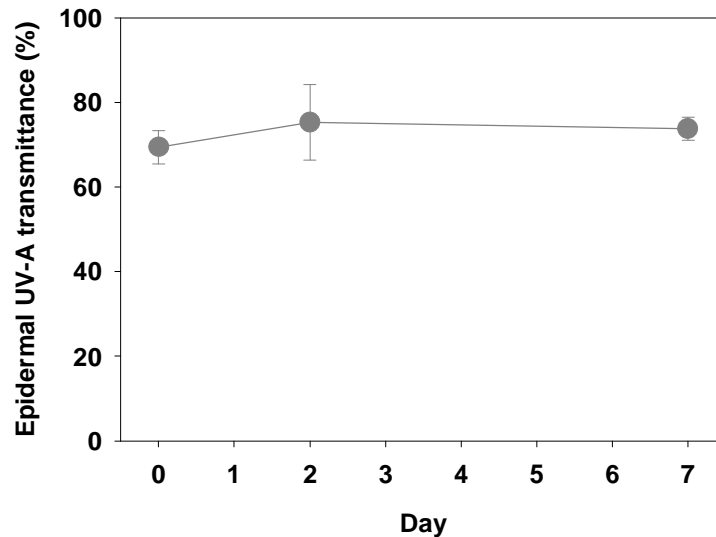


Figure 2.28: Timeplot of epidermal UV-A transmittance as measured with the UV-A-PAM in leaves of *A. thaliana* Col plants which were held at 9 °C air temperature and in absolute darkness. No significant change in transmittance was detected within seven days (n = 6). A one-way ANOVA proved that there are no statistically significant differences.

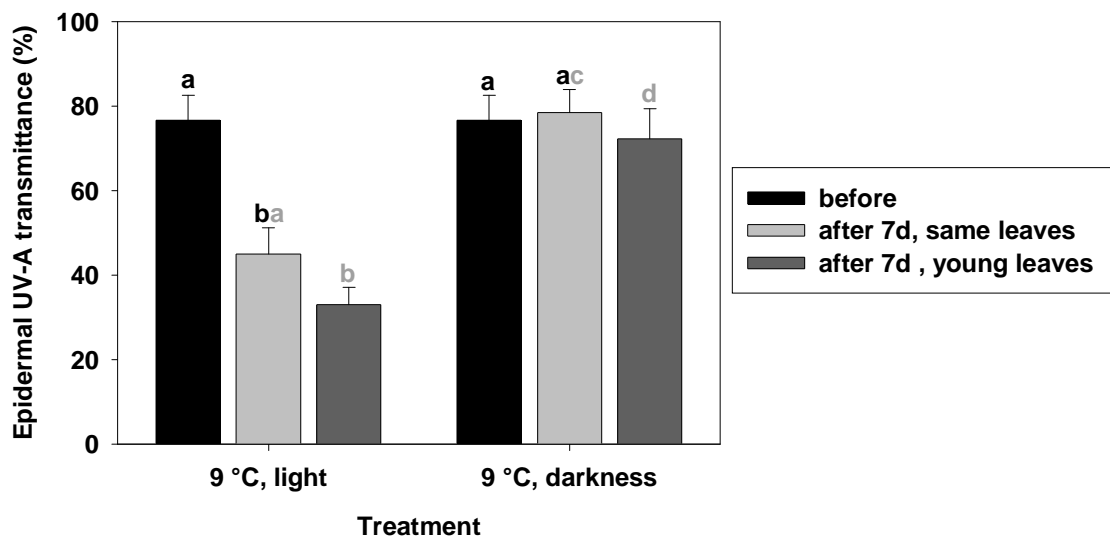


Figure 2.29: Epidermal UV-A transmittance in rosette leaves of *A. thaliana* Col before (black columns, n = 15) and after one week (grey columns, n = 15) at low temperature in light (left columns) or in darkness (right columns). After the treatment also new and young leaves were measured (dark grey columns, n = 15). Statistically significant differences were identified using two-way ANOVAs and tukey-tests as post-hoc tests. They are indicated by different letters of same colour. P was always high with  $P < 0.001^{***}$ .

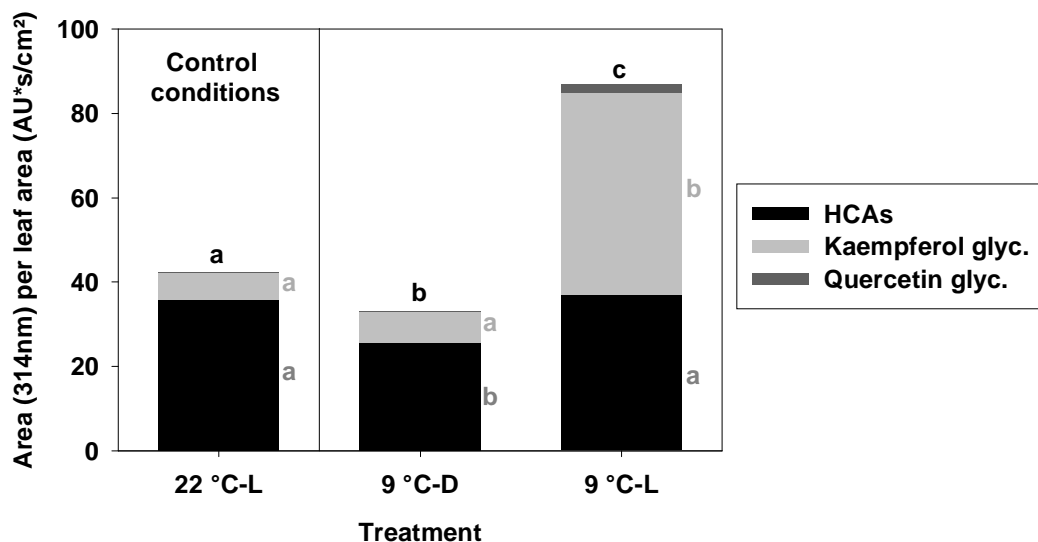


Figure 2.30: HPLC results of young rosette leaves of *A. thaliana* Col which were kept at control conditions (22 °C-L, left column), at low temperature conditions (9 °C-L, right column) and at low temperature without light (9°C-D, middle column; n = 8 for all data points). All total peak areas were significantly different to each other (t-test: P(ab) = 0.008\*\*, P(ac) and P(bc) < 0.001\*\*\*, black letters), whereas the sum of kaempferol glycosides and quercetin glycosides at 9 °C-L was significantly different to 22 °C-L and 9 °C-D, which in turn were not significantly different to each other (t-test: P < 0.001\*\*\*, grey letters). The amount of HCAs at 9 °C-D was significantly lowered in comparison to 22 °C-L and 9 °C-L (t-test: P < 0.001\*\*\*, dark grey letters) which were not different to each other.

HPLC analysis revealed that the flavonol compositions was also identical (data not shown). Few quercetin glycosides were accumulated in leaves which were illuminated under low temperature.

In summary it can be concluded that light played a crucial role in the accumulation of flavonoids at low temperature. In complete darkness and low temperature leaves were identical “control leaves”, thus, low temperature alone was successful in inducing flavonol accumulation in those leaves. Results of HPLC analysis clearly indicate that low temperature alone did not result in any additional accumulation of flavonoids in the leaf. It might have an effect on HCAs as they were decreased in darkness and low temperature, due to a low temperature typical decrease in sinapoylglucose (1) and absence of 5-hydroxy feruloylglucose.

### 2.1.4.3 The effect of single-period short-term low temperature treatments on epidermal flavonoid accumulation

Low temperature led to an accumulation of flavonol derivatives in the upper epidermis of rosette leaves of *A. thaliana* Col plants only if plants were illuminated with long-day light. Results could demonstrate that at an irradiance of  $120 \mu\text{mol}/\text{m}^2\text{s}$  only long-day conditions were able to induce this accumulation (refer to figure 1.9 in part one). Consequently the question arose if a low temperature treatment of less than 24 hours per day would also be capable in inducing an epidermal flavonol accumulation. Therefore *Arabidopsis* plants were exposed to low temperature for one single period of 1 up to 24 hours and then transferred back to control conditions. Epidermal UV-A transmittance was followed over four days. No flavonoids were accumulated in their leaves. Only leaves of plants, which were treated with prolonged low temperature conditions did accumulate flavonoids. The 24 hours treatment showed an initial effect. Of course, induction stopped after return to high temperature (figure 2.31).

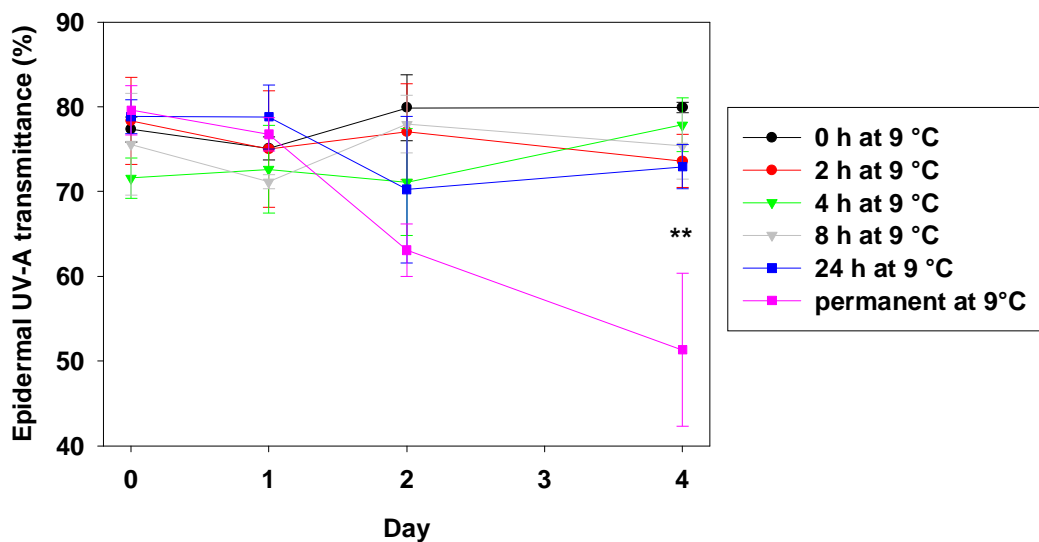


Figure 2.31: Low temperature induction experiment with *A. thaliana* Col. Plants were incubated either with low temperature conditions for a single period of 2, 4, 8 and 24 hours or kept permanently at control conditions (“0 h at 9 °C”) or at low temperature conditions (“permanent at 9 °C”). Light regime was  $120 \mu\text{mol}/\text{m}^2\text{s}$  18 hours per day, i.e. long-day conditions. Thus, the 24 h treatment consisted of a 18 h light period and a 6 h dark period at 9 °C. After the single treatment plants were kept at control conditions with 22 °C air temperature. Epidermal UV-A transmittance was measured before the treatment (day 0) and was followed over the next four days ( $n = 3$  for 0, 2, 4, 8 and 24 h and  $n = 6$  for permanent at 9 °C). At day 4 epidermal transmittance after the permanent low temperature treatment was significantly different to all other short-term treatment (t-tests:  $P \leq 0.01^{**}$ ). At day 2 transmittance of this treatment was significantly different to means of a low temperature treatment for 0 h, 2 h and 8 h (t-tests:  $P < 0.001^{***}$  for 0 and 8 h and  $P = 0.004^{**}$  for 2 h).

### 2.1.5 Indications for a metabolization of flavonoids

Until now, only the start and the end of induction experiments were investigated and the response of leaves, which were present at start and those, which developed throughout the experiment. However, transmittance data exist of “old” leaves, i.e. those leaves, which were measured before the transfer to new conditions. Figure 2.32 shows the timeplot of epidermal UV-A transmittance for those leaves of the light curve experiment at 22 °C (figure 2.5) which were exposed to only 50  $\mu\text{mol}/\text{m}^2\text{s}$ . A significant increase in transmittance was detected after five days, indicating a loss of a certain fraction of epidermal flavonoids.

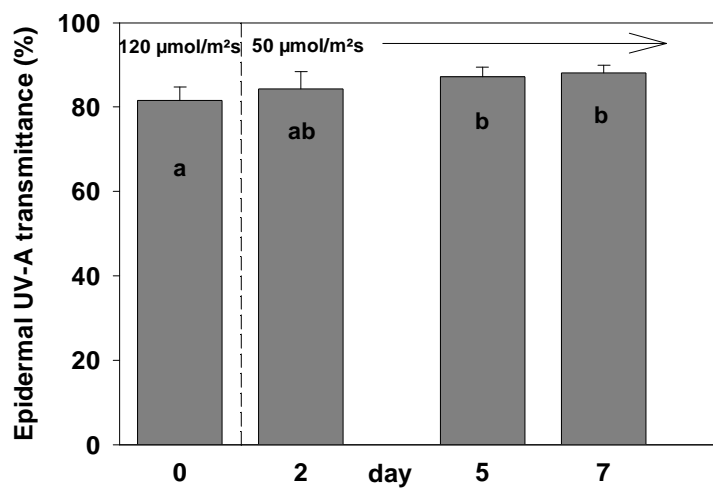


Figure 2.32: Timeplot of increase in epidermal UV-A transmittance in same rosette leaves of *A. thaliana* after transfer from control irradiance of 120  $\mu\text{mol}/\text{m}^2\text{s}$  to 50  $\mu\text{mol}/\text{m}^2\text{s}$ . Significantly increased transmittance values were detected after five days at 50  $\mu\text{mol}/\text{m}^2\text{s}$  (one-way ANOVA among days,  $n = 9$  for every day).  $P < 0.001^{***}$  for day 0 vs. day 7 and  $P = 0.002^{**}$  for day 0 vs. days 5.

In addition, figure 2.33 shows some data on measurements, which were performed with plants which were treated with UV-B for six days and then allowed to “recover” for one week at control conditions. Leaves, which were present at the start of the UV-B induction increased significantly their UV-A transmittance slightly upon a shift back to control conditions, but did not reach their initial start values (one-way ANOVA and tukey:  $P < 0.001^{***}$ ). New leaves, which developed on plants during the recovery period had nearly as high transmittance values as non-induced leaves. However, there was still a statistically significant difference between the two means. In conclusion, these results suggest firstly, that the epidermal UV-A shield could be dynamically regulated at least partly depending on leaf age, because relatively old leaves retained a reduced capability to decrease the epidermal UV-A shield. Secondly, the accumulation of flavonoids was a single leaf process, meaning that each leaf sensed environmental

conditions individually and regulated flavonoid content accordingly, excluding a putative whole plant response. This was already observed by the leaf age dependency of epidermal UV-A transmittance.

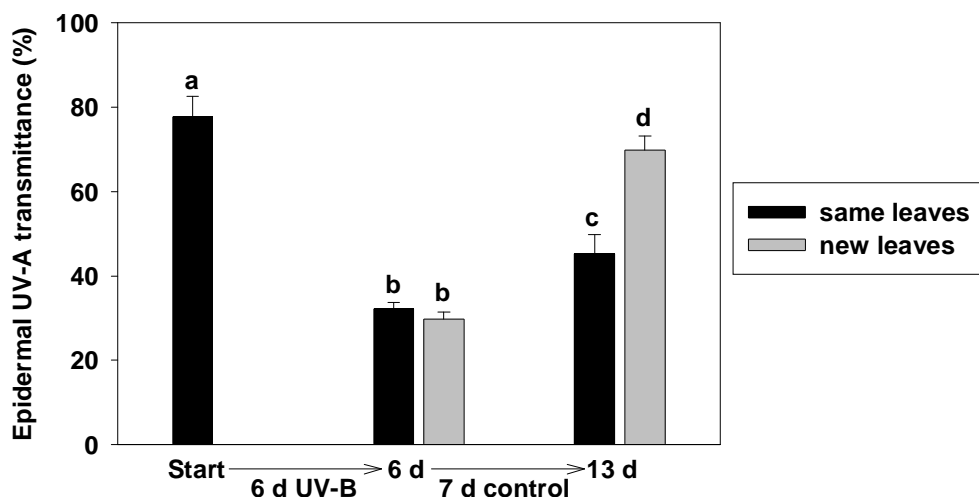


Figure 2.33: Recovery of epidermal UV-A transmittance after a six day UV-B treatment in *A. thaliana* Col (n = 18 for all columns with the exception of data point for “same leaves” after 13 days, where n = 17). Statistically significant differences are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$ .

#### 2.1.6 Validation of epidermal UV-A transmission measurements and HPLC analysis of UV-A absorbing compounds

Epidermal UV-A transmission measurements and HPLC analysis were mainly performed in this work. Whereas transmission measurements give information on the relative amount of UV-A absorbing compounds in the upper adaxial leaf epidermis, HPLC detects UV-A absorbing compounds in the total leaf. In order to compare both methods, a subsequent plot of peak sums of kaempferol and quercetin derivatives with epidermal UV-A absorbance from light curve experiments performed at 9 and 22 °C was produced and gave a good and positive correlation between these two parameters (figure 2.34).

Until now, it was always assumed that firstly, UV-A absorbing compounds in *A. thaliana* consist of only quercetin and kaempferol glycosides, and secondly, that UV-A absorbing compounds primarily exist in the upper epidermal layer of rosette leaves. With HPLC-MS no other flavonols than kaempferol and quercetin glycosides were identified. The figure 2.34 demonstrates that UV-A-PAM and HPLC gave very similar results pointing to an accumulation of flavonols mainly in the upper epidermis. Thus, both assumptions can be positively answered.

Figure 2.34 also indicates that a certain UV-A shield of about 0,1 absorbance (which is about 80 % transmittance) must exist even if no flavonols were accumulated according to HPLC analysis. This UV-A shield must be constitutive and made up by other UV-A absorbing compounds. These compounds might be HCAs which also have some absorption in the UV-A region of the light spectrum and are localized in the epidermis.

A similar regression was observed for values obtained before and after the UV-B treatment (not shown). However, only very low (from the control treatment) and very high values (from the UV-B treatment) were obtained because a UV-B curve was not analysed by HPLC. Nevertheless, a constitutive UV-A shield of about 0.1 absorbance was also detected.

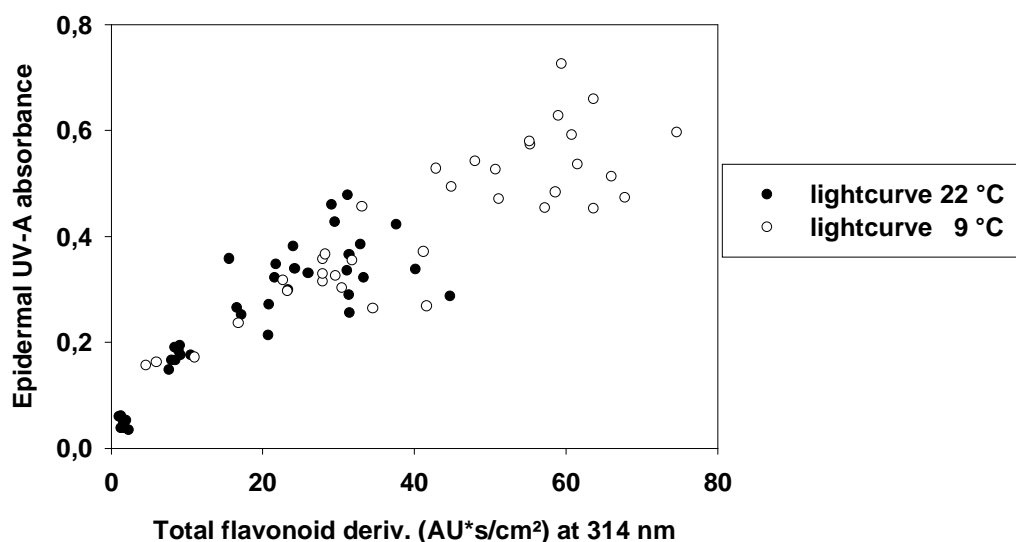


Figure 2.34: Correlation of total flavonoids as measured with HPLC with epidermal UV-A absorbance as measured with the UV-A-PAM of lightcurves at 22 and 9 °C air temperature (n = 40 and 35 for 22 °C and 9 °C, respectively).

### 2.1.7 Localisation of flavonoids in rosette leaves of *A. thaliana* Col

The previous paragraphs presented results mainly from epidermal UV-A transmittance measurements which are indirect as they give only relative values for the amount of UV-A absorbing compounds in the upper epidermal layer. HPLC results, which were also described in detail, are also somehow indirect as they give only the amount of pigment per leaf area and cannot provide with any information on the localisation of flavonol glycosides within the leaf and the cells. In order to get some information about the localisation of low temperature-induced flavonoids in the leaf on a tissue and cellular level whole leaves and cross-sections were investigated further with CLSM

technique applying the flavonoid sensitive dye Naturfarbstoffreagenz A (Nat. A) which was capable in visualizing flavonoids.

In non-induced control leaves as well as in treated leaves stomata of the abaxial epidermis gave strong fluorescence signals, indicating that even under control conditions in these cells flavonol glycosides and HCAs were accumulated (image 2.3).

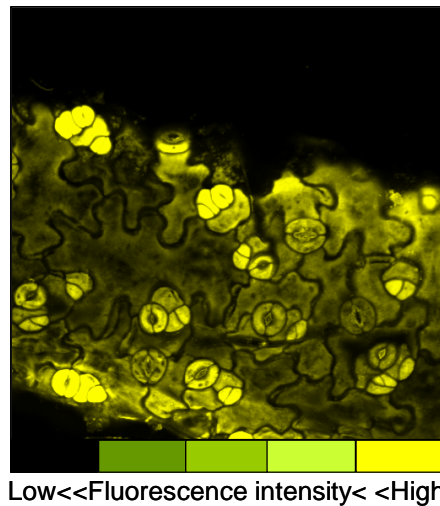


Image 2.3: Strip of abaxial epidermis of a control rosette leaf of *A. thaliana* Col treated with Nat. A and analysed in the CLSM. The image presents Nat. A fluorescence at 560-600 nm. High fluorescence signals were detected in stomatal cells indicating a high flavonoid content which was present constantly in control as well as in treated leaves. In contrast, abaxial epidermal cells of control leaves only displayed low fluorescence signals.

As it can be seen on the images below, substances bound to Nat. A were visible in cross-sections of non-induced leaves only in the stomata (image 2.3 and 2.4), whereas in low temperature treated leaves an accumulation of flavonoids was visible especially in cells of the upper epidermis and of the upper palisade parenchyma (image 2.5). Additionally, flavonoids appeared to accumulate also around nuclei of the upper parenchymal cells.

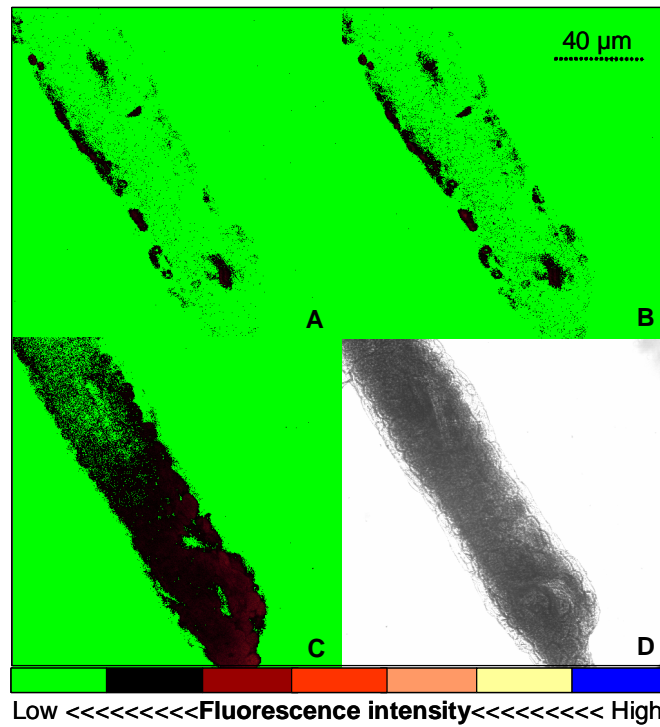


Image 2.4: Cross-section of control rosette leaf of *A. thaliana* Col treated with Nat. A and analysed in the CLSM. Images A and B present Nat. A fluorescence (emission at 515-550 nm and 560-600 nm, respectively), image C shows chlorophyll fluorescence (emission at 660-720 nm) and D is an image of transmitted white light through the cross-section.

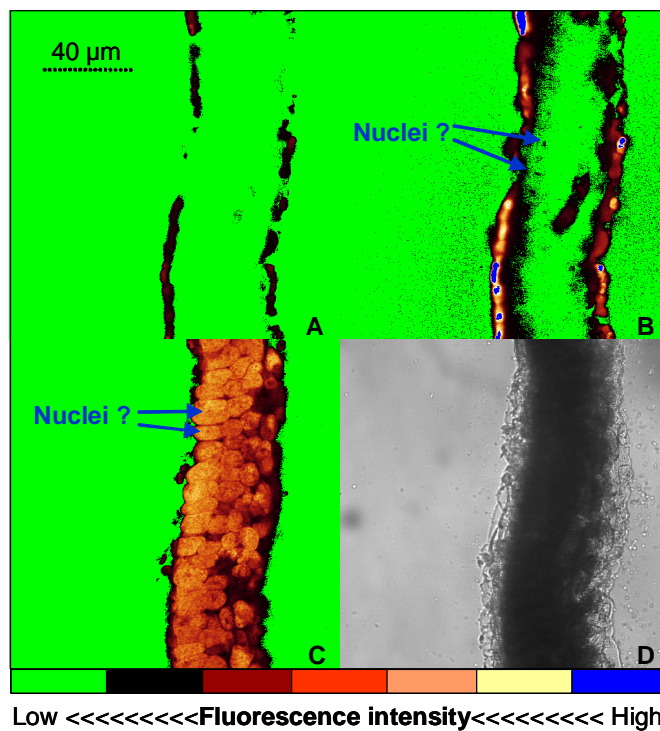


Image 2.5: Cross-section of low temperature-induced rosette leaf of *A. thaliana* Col treated with Nat. A reagent and analysed in the CLSM. Plants were treated with low temperature conditions



for eight days. Images A and B presents Nat. A fluorescence (emission at 515-550 nm and 560-600 nm, respectively), image C shows chlorophyll fluorescence (emission at 660-720 nm) and D is a image of transmitted white light through the cross-section.

The abaxial epidermis also contained a small amount of these substances and also anthocyanins, visible without any dye (image 2.6 below).

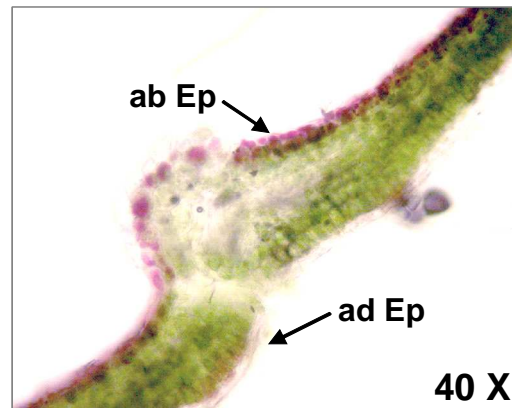


Image 2.6: Microscopy photography of a cross-section of a rosette leaf of *A. thaliana* Col treated with low temperature for several days (image from Prof. Dr. Christine Desel, University of Kiel, Germany). The abaxial epidermal layer (ab Ep) was filled with violet-coloured anthocyanins, whereas only a small amount of these substances were visible in the adaxial epidermis (ad Ep). No additional anthocyanins were detected in the mesophyll.

## 2.2 Discussion (Second part)

### *Principles of flavonoid accumulation after treatment with low temperature in comparison to treatment with high irradiances or supplemental UV-B radiation*

Measurements of epidermal UV-A transmittance with the UV-A-PAM are non-destructive allowing repeated measurements on identical leaves at different time points throughout the experiments. Time plots of epidermal UV-A transmittance showed different kinetics for high irradiances or UV-B and low temperature. Whereas high light and UV-B led to an abrupt decrease in transmittance reaching a minimum early after two days of incubation, transmittance decreased steadily over a period of at least seven days at low temperature conditions. End values were similar for UV-B and low temperature, namely about 30 to 40 %, but were still 60 % for the high light treatment.

Adaxial UV-A transmittance was lowered significantly in leaves of *Vicia faba* when transferred from 21 °C to 9 °C air temperature at a PPFD equal or higher than 125  $\mu\text{mol}/\text{m}^2\text{s}$  (Bilger et al. 2007). Start and end values of the low temperature treatment for *A. thaliana* Col were 78 and 30 %, respectively, thus a 48 % decrease in epidermal UV-A transmittance was observed. This value is in contrast to results for *A. thaliana* Ler as measured by Bilger and co-workers recently (2007), where a 70 % decrease in UV-A transmittance was measured after the low temperature treatment. However, the low temperature treatment was possibly much longer for *A. thaliana* Ler as it was grown from seeds at 9 °C air temperature and was not only transferred to low temperature conditions. In addition, light conditions were possibly much stronger than in this work because experiments were performed in the greenhouse where illumination was confined to at least 125  $\mu\text{mol}/\text{m}^2\text{s}$  under overcast weather conditions.

Epidermal UV-A transmittance was also strongly reduced upon UV-B treatment in young leaves of *Vicia faba* (Bilger et al. 2001). Similar to results obtained with *A. thaliana* Col in this work, transmittance declined from about 105 % to about 70 % within six days and an abrupt decrease was observed between the first and second day of the UV-B treatment. A strong decline in epidermal UV-A transmittance from 0.8 to 0.2 over a period of seven days in leaves of grape plants (*Vitis vinifera*) which were transferred from a shaded environment to solar radiation of maximum 1500  $\mu\text{mol}/\text{m}^2\text{s}$  was observed earlier (Kolb et al. 2001). In contrast, epidermal blue-green (BG)-fluorescence did not decrease indicating that chlorophyll fluorescence was not reduced by blue light absorbing anthocyanins.

End values of UV-A transmittance of high light treated leaves were higher than end values obtained after the low temperature or UV-B treatment. However since epidermal UV-A transmittance at 22 °C was irradiance dependent, it could be very possible that with a higher irradiance even lower values than 60 % could be reached. Unfortunately, higher irradiances than 350  $\mu\text{mol}/\text{m}^2\text{s}$  could not be tested because this PPFD was already the maximum intensity reached by the installed lamps.

A significant decrease in epidermal UV-A transmittance at low temperature conditions was only observed when low temperature was extended. A single treatment with low temperature up to 24 hours did not result in epidermal flavonoid accumulation indicating the need for a permanent signal in order to induce epidermal flavonoid accumulation. These observations point to a complex regulation of epidermal UV-A transmittance by different external stimuli including temperature, irradiance, light quality and UV-B. The close interplay of temperature and irradiance also indicates that a temperature sensitive light sensor must exist which is stimulated more at low air temperature.

A dose-response relationship for epidermal UV-A transmittance was measured for different PAR, three different temperatures and different UV-B dosage. The influence of the temperature gradient on the low temperature-induced reduction in epidermal UV-A transmittance in leaves of *Vicia faba* was investigated by Bilger et al. recently (2007). A reduction by only 3 °C from control temperature already led to markedly lowered UV-A transmittance values. A dose-response relationship for UV-B up to 150 mW/m<sup>2</sup> and flavonoid accumulation (measured as absorption at 330 nm) in aerial tissues of *A. thaliana* Col was observed earlier by Lois (1994). Higher UV-B intensities led to excessive damage to plants resulting in decreased flavonoid contents. In my work, *A. thaliana* Col plants were still capable to accumulate UV-A absorbing compounds when irradiated with about 300 mW/m<sup>2</sup> (figure 2.16), which is twice as much as 150 mW/m<sup>2</sup>. However, Lois' plants were very small and young and vulnerable when they had to cope with not only high intensity UV-B for 24 hours per day but also with high intensity white light for 24 hours per day, all of which could explain the severe damage of this treatment to plants and the resulting decreased flavonoid contents.

HPLC analysis allowed not only to measure flavonoid accumulation but also the amount and composition of five different UV-B absorbing HCAs. No study exists in which the composition of HCAs and flavonoids was investigated after treatment by various external factors. While the amount of total HCAs did not change significantly after any treatment, a strong accumulation of flavonoids was observed after every treatment (figure 2.35). With the exception of the UV-B treatment, where half of flavonoids was contributed by quercetin glycosides, mostly kaempferol glycosides accumulated. At control conditions no quercetin glycosides and few (6 to 9 AU\*s/cm<sup>2</sup>) kaempferol glycosides were detected. This observation was made earlier by Veit & Pauli (1999). The main kaempferol was always kaempferol rhamnopyranoside-rhamnopyranoside which made up about 50 % of all kaempferol glycosides.

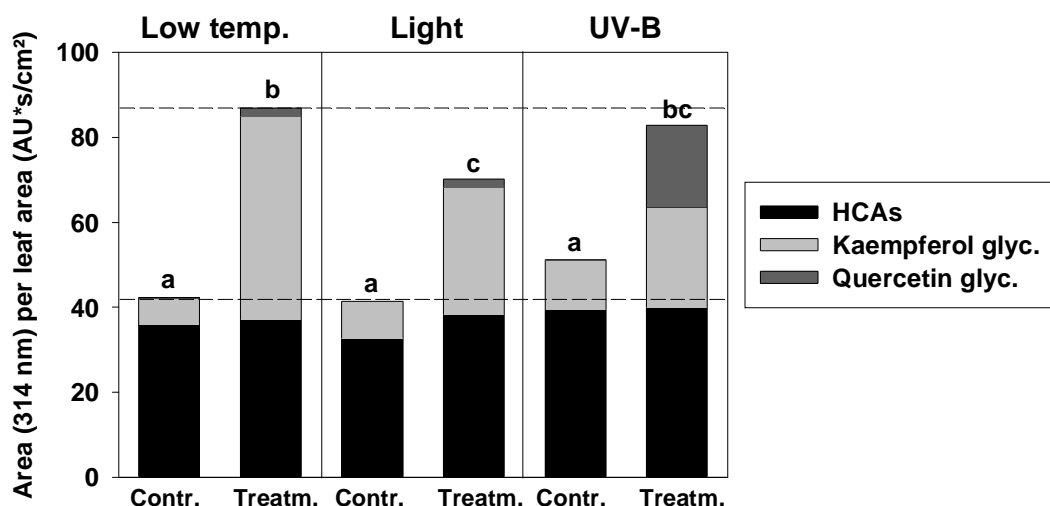


Figure 2.35: Main pigment composition in rosette leaves of *A. thaliana* Col after seven days at control conditions (Contr.) or treatments (Treatm.) with low temperature (n = 8), high light (n = 8 for control and n = 6 for high light) or UV-B (n = 15 for control and n = 18 for UV-B; exposure was only six days). Means of total pigments were compared with a two-way ANOVA and a tukey-test as post-hoc-test. Significant differences are indicated by different letters with  $P < 0.001^{***}$  with the exception for treatm.(light) vs. treatm.(low temp.) with  $P = 0.023^*$ .

All three conditions led to an increase in total flavonoids, total kaempferol glycosides and total quercetin glycosides. The question arose to which degree the effect of the three inducing conditions was similar and whether it could be concluded that equal signal transduction pathways were used by these three conditions. After all treatments the main kaempferol was again kaempferol rhamnopyranoside-rhamnopyranoside which made up 50 % of total kaempferol glycosides. The amount of quercetin glycosides was slightly increased after treatment with high irradiances or low temperature up to 2 AU\*s/cm<sup>2</sup>. In contrast, supplemental radiation with UV-B at 22 °C led to a severe accumulation of quercetin glycosides which made up 50 % of total flavonoids. The main quercetin was the quercetin derivative (3) which is presumably a quercetin rhamnopyranoside-rhamnopyranoside and made up 50 % of all quercetin glycosides. Thus, it appears that at least UV-B used a signal transduction pathway that was distinct to the pathway used by low temperature or high light. Interestingly, low temperature alone, i.e. low temperature and darkness, did not result in any flavonoid accumulation; control levels of kaempferol glycosides and no quercetin glycosides were detected. The main kaempferol with 50 % share on total kaempferol glycosides was again the kaempferol rhamnopyranoside-rhamnopyranoside.

In comparison, increased levels of quercetin glucopyranoside-rhamnopyranoside (rutin) and free quercetin were detected in half-cut leaves of tartary buckwheat (*Fagopyrum tataricum*) exposed to either UV-B (0.34 J/cm<sup>2</sup> for 30 min.), freezing (-5 °C for 5 min.) or drought stress (no water for 30 min.) after only 24 hours, with UV-B having the strongest effect on rutin and quercetin accumulation (Suzuki et al. 2005). More than half

of the rutin (53.1 %) was detected in the upper leaf epidermis of field grown tartary buckwheat plants.

The effect of UV-B on flavonoid content in *A. thaliana* was investigated by several scientists during the last 15 years. Li and co-workers (1993) could demonstrate an increase in leaf phenolic content, indicated as relative summed peak areas at 330 nm absorption, after supplemental radiation with UV-B (7.1 kJ/m<sup>2</sup>/day), which was maximum after only two days of treatment, whereas CHS mRNA level displayed maximum values early after six hours. It was also discovered, that beside kaempferol derivatives also two HCAs (sinapoylmalate and sinapoylglucose) were accumulated after UV-B treatment, an observation which contrasts my results (Chapple et al. 1992). No quercetin glycosides were found to be increased after UV-B radiation. A dose-dependent accumulation of both, sinapate esters and flavonoids in leaves of *A. thaliana* Ler after irradiation with two different UV-B dosages (1.5 and 2.8 kJ UV-B<sub>BE</sub>) was detected by Sheahan (1996). A UV-B induced, strong increase in kaempferol and quercetin glycosides in leaves of *Brassica napus* was observed for the first time by Olsson et al. (1998). An extremely strong increase in the ratio of quercetin glycosides in relation to kaempferol glycosides was also detected. Few years later, Ryan and co-workers (2001, 2002) report also on the strong accumulation of quercetin glycosides in *Arabidopsis* and *Petunia* after UV-B radiation and suggest a crucial role for the flavonoid-3-hydroxylase (F3H) which is responsible for the formation of dihydroquercetin from dihydrokaempferol. The amount of flavonoids was doubled and the ratio of quercetin glycosides to kaempferol glycosides was increased in comparison to control leaves of *A. thaliana*. Also and in agreement with my results, no change in the amount of HCAs was detected. A strong accumulation of flavonoids, measured as absorption at 330 nm, in *Arabidopsis* after UV-B treatment was observed recently by Kalbina & Strid (2006). No comparative pigment analysis of high light treated leaves of *A. thaliana* was performed until to date.

In summary, a strong accumulation of flavonoids in leaves of *Arabidopsis* after UV-B treatment was observed earlier by several scientists (see review of Hahlbrock & Scheel 1989, Li et al. 1993, Sheahan 1996, Ryan et al. 2001, Kalbina & Strid 2006) and is in agreement with my results. UV-B leads to a predominant accumulation of quercetin glycosides and a characteristic increase in the ratio of quercetin glycosides : kaempferol glycosides (Olsson et al. 1998, Veit & Pauli 1999, Ryan et al. 2001, Ryan et al. 2002), which was also observed in this work.

Light curves at 22 and 9 °C air temperature were also performed and analysed by HPLC. A light-dependent increase in total kaempferol glycosides was detected under both temperatures with similar gradients. However, the low temperature curve for kaempferol glycosides was always on a higher level of about 25 to 30 units (figure 2.36). Maximum levels were reached at a PPFD of 250 µmol/m<sup>2</sup>s with 60 and 29 units at 9 and 22 °C, respectively.

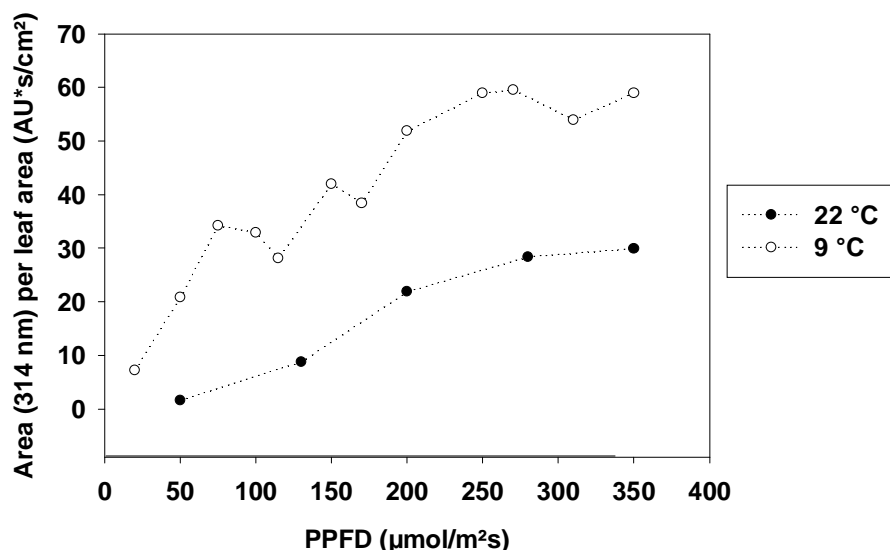


Figure 2.36: Increase in total kaempferol content with increasing PPFD at different temperatures of 9 or 22 °C. Data derive from light curve experiments at 9 and 22 °C. (22 °C: n = 9 for 50 and 280 µmol/m²s, n = 8 for 130 and 200 and n = 6 for 300 µmol/m²s; 9 °C: n = 3 for all data points).

A light-dependent increase in total quercetin glycosides was also observed. However, quercetin glycosides appeared at a PPFD higher than 50 and 130 µmol/m²s at 9 and 22 °C, respectively. The maximum amount of quercetin glycosides in these experiments was small in comparison to values obtained after UV-B radiation, where 19 AU\*s/cm² were detected.

An induction and regulation of flavonoid biosynthesis at low temperature and light was demonstrated. Measurements of epidermal UV-A transmission as well as HPLC analysis showed this clearly. The induction for low temperature and high light appeared to be identical whereas UV-B led to a more pronounced accumulation of quercetin glycosides besides of kaempferol glycosides.

In summary it can be concluded that low temperature and high irradiance displayed similar accumulation patterns for kaempferol glycosides and quercetin glycosides and that low temperature might have an additive effect on a light-induced flavonol accumulation. However, low temperature alone was not effective in inducing flavonoid accumulation. Thus, interactive signal transduction pathways for low temperature and light could be assumed. By contrast, high amounts of quercetin glycosides were accumulated only after a UV-B treatment, indicating a completely different signal transduction pathway under UV-B radiation.

Another aspect was the question if epidermal screening could be reduced by metabolizing flavonoids by the plant. A light-dependent relationship of epidermal flavonoid content was shown for 22 °C air temperature which included a lower flavonoid content at 50  $\mu\text{mol}/\text{m}^2\text{s}$  in comparison to the content at the control irradiance of 130  $\mu\text{mol}/\text{m}^2\text{s}$  (figure 2.5 and 2.6). However, as only youngest leaves were measured and sampled after exposure, one could argue that new leaves, which were “born” at the according irradiance, did accumulate as much flavonoids as “needed” at the present irradiance without any real regulation. However, a timeplot of transmittance data for “old” leaves, i.e. those leaves, which were measured before the transfer to new conditions of 50  $\mu\text{mol}/\text{m}^2\text{s}$  was presented in figure 2.32. A significant increase in transmittance was detected after five days, indicating a loss of a certain fraction of epidermal flavonoids and proving the potential for a metabolization of epidermal flavonoids at low irradiances. This is fairly astonishing because no indications exist in the current literature pointing to the presence of such a break-down of epidermal flavonoids (Bilger et al. 2007). Also, no mechanism is known by which vacuole-deposited flavonoids could be degraded. Another possibility could be a “dilution” effect during increase in leaf growth without increasing the flavonoid content. At start of timeplot experiments always those three leaves were chosen for analysis of epidermal UV-A transmittance which were youngest and fully developed. It could be very well possible, that at least the youngest of those three leaves increased their leaf area throughout the experiment. However, it was observed that essentially all leaves were found to reduce their epidermal UV-A shield (data not shown). Additionally, another experiment was presented in chapter 2.1.5 where the partial but significant recovery of epidermal UV-A transmittance after treatment with UV-B radiation was documented for “old” leaves (figure 2.33). Those plants were first grown at control conditions for 24 days, then treated with supplemental UV-B for six days and finally transferred back to control conditions for additional seven days. Thus, those leaves were young and already fully developed at start of the UV-B exposure but even when they were 13 days older, epidermal UV-A transmittance was again increased significantly, thus invalidating a potential “dilution”-effect by increase in leaf area.

Reduced absorbance values at 330 nm of leaf extracts of *A. thaliana* were observed after irradiation with high UV-B dosages and were explained by excessive UV damage resulting in an impairment of cellular functions (Lois 1994). However, Lois also observed a gradual decrease in total phenolics with increasing leaf age for individual leaf pairs including cotyledons independently of a UV-B treatment. As cotyledons do not increase their leaf size during plant growth and stay as small as they were at the seedling stage, a dilution effect appears again as unlikely. Conclusively, an unknown mechanism must exist in *Arabidopsis* by which vacuole-deposited flavonoids can be metabolized in a way that they do not contribute any more to the epidermal UV-A shield. More information on this phenomenon could possibly be obtained by following pigment composition via HPLC-MS analysis in those leaves.

*Principles of HCA composition after treatment with low temperature in comparison to treatment with high irradiances*

The total amount of HCAs did not change after treatment with either low temperature or high irradiances or supplemental UV-B (figure 2.35). However, their composition in leaves treated with low temperature or high light was different as compared to control plants. No change in composition was observed after a UV-B treatment. This last aspect is especially interesting since Landry et al. (1995) and Sheahan (1996) suggested after having investigated *Arabidopsis* mutants lacking flavonoids (tt5, tt4) or sinapate esters (fah1) that HCAs might be the better UV-B protectants than flavonoids. Sinapate esters (sinapoylmalate and sinapoylglucose) were only weakly accumulated in leaves of WT *Arabidopsis* after UV-B treatment in comparison to a strong flavonoid accumulation as shown in the works of Li et al. (1993) and Sheahan (1996). Thus, although being the better UV-B protectants, sinapate esters/ HCAs were only weakly accumulated in comparison to flavonoids according to the investigations of Li and Sheahan and were not accumulated under the conditions tested in this work.

In contrast, a strong accumulation of soluble phenolic acids upon cold acclimation (2 °C) was observed in leaves of winter oilseed rape (*Brassica (B.) napus*, Solecka et al. 1999). Similar to the accumulation of flavonoids observed in this study, the rate of HCA accumulation was dependent on temperature and a transient freezing of leaves of *B. napus* led to an extremely strong and rapid accumulation of total phenolic acids. Total phenolic acids consisted of four different HCAs, namely caffeic, p-coumaric, ferulic and sinapic acid. Although *B. napus* belongs to the same plant family as *A. thaliana*, both species may show a different response to temperature with respect to phenolic acid biosynthesis.

Table 2.3 summarizes changes in HCA composition after treatments with low temperature, high light and supplemental UV-B and which will be discussed in the following paragraphs.

High light led to a significant increase in the precursors of sinapoylmalate, sinapoylglucose (1) and feruloylglucose, whereas the amount of sinapoylmalate was significantly decreased at 350  $\mu\text{mol}/\text{m}^2\text{s}$ . A light curve experiment performed at 22 °C showed a light-dependent increase for the two precursors, so that lower values were obtained for PPFDs smaller than 120  $\mu\text{mol}/\text{m}^2\text{s}$ , which was the “control”-PPFD. The level of sinapoylmalate was found to be increased with increasing irradiance between 50 and 280  $\mu\text{mol}/\text{m}^2\text{s}$ .



Table 2.3: Summary of characteristics of HCA composition as observed after treatments with low temperature, high light (350  $\mu\text{mol}/\text{m}^2\text{s}$ ) and supplemental UV-B radiation in comparison to control conditions.

Parameter	Low temperature	High light	Suppl. UV-B
Total HCAs	+/- 0	+/- 0	+/- 0
HCA composition	changed	changed	not changed
Sinapoylmalate	↑	↓	-
Fluence rate-dependency of sinapoylmalate	no, always ↑	yes, up to 280 $\mu\text{mol}/\text{m}^2\text{s}$ : ↑	not investigated
Sinapoylglucose (1)	↓	↑	-
Feruloylglucose	missing (↓)	↑	-
Fluence rate-dependency of sinapoylglucose (1)	weakly ↓, always ↓ (not at 20 $\mu\text{mol}/\text{m}^2\text{s}$ )	yes, ↑	not investigated
Fluence rate-dependency of feruloylglucose	no, present at higher PPFDs	yes, ↑	not investigated
Sum of sin.glc (1), (2), (3)	↓	fluence-rate dependent ↑	-

At low temperature conditions, the total amount of HCAs was not changed, but very well the composition. The main HCA sinapoylmalate was significantly increased, whereas the precursor sinapoylglucose (1) was significantly reduced and feruloylglucose was missing. This finding is clearly opposite to results obtained from a high light treatment. A light curve experiment at low temperature conditions was also analysed by HPLC. There, the pigment sinapoylmalate was always increased in comparison to control levels, especially at the lowest irradiance of 20  $\mu\text{mol}/\text{m}^2\text{s}$ , indicating a general low temperature effect. The first precursor sinapoylglucose (1), which was strongly fluence rate-dependently increased at 22 °C, was found to be weakly fluence rate-dependently decreased at 9°C and was always lower than control levels with the exception of 20  $\mu\text{mol}/\text{m}^2\text{s}$  (figure 2.37). This observation is very surprising and difficult to find a plausible explanation for. The second precursor of sinapoylmalate, 5-hydroxy feruloylglucose, which was found to be positively accumulated with increasing irradiance at 22 °C, was not present at low PPFDs of 20 and 50  $\mu\text{mol}/\text{m}^2\text{s}$  and low temperature and displayed control levels at higher irradiances and low temperature.

In summary it can be stated, that HCA composition might be differentially regulated at warm and low temperatures. A positive fluence rate-dependent accumulation was detected for the two precursors of sinapoylmalate at 22 °C. Sinapoylmalate itself was fluence rate-dependently increased up to an irradiance of 280  $\mu\text{mol}/\text{m}^2\text{s}$  and so did the total amount of HCAs (figure 2.7) . Unfortunately, pigment composition at 350  $\mu\text{mol}/\text{m}^2\text{s}$  did not follow this light-dependent relationship as again reduced amounts of total HCAs and sinapoylmalate were measured. At 9 °C air temperature sinapoylmalate

was always increased in comparison to levels at 22 °C and a weak fluence rate-dependent decrease was observed for sinapoylglucose (1). The sum of sinapoyl glycosides (1), (2) and (3) was always and fluence rate-independently reduced in low temperature treated leaves. 5-OH feruloylglucose was not present at very low PPFDs but was found at higher PPFDs in a light independent manner and at control levels. At 9 °C, the total amount of HCAs was not changed with increasing irradiance (figure 2.24).

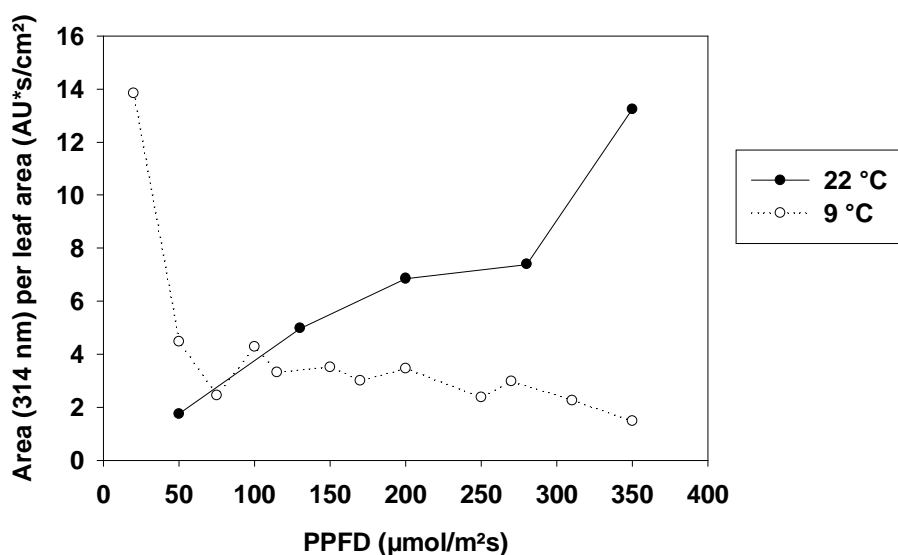


Figure 2.37: Level of sinapoylglucose (1) content at increasing PPFD at 9 or 22 °C air temperature. Data derive from light curve experiments at 9 and 22 °C. (22 °C: n = 9 for 50 and 280 µmol/m²s, n = 8 for 130 and 200 and n = 6 for 300 µmol/m²s; 9 °C: n = 3 for all data points).

So it appears as if at 22 °C the biosynthetic pathway to sinapoylmalate might be initially accelerated with increasing PPFD up to 280 µmol/m²s, but that at 350 µmol/m²s the last enzyme of sinapoylmalate biosynthesis, sinapoylglucose: malate sinapoyl transferase (SMT), would be inhibited, so that precursors would accumulate even more and reduced amounts of sinapoylmalate would be detected (figure 2.38). In contrast, at 9 °C more sinapoylmalate might generally be synthesized from the precursors by the enzyme SMT (At2g22990, SNG1) without accelerating the whole biosynthetic pathway resulting in a lower amount of total sinapoyl glycosides possibly in a light independent manner. The early precursor 5-hydroxy feruloylglucose was obviously only present when light was present, indicating that light was essential for activation of the according enzyme(s). However, a light-dependent regulation was only observed at 22 °C.

Results of gene expression studies via microarray analysis on high light or low temperature treated leaves of *A. thaliana* will be shown in part IV of this work. At this point of the work, the short information must be given, that gene expression results

could not confirm this last hypothesis (figure 4.4 and table 4.10). A potential post-translational regulation of the essential biosynthetic enzymes must also be considered.

Differences in HCA composition after the different treatments could also be explained by a growth effect. The content of sinapoylmalate changes extremely with plant age in *Arabidopsis* plants grown at 23 °C under continuous PAR of 100  $\mu\text{mol}/\text{m}^2\text{s}$ , which is similar to control conditions established in this work (Chapple et al. 1992). A strong increase up to the third week is followed by a slow decline over the following six weeks of plant life. Thus, it is possible that sinapoylmalate content was at its maximum when I started my experiments and after one week of exposure its content would have been halved provided that control conditions prolonged. As high irradiance and low temperature appeared to have an accelerating or retarding effect on plant growth, respectively, lower and higher sinapoylmalate contents would be expected in comparison to control conditions, as was indeed observed. The applied UV-B treatment did not have any visible effect on plant growth and development by visible inspection, so that a sinapoylmalate concentration as under control conditions observed would be expected, which was also true. However, the sinapoylmalate concentration was not consistently lowered at increased irradiances at 22 °C, so that this assumption must remain speculative.

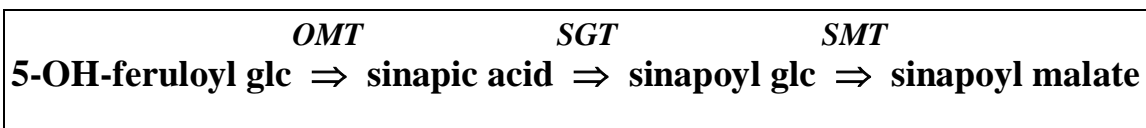


Figure 2.38: Schematic biosynthetic pathway of sinapoylmalate biosynthesis in *A. thaliana* adopted from Chapple et al. 1992 (glc = glucose, OMT = O-methyl transferase, SGT = UDP-glc sinapoyl transferase, SMT = sinapoyl glc: sinapoyl malate transferase).

#### *Validation of epidermal UV-A transmission measurements and HPLC analysis*

Epidermal UV-A transmission measurements and HPLC analysis were the main methods applied in this work. Whereas transmission measurements with the UV-A-PAM are non-destructive allowing repeated measurements on same leaves throughout the course of experiments and giving a relative value of the epidermal protection against UV-A radiation, HPLC analysis is destructive but gives detailed information on pigment amount and composition per sampled leaf area. In this work, chromatograms were recorded and analysed at 314 nm absorption, thus only those pigments were detected which dissolved in the extraction buffer and which had a specific absorption at 314 nm. Thus, it was not possible to detect any blue-light absorbing anthocyanins and their glycosides with both applied methods, though they were present sometimes in leaf extracts. However, experimental conditions were set up in a manner, that rosette leaves did not turn purple at all, especially not the adaxial side of the leaves. This was

important as any blue light absorbing compounds in the adaxial leaf side would artificially increase epidermal UV-A transmittance which is in fact the relation of fluorescence intensity derived from UV-A illumination ( $F(\text{UV-A})$ ) divided by the fluorescence intensity derived from blue light illumination ( $F(\text{BL})$ ). Additionally, it appeared as if a strong accumulation of anthocyanins was not primarily induced in *A. thaliana* by low temperature, high irradiance and supplemental UV-B radiation, but was induced by either drought, high temperature or nutrient deficiency (data not shown) and especially only in the shaded, abaxial leaf epidermis of *A. thaliana* (image 2.6). One argument for the proposition that anthocyanins do not accumulate upon UV-B treatment, are the reported absorption spectra of leaf extracts from non-treated and UV-B treated *Arabidopsis* plants which revealed a significant increase in absorption between 250 and 370 nm, where HCAs and flavonoids absorb, but not at 530 nm, where anthocyanins absorb (Lois & Buchanan 1994). In contrast, a clear accumulation of anthocyanins (absorption at 550 nm) by UV-B radiation was observed in leaves of *Lactuca sativa* (Krizek et al. 1998)

Unfortunately, very little information exists on anthocyanin accumulation in *Arabidopsis* rosette leaves. The flavonoid biosynthetic pathway is resolved and enzymes responsible for the formation of anthocyanins are known (Shirley et al. 1995, Noh & Spalding 1998, Solfanelli et al. 2006). However, frequently scientists did not distinguish between flavonoid accumulation and anthocyanin accumulation especially when investigating gene expression of earlier key biosynthetic enzymes like PAL or CHS (Tsukaya et al. 1991, Leyva et al. 1995, Yamamoto et al. 1998). Also, anthocyanin accumulation in *Arabidopsis* seedlings (which exhibit an “anthocyanin ring“ at the upper end of the hypocotyl, Yamamoto et al. 1998) was explored but not in rosette leaves, and if, anthocyanin content was indicated as the absorption value at 535 nm and no HPLC analysis was performed (Tsukaya et al. 1991, Shirley et al. 1995, McKown et al. 1996, Noh & Spalding 1998). Thus, until to date and surprisingly, it is not clear which and where anthocyanins occur in leaves of *Arabidopsis*. The biochemical pathway database “AraCyc” ([www.arabidopsis.org/tools/aracyc](http://www.arabidopsis.org/tools/aracyc)) lists pelargonidin glucoside and cyanidin glucoside as common anthocyanins in vascular plants (Mueller et al. 2003). However, an accumulation of anthocyanins in *Arabidopsis* plants was reported to occur during cold acclimation under low temperature like 4 °C or after illumination, which might be induced by sucrose (McKown et al. 1996, Solfanelli et al. 2006). Also, anthocyanin accumulation, measured as absorption at 570 nm, during cold acclimation at 2 °C was reported for leaves of *Brassica napus* (Solecka et al. 1999). A diurnal fluctuation in soluble sugars like sucrose, glucose and fructose in rosette leaves of *A. thaliana* under illumination with 90  $\mu\text{mol}/\text{m}^2\text{s}$  at 22 °C was measured by Solfanelli and colleagues (Solfanelli et al. 2006). Parallel determination of mRNA levels of several genes of the flavonoid and anthocyanin synthesis revealed a weak diurnal fluctuation for PAP1, for all anthocyanin biosynthetic genes like *DFR*, *UF3GT*, *LDOX* and for *F3H* and *F3'H* of the flavonoid biosynthesis pathway. Genes coding for key enzymes of flavonoid biosynthesis like *PAL*, *CHS*, *CHI*, *FLS*, *4CL* and *C4H* displayed decreasing mRNA levels during the day. These results point to a sugar-regulated

accumulation of anthocyanins via PAP1, but they also support my hypothesis, that flavonoids and anthocyanins might be differentially regulated in rosette leaves of *A. thaliana*. However, the irradiance of 90  $\mu\text{mol}/\text{m}^2\text{s}$  applied in the cited work was low and was not sufficient to induce flavonoid accumulation in my work. It is also reported that anthocyanins accumulate at high irradiances of 150 and 175  $\mu\text{mol}/\text{m}^2\text{s}$  and warm temperatures of 21 °C in *A. thaliana* as detected by increased *DFR* mRNA levels and increased absorption at 530 nm (Jackson et al. 1995).

Light curve experiments performed at 22 and 9 °C gave a broad range of different epidermal UV-A transmittance values and according peak sums of total flavonoids as measured with HPLC. In order to prove whether epidermal UV-A transmittance is a good measure for flavonoid accumulation, results of both methods were compared. Thus, a correlation between these two parameters was performed in order to validate the epidermal UV-A transmission measurements. A good and positive correlation was observed for both temperatures (figure 2.34). Regression pattern was observed to be very similar and also a broad area of overlapping datapoints was observed. This was also observed for results from UV-B treatments (not shown). However, it was striking that firstly, variation increased with increasing UV-A absorbance and that secondly, a certain UV-A shield of about 0.1 absorbance must exist even when only very few flavonoids were accumulated. For information, no epidermal UV-A absorbance (i.e. 100 % UV-A transmittance) was detected in either shaded abaxial epidermal layers of control leaves (will be shown in third part of this work in figure 3.7) or when the epidermis was peeled off (paragraph III 3.1). Therefore, epidermal cell wall located, constitutive UV-A absorbing compounds like specific HCAs which show some absorption also in the UV-A region of the spectrum and also show some absorption at 375 nm (not shown), where UV-A transmittance was measured, could be responsible for this fraction of UV-A protection (Hutzler et al. 1998, Meyer et al. 1998, Ruegger et al. 1999, Franke et al. 2002, Nair et al. 2004). Alternatively, a potential co-accumulation of anthocyanin glycosides in the epidermal vacuole along with flavonoid accumulation could be the reason for the relatively large variation in epidermal UV-A absorption at higher flavonoid contents. Red pigmented extracts were frequently observed from leaves which were irradiated with high intense white light. In addition and as mentioned before, anthocyanins in the upper epidermis would artificially increase epidermal UV-A transmittance due to their strong absorbance of the blue light reference beam of the UV-A-PAM. In summary, it can be concluded that altogether a good agreement of both methods was observed which also proved that the epidermal UV-A absorption in *A. thaliana* Col was mainly made up by kaempferol and quercetin glycosides as detected by HPLC and HPLC-MS and that these flavonoids accumulate mainly in the upper epidermis layer. At higher concentration of flavonoids an accumulation of these compounds in the mesophyll layer must also be considered and was visible in leaf cross sections of *A. thaliana* which were treated with Naturstoffreagenz A (image 2.5).

A similar comparison of both methods was presented earlier for developing rye primary leaves of *Secale cereale* and for low temperature (11 °C) treated leaves of *Vicia faba*

(Burchard et al. 2000, Bilger et al. 2007). A linear relationship between the calculated epidermal UV-A absorbance and the extract absorbance at 366 nm was detected with a small fraction of constitutive epidermal absorbance when no UV-A absorbing compounds were found in the extracts (Burchard et al. 2000, figure 4 (a), Bilger et al. 2007, figure 2). Also, a (nearly) linear relationship was observed for my dataset.

The observation that flavonoids accumulate primarily in vacuoles of the adaxial epidermis was also tried to confirm by CLSM technique applying the polyphenol-sensitive fluorophore Naturstoffreagenz A (Nat. A) on control and low temperature treated leaves of *A. thaliana* Col (chapter 2.1.7). Nat. A was applied earlier and with success on primary leaves of *A. thaliana* (Sheahan & Rehnitz 1993), on stigmas of *Petunia hybrida* (Vogt et al. 1994), on needles of *Pinus sylvestris* (Schnitzler et al. 1996) and on leaves of several other species (Hutzler et al. 1998: *Vicia faba* and *Secale cereale*, Gould et al. 2000: *Quintinia serrata*, Neill et al. 2002: *Elatostema rugosum*, Gould et al. 2002: *Pseudowintera colorata*) and is sensitive to lignin and flavonol derivatives (Schnitzler et al. 1996). An apparently constitutive presence of polyphenols in stomatal guard cells was visible also in control leaves in the CLSM (image 2.3 and 2.4) which was also reported earlier for *Vicia faba* (Weissenböck et al. 1984, Hutzler et al. 1998) and *Pisum sativum* (Weissenböck et al. 1986). A low concentration was also detected in the adaxial epidermis, which is consistent with a certain UV-A shield at control conditions. Low temperature treated leaves displayed strong fluorescent signals in adaxial epidermal cells and to a lesser extent in abaxial epidermal cells (figure 2.5). This finding is in some contrast to epidermal UV-A absorption measurements where values near zero were detected in the abaxial epidermis (will be shown in third part, figure 3.7). However, no information is available if Nat. A also binds to anthocyanins which accumulate sometimes in the abaxial epidermis (figure 2.6). Interestingly, substances bound to Nat. A were also detected to be accumulated around nuclei of cells of the upper palisade parenchyma, an observation which was made earlier (Sheahan 1996, Hutzler et al. 1998, Peer et al. 2001) and is supported by the nuclear detection of flavonoid biosynthetic enzymes like CHS and CHI recently (Saslowsky et al. 2005).

### **3 Third part: Which signal mediates the low temperature response? Potential roles of oxidative stress, photosynthesis and light perception**

#### **3.1 Results**

In the chapter before it was shown that low temperature induced strongly flavonoid synthesis. From this observation the question arose which signal would mediate the low temperature response. Very essential for investigating this question was the observation that at low temperature in darkness no flavonoids were accumulated (figure 2.30 and paragraph 2.1.4.2). Thus, the mechanism of signal transduction had to rely on an interaction between light and temperature. As sensors several models came into question and will be presented in the following chapters. Oxidative stress products like reactive oxygen species (ROS, chapter 3.1.1), the reduction state of the psETC (chapter 3.1.2) or the role of light perception (chapter 3.1.3) and photoreceptors (chapter 3.1.3.3) were favoured as potential signal carriers. On the other hand, a low temperature induced calcium signal (Monroy et al. 1993, Örvar et al. 2000) could be excluded due to their independence of irradiation and on the basis of the missing effect of short-term low temperature treatments (figure 2.31). The possibility that sugar levels were involved in flavonoid accumulation at low temperature (Tsukaya et al. 1991, McKown et al. 1996, Mita et al. 1997, Solfanelli et al. 2006) could not be investigated due to time limitations.

##### **3.1.1 Oxidative stress/ ROS formation as a potential signal carrier**

Plants suffer from oxidative stress when an abrupt decrease in air temperature without a reduction in irradiance occurs (Feierabend et al. 1992, Dat et al. 2000). This stress may be visible in the accumulation of reactive oxygen species (ROS) like superoxide and hydrogen peroxide (Feierabend et al. 1992, Dat et al. 2000).

###### **3.1.1.1 Visualizing oxidative stress products in leaves of *A. thaliana* Col using NBT**

An increased formation of hydrogen peroxide and superoxide in the leaf tissue after a low temperature treatment of 27 hours could be detected with the dyes diaminobenzidin (DAB, data not shown) and nitrotetrazolium blue chloride (NBT). Accumulated hydrogen peroxide and superoxide were visible under the microscope as brown and blue spots, respectively, which were homogeneously spread over the leaf lamina. Loading of leaves with dye was achieved by the uptake of dye solution via the transpiration stream. Leaves kept at control conditions for 27 hours did not show any coloured precipitates spread over the leaf lamina. Only leaf veins were coloured, which was explained by ROS formation during the process of lignification. Results achieved with NBT are presented in image 3.1.

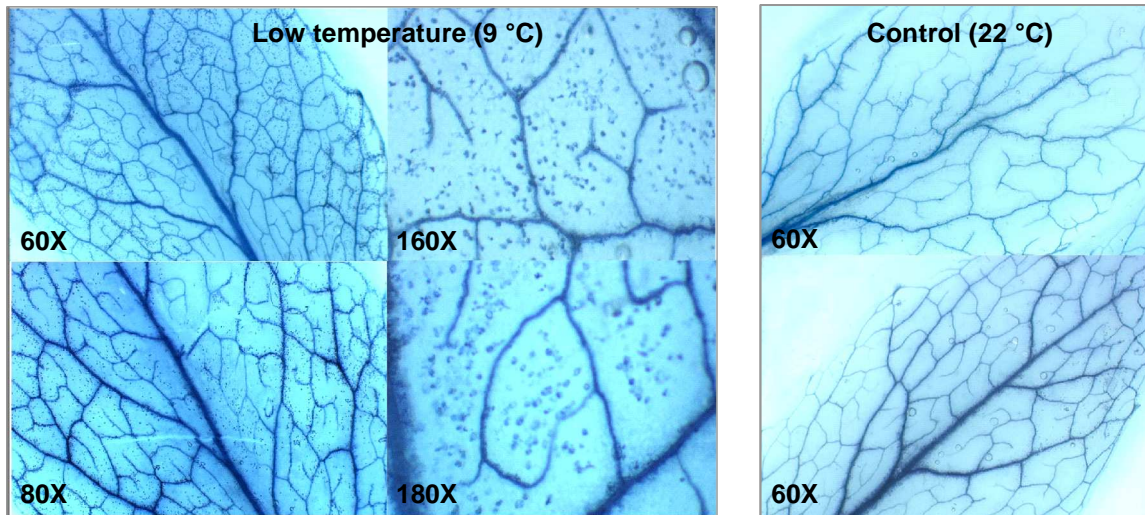


Image 3.1: ROS accumulation in leaves of *A. thaliana* Col after low temperature treatment (left images) and in comparison to control conditions (right images). Superoxide accumulation was shown by NBT and was visible only in low temperature treated leaves as blue spots. Leaf veins were constitutively coloured due to ROS formation during the process of lignification.

Precipitated dye was detectable at low temperature also in darkness (image 3.2). This result is in clear contrast to flavonoid accumulation under low temperature which was completely missing in darkness.

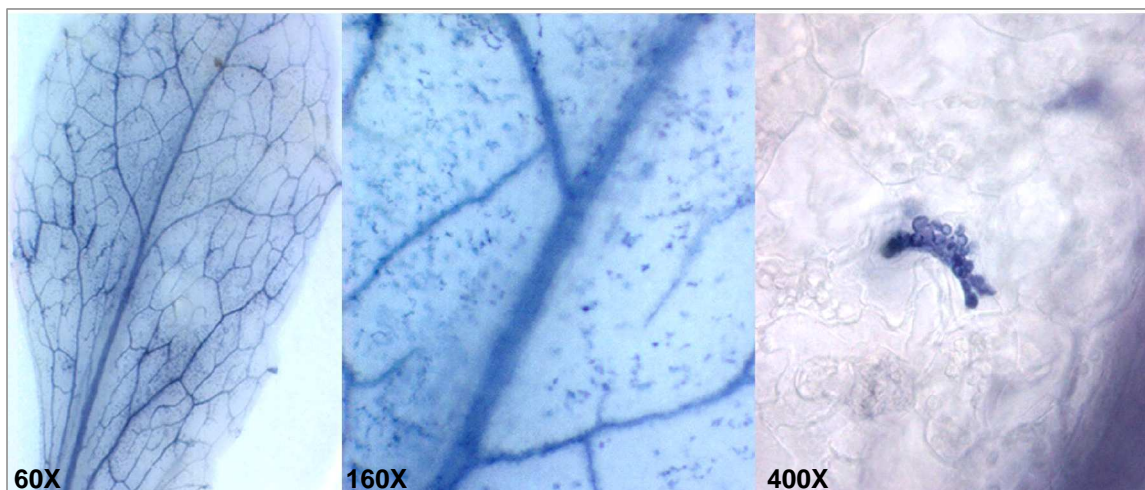


Image 3.2: ROS accumulation shown by NBT in leaves of *A. thaliana* Col after low temperature treatment in complete darkness. Strong superoxide accumulation was also visible in these leaves as blue spots.

Unfortunately, with these methods it was not possible to identify clearly the sources of ROS accumulation within the cells at low temperature in light and darkness.



### 3.1.1.2 The role of ROS in the induction of epidermal UV-A protection

In order to prove directly whether oxidative stress products might induce flavonoid accumulation, leaves of *A. thaliana* were brushed with different ROS generating solutions twice a day over a period of three days and epidermal UV-A transmittance measurements were performed. However, this treatment did not result in a significant decrease in epidermal UV-A transmission as measured with the UV-A-PAM in comparison to control leaves (figure 3.1).

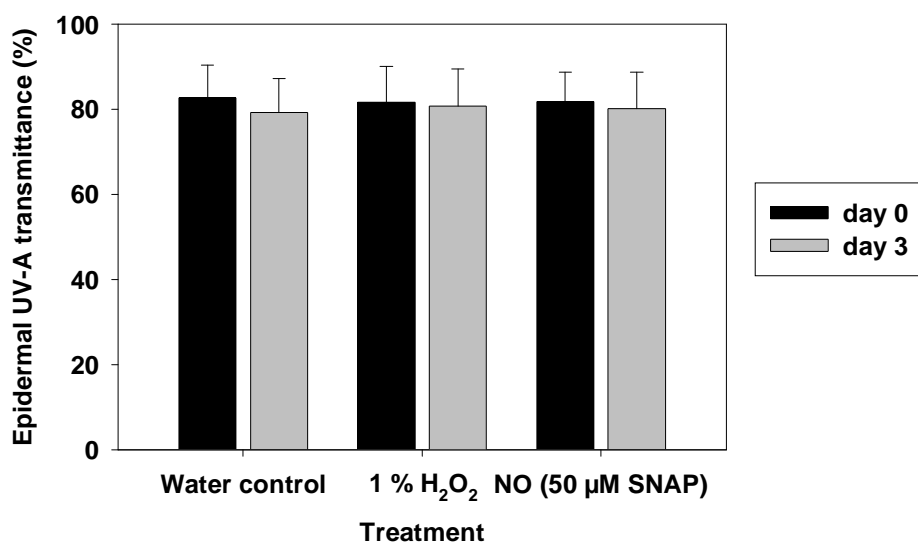


Figure 3.1: Epidermal UV-A transmittance in rosette leaves of *A. thaliana* Col before (black columns) and after a three day treatment (grey columns) with ROS- inducing solutions or with tap water (n = 12, 14 and 15 for SNAP, water and H<sub>2</sub>O<sub>2</sub>, respectively). A two-way ANOVA proved that there is no statistically significant difference:

Another experiment was performed in which ROS-generating solutions were allowed to be taken up into rosette leaves via the transpiration stream. After a two day incubation in different ROS-generating solutions or in tap water as negative control no marked decrease in epidermal UV-A transmittance was measured with the exception of those leaves which were incubated with tap water at low temperature conditions (not significant, ns) or with SIN-1 at 22°C (highly significant\*\*\*, see figure 3.2). A small but significant decrease in epidermal UV-A transmittance was also observed for SNP-treated leaves.

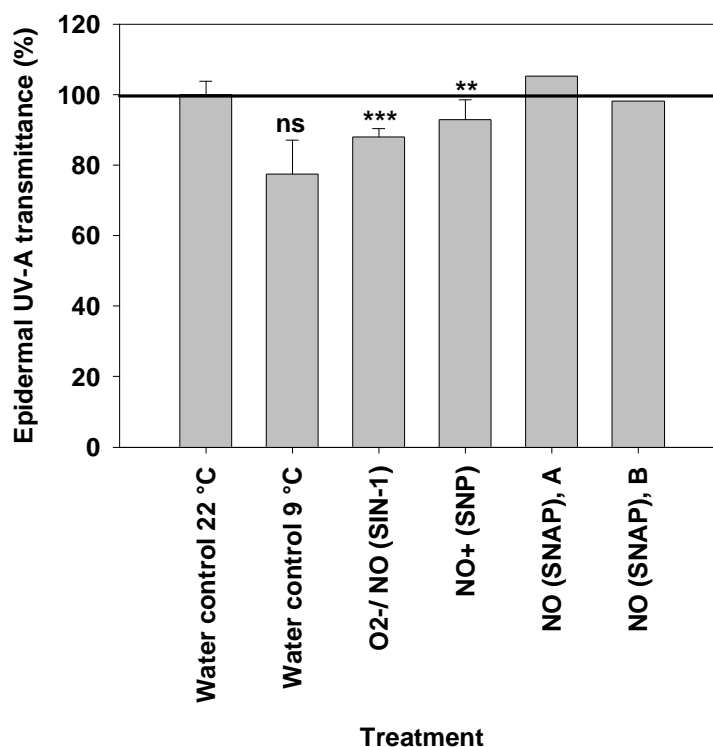


Figure 3.2: Change in epidermal UV-A transmittance in relation to a 22 °C water control in rosette leaves of *A. thaliana* Col after a two day treatment with ROS- inducing solutions or with tap water at low temperature (n = 8, 3, 5 and 7 for water (22 °C), water (9 °C), SIN-1 (80 μM) and SNP (0.5 mM), respectively). Concentration of SNAP was 50 μM. The decrease of the water sample at 9 °C was due to significantly different variances as tested with an F-test found not to be significant in the t-test (ns; t-test, P = 0.075). However, significant decreases were observed for treatments with SIN-1 (t-test, P < 0.001\*\*\*) and SNP (t-test, P = 0.016\*\*).

In order to check whether this procedure led to a potential accumulation of UV-A absorbing compounds in non-epidermal deeper tissues, whole leaf extracts were prepared and their absorption was measured in a photometer. Distinct wavelengths were analysed, namely 314, 360 and 540 nm, at which HCAs and flavonoids and anthocyanins (only 540 nm) display absorbance peaks. Absorption values of leaves which were treated with tap water at control conditions were similar to values of intact control plants and were set to 100 % (data not shown). Leaves treated with tap water at low temperature conditions had strongly and significantly increased absorption values at 314 and 360 nm, which are wavelengths where HCAs and flavonoids absorb (figure 3.3). This positive result was especially remarkable as it demonstrates that after a short incubation of two days cut leaves were capable in inducing flavonoid accumulation. Among all leaves which were treated with ROS generating solutions at control conditions only SIN-1- treated samples displayed slightly increased absorption values of 119 %. All other samples had values near or below 100 %. All tested solutions

generated NO $\cdot$ -radicals (SIN-1, SNAP) or the NO $^+$  ion (SNP) in light and SIN-1 was the only solution which additionally released O $_2^{\cdot-}$ -radicals (de Groot et al. 1993). NO $\cdot$  and O $_2^{\cdot-}$  react to form peroxynitrite (ONOO $\cdot$ ) which in turn may oxidize proteins (Jasid et al. 2006). However, these increases in comparison to the negative water control were not significant (t-test, P = 0.419 for 314 nm and P = 0.269 for 360 nm). Thus, a potential role of superoxide or a combined effect of nitric oxide and superoxide, which were both released by SIN-1, in the accumulation of flavonoids could not be completely excluded.

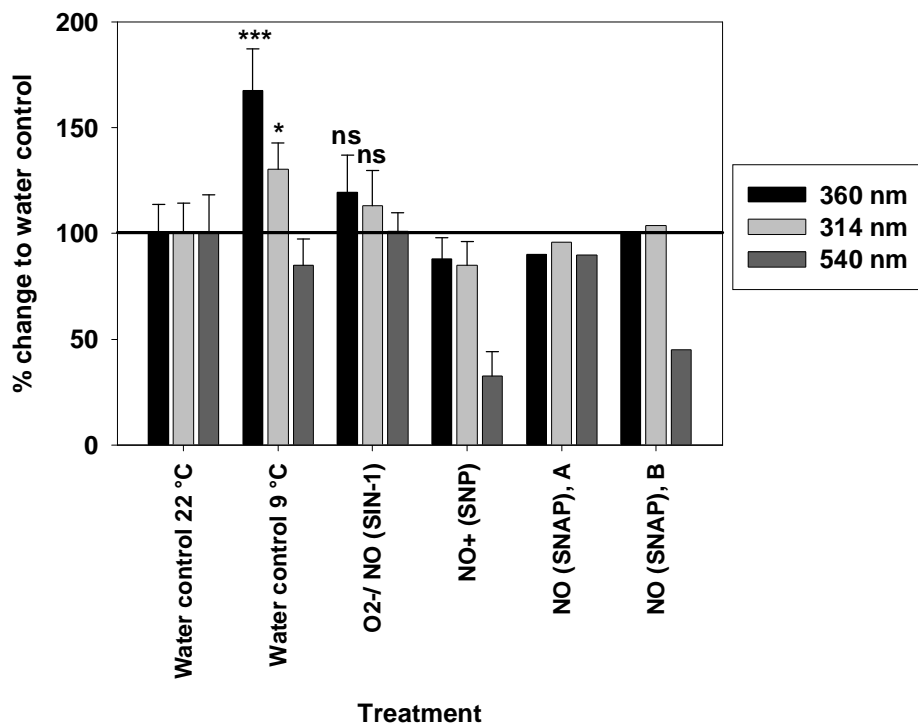


Figure 3.3: Change in absorption at 360, 314 and 540 nm per leaf area in comparison to control samples after treatment with different ROS-generating solutions for two days in leaf extracts of *A. thaliana* Col (n = 7 for 22 °C water control and SNP, n = 4 for SIN-1, n = 3 for 9 °C water control and n = 2 for SNAP, of which individual results A and B were presented). Solutions were taken up into rosette leaves via the transpiration stream. Concentrations were as follows: SIN-1: 80  $\mu$ M, SNP: 0.5 mM and SNAP: 50  $\mu$ M. SIN-1 and SNAP generated NO $\cdot$ -radicals when illuminated, and SIN-1 additionally released O $_2^{\cdot-}$ -radicals. SNP released the NO $^+$  ion. The positive control displayed 68 % higher absorption at 360 nm than the negative control (t-test, P < 0.001\*\*\* for 360 nm, P = 0.021\* for 314 nm). The SIN-1 derived increase in absorption was not significant (t-test, P = 0.269 for 360 nm, P = 0.419 for 314 nm). Values for 540 nm were not tested.

As, with the exception of a weak induction of epidermal flavonoid accumulation by the combined effect of superoxide and NO-radical, quasi no effect was observed in the experiments just described, the question arose if the application procedures were really effective in treating leaves with ROS. Two arguments may allow to answer this question positively. Firstly, several other leaves in the second experiment were treated with

water-dissolved riboflavin (250  $\mu\text{M}$ ). Riboflavin decays under illumination and releases superoxide radicals (Beauchamp & Fridovich 1971). Those treated leaves, which took up riboflavin via the transpiration stream, were bleached by the reactive oxygen which was released from riboflavin and could thus not be analysed with the UV-A-PAM. But this strong effect proved that ROS generating solutions could enter the tissues. Secondly, in order to prove whether the substances were taken up into the leaf tissue, NBT loaded leaves were treated with 1 mM riboflavin or with 40  $\mu\text{M}$  SIN-1, which generates superoxide and nitric oxide peroxide in light. For both solutions ROS could be visualized in leaves after a 24 hours treatment and uptake and release of ROS into the leaf tissue was thereby confirmed (image 3.3).

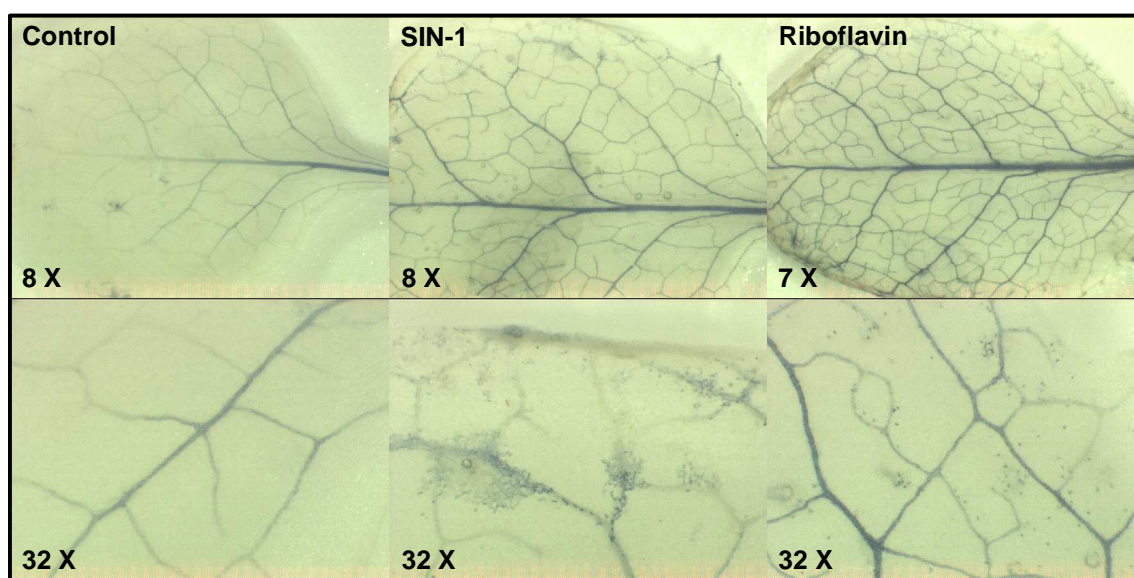


Image 3.3: Images presenting young rosette leaves of *A. thaliana* Col which were loaded with NBT prior treatment with ROS-inducing solutions of SIN-1 and riboflavin in order to demonstrate a positive release of ROS in the leaf tissue by brushing leaves with these solutions. Leaves were fixed in bleaching solutions after treatments and accumulated ROS were visible as blue spots. Control leaves were treated the same way but with tap water. Control leaves displayed blue coloured leaf veins as a result of lignification and which was an indication of successful uptake of dye. Many blue spots were visible in treated leaves indicating a strong accumulation of ROS in the leaf tissue.

### 3.1.1.3 Summary: ROS and epidermal UV-A protection

In summary it can be stated that ROS accumulation was shown at low temperature in light and in darkness with the dye NBT which is mainly sensitive for superoxide radicals. Findings, that ROS also accumulated in darkness and low temperature were in clear contrast to results of low temperature induction experiments where flavonoid accumulation was missing in complete darkness. Thus, a role of ROS appeared to be rather unlikely. However, ROS induction experiments were performed to investigate a

potential role of ROS more intensively. Two different procedures of applying ROS on or in leaves were performed and checked for effectiveness by different control experiments. But also here no definite results were obtained. Only a solution of SIN-1, which generates mainly superoxide radicals but also other ROS like nitric oxide had a weak effect on epidermal UV-A transmittance and absorption at wavelengths of 314 and 360 nm, where HCAs and flavonoids have a strong absorption, so that a role of at least superoxide in the induction of flavonoid accumulation could not be completely excluded.

### 3.1.2 Photosynthesis/ Reduction state of the primary electron acceptor in PS II, $Q_A$ as potential signal carrier

The reduction state of the psETC was determined measuring the reduction state of the primary electron acceptor in PS II, plastoquinone ( $Q_A$ ) with a PAM fluorometer. Measurements were based on chlorophyll fluorescence. The reduction state was measured and indicated as  $1-q_L$  (Kramer et al. 2004).

In the literature it was described that  $1-q_L$  is increased with decreasing temperature and with increasing irradiance and thus, would follow the same trend as flavonoid accumulation did (Huner et al. 1998). It is also the redox sensor for the signal transduction pathways for gene expression of chloroplast-encoded photosynthetic genes (Escoubas et al. 1995, Pfannschmidt et al. 1999) and for some nuclear-encoded antioxidative genes (Karpinski et al. 1997). These observations led to the hypothesis that  $1-q_L$  might be a signal carrier also for induction of transcription leading to flavonoid accumulation.

#### 3.1.2.1 The reduction state of $Q_A$ of *A. thaliana* Col in low temperature

The conditions of the low temperature treatment as chosen in this work did result in a rapid increase in the reduction state of  $Q_A$  in leaves of *A. thaliana* Col when determined as  $1-q_L$  (squares in figure 3.4) which stayed high over a period of nine days. Old leaves did not acclimate to low temperature as no decrease in  $1-q_L$  was observed later. Only new and young leaves showed acclimation of the reduction state. In contrast, this low temperature treatment always resulted in a significant decrease of epidermal UV-A transmittance (see triangles in same figure 3.4) and thus a significant accumulation of flavonoids.

So far it could be proven that in old rosette leaves of *A. thaliana* the reduction state of the psETC was increased upon a shift to low temperature conditions. An acclimation of the reduction state was only observed in young leaves. However, old and young leaves did both accumulate flavonoids in their epidermis during the low temperature treatment. Thus, the observation that young leaves with a reduced reduction state did accumulate

more flavonoids than old leaves did not follow the proposed trend of flavonoid accumulation at low temperature and was therefore an argument against  $1-q_L$  as signal carrier for flavonoid induction.

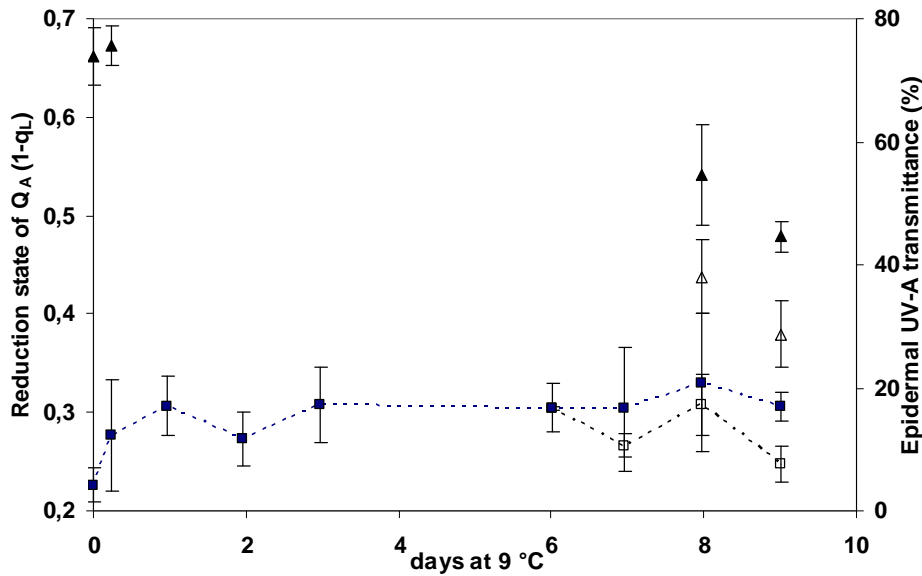


Figure 3.4: Change of reduction state of plastoquinone  $Q_A$  (squares), measured as  $1-q_L$  and epidermal UV-A transmittance values (triangles) in leaves of *A. thaliana* Col during a low temperature treatment for nine days ( $n = 3$ ). Chlorophyll fluorescence measurements were performed in the lab with a PAM101 and samples were cooled to 9 °C during the reduction state measurements. Open symbols display results of newly developed leaves, whereas closed symbols are results of leaves which were already present at start of the experiment. At each timepoint leaves of one treated plant were harvested and analysed. Higher standard error bars at the end of the experiment could be explained by higher reduction state values and transmittance values of old leaves.

### 3.1.2.2 Antisense *Solanum tuberosum* at low temperature: change in epidermal UV-A transmission and the reduction state of $Q_A$

In order to investigate the hypothesis of an involvement of  $1-q_L$  in the accumulation of flavonoids in a different, more “active” manner, I wanted to test whether an artificially increased reduction state of plastoquinone A could be correlated with an increased amount of epidermal flavonoids. For this purpose antisense potato plants (*Solanum* (*S.*) *tuberosum* Désirée) with reduced amounts of ferredoxin were analysed for epidermal UV-A transmittance. The plants were kindly donated by Dr. Jan Backhausen (University of Osnabrück, Germany) and are presented in image 3.4. The reduced amount of ferredoxin in the leaves was linked with a significantly increased reduction state of the psETC, measurable as increased  $1-q_L$  even at control conditions (figure 3.5).

After one hour at low temperature conditions  $1-q_L$  was increased in WT and remained high in antisense *FdA6* and *FdA7* plants.

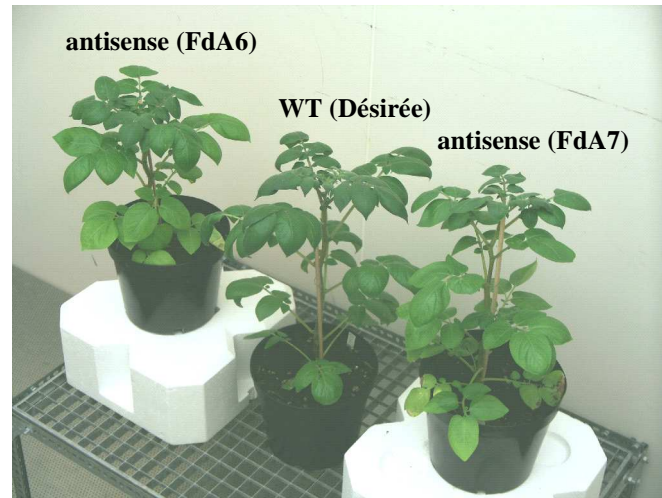


Image 3.4: *Solanum tuberosum* Désirée plants with different ferredoxin (Fd) content. Antisense plants display only 40 % (*FdA6*) or 50 % (*FdA7*) of normal ferredoxin content per leaf area.

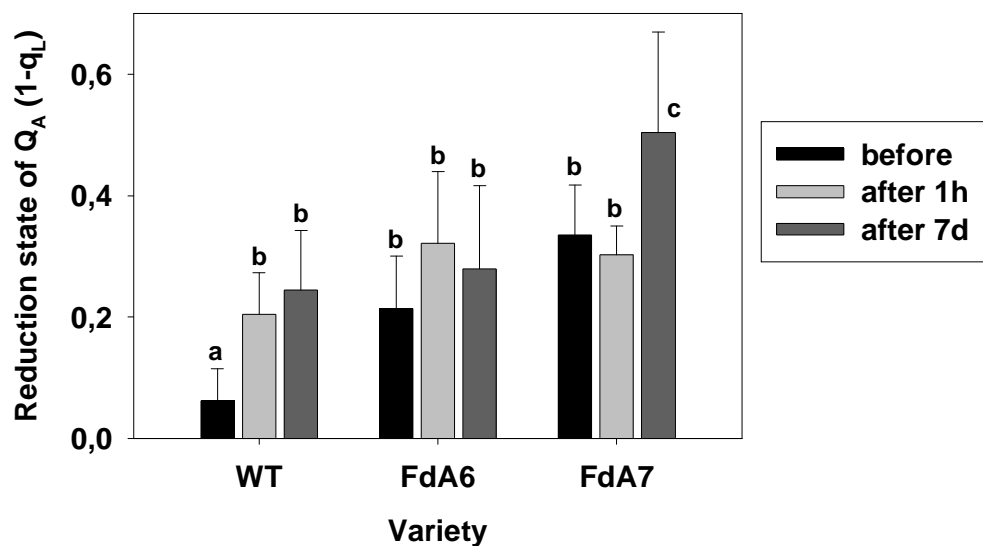


Figure 3.5: Reduction state of plastoquinone A ( $Q_A$ ) measured as  $1-q_L$  in WT and antisense (*FdA6* and *FdA7*) plants of *S. tuberosum* Désirée before, after one hour and after seven days at low temperature conditions. Results were tested with a two-way ANOVA and a tukey-test as post-hoc for significant differences. WT(before) vs. WT (1h),  $P = 0.024^*$ ; WT(before) vs. WT(7d),  $P = 0.003^{**}$ ; *FdA7*(before) vs. *FdA7*(7d),  $P = 0.004^{**}$ ; *FdA7*(1h) vs. *FdA7*(7d),  $P < 0.001^{***}$ ; WT(before) vs. *FdA6*(before),  $P = 0.015^*$ ; WT(before) vs. *FdA7*(before),  $P < 0.001^{***}$ ; WT(7d) vs. *FdA7*(7d),  $P < 0.001^{***}$ ; *FdA6*(7d) vs. *FdA7*(7d),  $P < 0.001^{***}$ .

Potato plants were exposed to low temperature and 200  $\mu\text{mol}/\text{m}^2\text{s}$  irradiance. In spite of different excitation pressures measured as  $1-q_L$ , UV-A transmission measurements on these plants revealed nearly identical reduced transmittance values of about 30 % (figure 3.6). No differences in transmission in transgenic potato plants were observed in comparison to WT plants. Thus, the hypothesis that an increased reduction state of plastoquinone A would result in an increased epidermal UV-A shield could not be confirmed again. Therefore, a role of the reduction state of the psETC in the induction of flavonoid biosynthesis appeared to be even more unlikely.

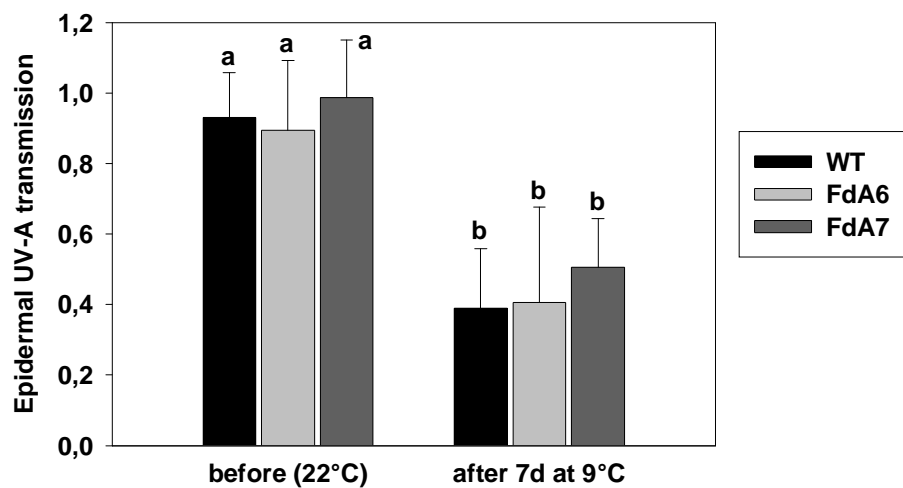


Figure 3.6: Relative epidermal UV-A transmission as measured with the UV-A-PAM in leaves of *S. tuberosum* (Désirée) before and after a seven day low temperature treatment at 200  $\mu\text{mol}/\text{m}^2\text{s}$  irradiance ( $n = 9$ ). A two-way ANOVA and a tukey-test as post-hoc test proved that transmission was significantly lowered after seven days in all varieties ( $P < 0.001^{***}$ ).

### 3.1.3 The role of light perception via light receptors in flavonoid accumulation

If ROS or the reduction state of the psETC, which both would be signals from the photosynthetically active mesophyll, would be involved in an induction of flavonoid accumulation, then signal transduction processes must run to the adaxial and abaxial epidermal sides of the leaf. Direction-bound signal transduction pathways seem to be unlikely, thus a potential signal for flavonoid accumulation must be conducted to the abaxial **and** adaxial leaf epidermis. Therefore, low temperature experiments were performed in which rosette leaves of *A. thaliana* Col were turned upside down, so that also the abaxial leaf side could be illuminated. Results are presented in the following chapter.



## 3.1.3.1 Epidermal UV-A transmittance of adaxial and abaxial leaf sides

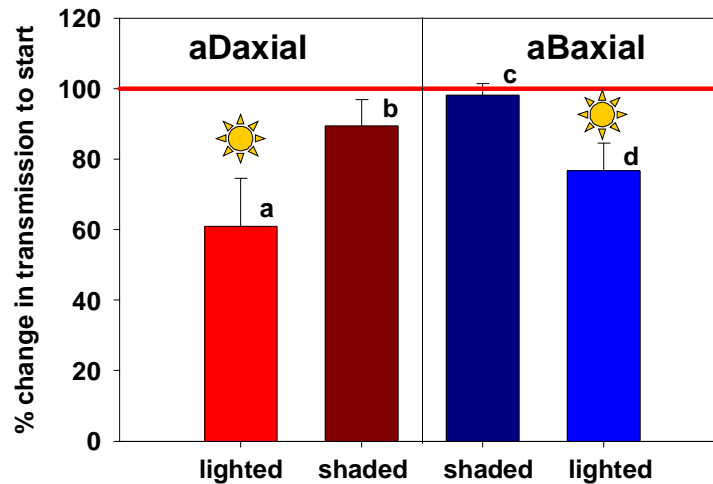


Figure 3.7: Change in epidermal UV-A transmission in comparison to start values of adaxial and abaxial leaf sides of rosette leaves of *A. thaliana* Col after a low temperature treatment of eleven days. Leaves of some plants were turned and fixed upside down so that abaxial leaf sides were illuminated while adaxial leaf sides were shaded.  $n = 54$  for non-turned leaves and  $n = 25$  for turned leaves. Results were tested for significance with a two-way ANOVA and a tukey-test. All results were highly significant to each other, with  $P < 0.001^{***}$ .



Figure 3.8: Illustration to figure 3.7. Image of *A. thaliana* Col whose rosette leaves were turned upside down and fixed by pins. Red arrows indicate adaxial leaf sides and blue arrows point to abaxial leaf sides. The darker colour always indicates shaded leaf sides.

As it can be seen on the two figures 3.11 and 3.12, low temperature conditions led to a strong accumulation of UV-A absorbing compounds only in illuminated leaf sides. Shaded leaf sides, either adaxial or abaxial, did not accumulate any or only few of these

compounds. However, illuminated abaxial leaf sides displayed a reduced capability to increase epidermal UV-A absorption as transmission was reduced to 77 % whereas illuminated adaxial leaf sides reached 61 %. These results prove again that sensing of light plays a crucial role in low temperature-induced flavonoid accumulation and that the sensor involved in this might be located in the epidermal layer(s).

### 3.1.3.2 Epidermal UV-A transmittance in darkness and low temperature

Many results presented so far clearly demonstrated that light was very essential for flavonoid accumulation at low temperature. For this reason several experiments followed in order to investigate this interaction in more detail.

The figure 2.30 in the second part of this work (chapter 2.1.4.2) showed that at low temperature in darkness no additional kaempferol or quercetin glycosides were accumulated. Additionally, in the second part of this work a light dependency of flavonoid accumulation also at low temperature conditions was described (chapter 2.1.3.2 and figure 2.25). In another experiment *Arabidopsis* plants were first treated with low temperature in darkness for 5 days and were then exposed to a low irradiance of only  $50 \mu\text{mol}/\text{m}^2\text{s}$  also in low temperature for further three and six days. In leaves of these plants epidermal UV-A transmittance was significantly decreased to a value of 70 % after six days (figure 3.9). Thus, as soon as leaves could sense some photons at low temperature they started to accumulate flavonoids in their epidermis.

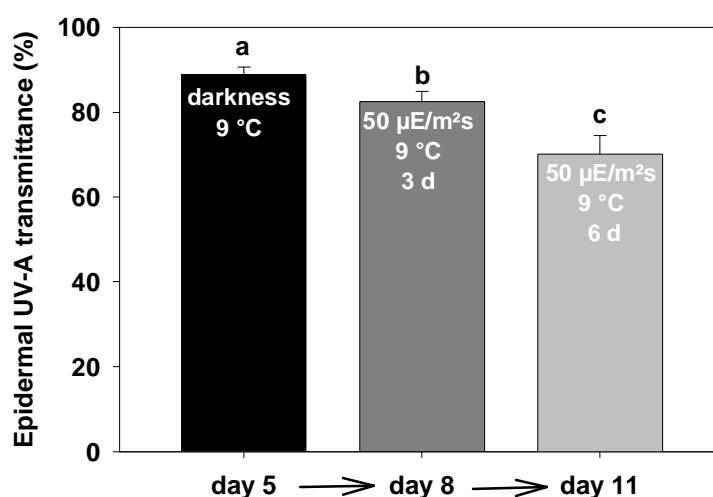


Figure 3.9: Epidermal UV-A transmittance in rosette leaves of *A. thaliana* Col after 5 days at low temperature in darkness (dark-blue column,  $n = 14$ ) and after further three and six days at  $50 \mu\text{mol}/\text{m}^2\text{s}$  irradiance (blue columns,  $n = 9$ ). All transmittance values were found to be significantly different to each other as tested with a one-way ANOVA and a tukey-post-hoc-test ( $P < 0.001$ \*\*\*).

One could argue that in darkness plants were not able to synthesize flavonoids as the required energy was missing due to missing of photosynthesis. But even if plants were allowed to do photosynthesis for six hours per day at 22 °C (plus an acclimation period of 30 minutes in darkness at 22 °C) no additional flavonoids were accumulated at all (figure 3.10). Transmission was even slightly increased to 86 % when followed over a period of four days. This result demonstrates that a potential lack of carbon fixation in darkness at low temperature could not be responsible for the lack of flavonoid accumulation.

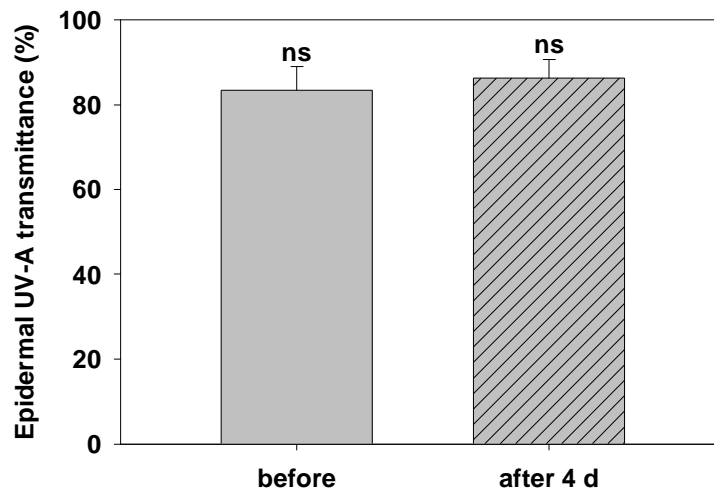


Figure 3.10: Epidermal UV-A transmittance in rosette leaves of *A. thaliana* Col before (n = 13) and after (n = 16) a four day treatment with 18 hours low temperature in darkness per day and with six hours control conditions per day. Prior to treatment with six hours control conditions, plants were allowed to warm up in darkness at 22 °C for 30 minutes. Averages were not significantly different (ns, t-test: p = 0.147).

### 3.1.3.3 The role of blue light in the induction of epidermal UV-A protection

In higher plants the blue light photoreceptors phototropin (phot) and cryptochrome (cry) regulate several processes in plant life. As flavonoids were found to accumulate in an intensity dependent manner in white light, it was hypothesized that at low temperature the signalling processes involved in blue light perception might be involved.

#### a) Results for *A. thaliana* WT Col

Therefore, *Arabidopsis* Col wild type plants were exposed to 270  $\mu\text{mol}/\text{m}^2\text{s}$  PPFD of either white or excluded blue light (ie yellow light) at either 9 or 22 °C air temperature for seven days and epidermal UV-A transmission was measured before and after

exposition. Before experiments started, all plants were grown at control conditions which was 22 °C and 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light. Figure 3.11 presents results of WT Col plants. Differences between start values across treatments are accidental.

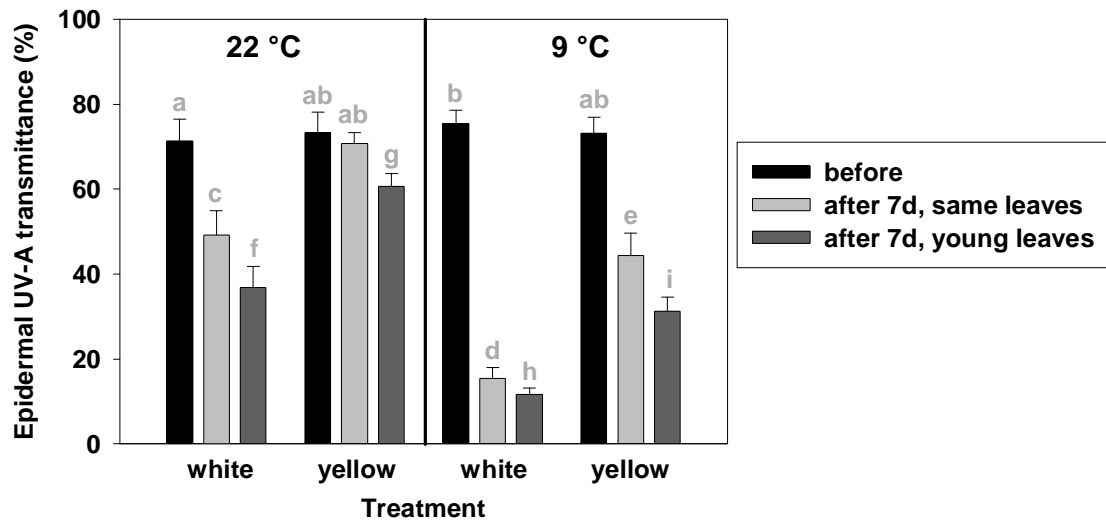


Figure 3.11: Epidermal UV-A transmittance in leaves of *A. thaliana* WT Col plants before (dark columns) and after a seven day treatment (grey columns) with either white (w) or yellow (y) light of same intensity (270  $\mu\text{mol}/\text{m}^2\text{s}$ ) at 22 °C and 9 °C (n = 18). Epidermal transmittance values of each timepoint were tested separately for significant differences with a one-way ANOVA and tukey-test. Additionally, values for old and young leaves at day 7 were also found to be statistically different in all cases. Comparisons of values for “before” and “day 7” showed that all were significantly different with the exception of 22/y(before) vs. 22/y(day 7, same leaves) which were not statistically different. Levels of significance were always high with  $P < 0.001$ \*\*\* with the exception of 22/w(before) vs. 9/w(before), where  $P = 0.007$ \*\*.

After a seven day exposure the light effect of 270  $\mu\text{mol}/\text{m}^2\text{s}$  was eliminated when blue light was excluded from the spectrum by a yellow foil in *Arabidopsis* WT Col (grey columns in figure 3.11) because control levels of epidermal UV-A transmittance were measured (compare with control plants displayed in figure 2.12 of the UV-B experiment which was also conducted in the growth cabinet). This was true for both temperatures. At low temperature only the temperature effect was visible in leaves which were treated with solely yellow light. For comparison refer to figure 2.18 in part two of this work where low temperature led to a decrease in epidermal UV-A transmittance from 75 to 40 %.

### b) Results for *A. thaliana* WT Ws

In this study the light receptor mutant *phot1phot2* of *Arabidopsis* which lacks phototropin 1 and 2, was used and thus the according background (BG) plants had to be analysed as well. Therefore *Arabidopsis* WT of the variety Ws were also treated with either white or yellow light at 22 and 9 °C air temperature. Differences in the behaviour of Col and Ws to different control and experimental conditions were comprehensively presented in part one of this work. Generally, Ws plants showed weaker responses to high light or low temperature when compared with Col plants. Figure 3.12 presents results of the different treatments. Responses were as for Col ecotypes with the exception of the treatment with yellow light at low temperature. Here transmittance was as high as start values meaning that both effects, the high light and also the low temperature effect, were missing.

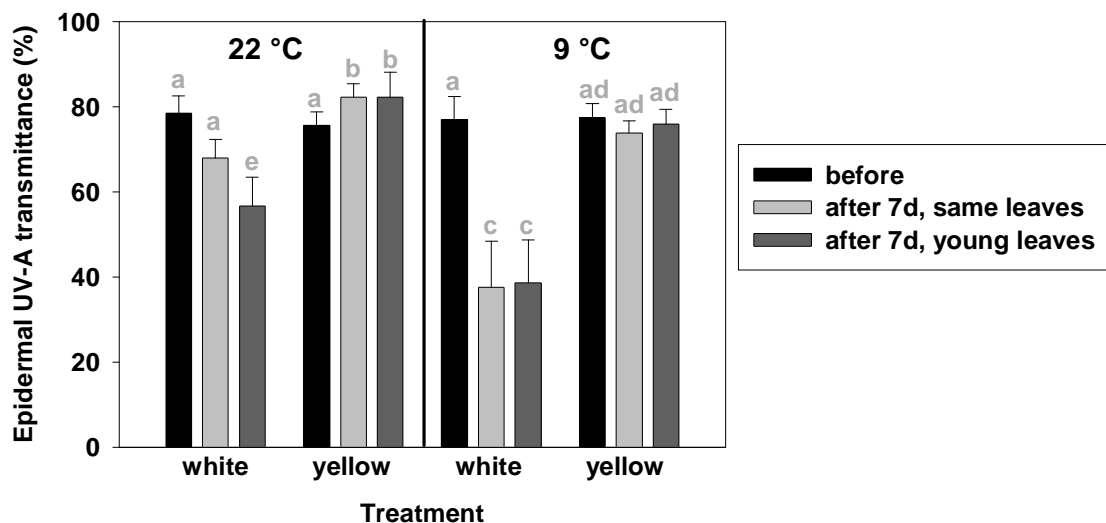


Figure 3.12: Epidermal UV-A transmittance in leaves of *A. thaliana* WT Ws plants before (dark columns) and after a seven day treatment (grey columns) with either white (w) or yellow (y) light of same intensity ( $270 \mu\text{mol}/\text{m}^2\text{s}$ ) at 22 °C and 9 °C ( $n = 15$  for all data points with the exception of measurements after 7 days of same leaves as start, where  $n = 16$  for treatments with 22 °C, white and yellow light, and of measurements after 7 days of young leaves, where  $n = 14$  for treatments with 22 °C, yellow light). Epidermal transmittance values of each timepoint were tested separately for significant differences with a one-way ANOVA and tukey-test. Results for old and young leaves at day 7 were found to be statistically different in only one case: 22/w(7 days, same leaves) vs. 22/w(7 days, young leaves) with  $P < 0.001^{***}$ . Comparisons of values for “before” and “day 7” showed that only for the treatments 22/y and 9/w significantly different means were detected. Levels of significance were always high with  $P < 0.001^{***}$  with the exception of 22/y(day 7, young leaves) vs. 9/y(day 7, young leaves), where  $P = 0.021^*$  and 22/y(before) vs. 22/y(day 7, same leaves), where  $P = 0.039^*$ .

c) Results for *A. thaliana* mutant *phot1phot2* (BG: Ws)

In a subsequent step blue light photoreceptor mutants of *Arabidopsis* were tested for their potential to accumulate epidermal flavonoids at the described conditions. These deletion mutants were lacking either cryptochrom 1 and 2 (*cry1cry2*, cc) or phototropin 1 and 2 (*phot1phot2*, pp). Firstly, results of the pp mutant will be presented in figure 3.13.

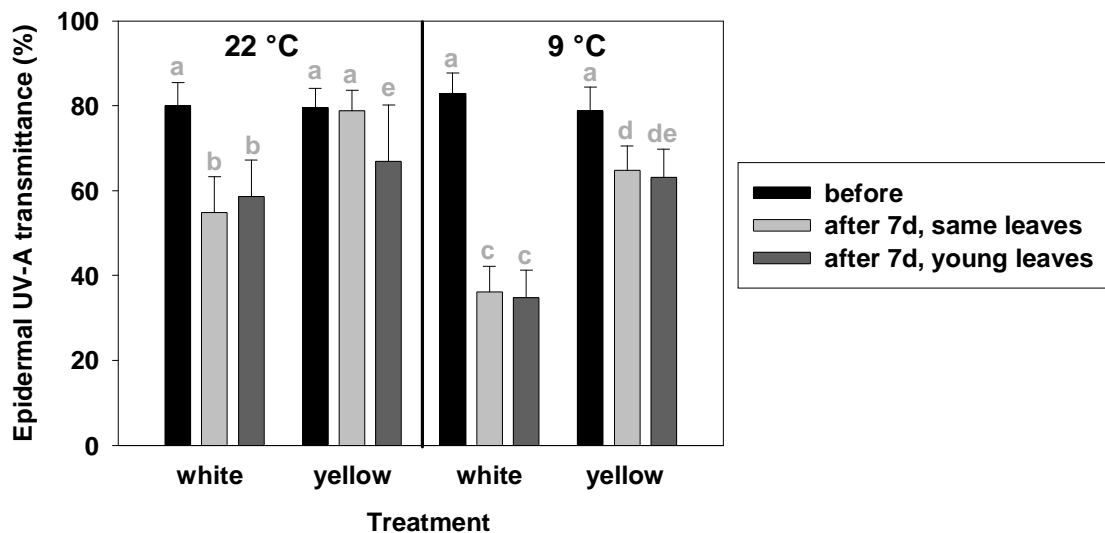


Figure 3.13: Epidermal UV-A transmittance in leaves of *A. thaliana* mutants *phot1phot2* which lack phototropins. The diagram presents transmittance values of before (dark columns) and after a seven day treatment (grey columns) with either white (w) or yellow (y) light of same intensity ( $270 \mu\text{mol}/\text{m}^2\text{s}$ ) at  $22 \text{ }^\circ\text{C}$  and  $9 \text{ }^\circ\text{C}$  ( $n = 15$  for all data points with the exception of measurements after 7 days of same leaves as start, where  $n = 9, 13$  and  $16$  for treatments with  $22 \text{ }^\circ\text{C}$ , yellow light,  $22 \text{ }^\circ\text{C}$ , white light and  $9 \text{ }^\circ\text{C}$ , yellow light, respectively). Epidermal transmittance values were tested for significant differences with a one-way ANOVA and tukey-test. Results for old and young leaves at day 7 were found to be statistically different in only one case:  $22/\text{y}(7 \text{ days, same leaves})$  vs.  $22/\text{y}(7 \text{ days, young leaves})$  with  $P < 0.001^{***}$ . Comparisons of values for “before” and “day 7” showed that significantly different means were detected for the treatments  $22/\text{w}$ ,  $9/\text{w}$  and  $9/\text{y}$  but not for  $22/\text{y}$  ( $P = 0.772$ ). Levels of significance were always high with  $P < 0.001^{***}$  with the exception of  $22/\text{w}(\text{day 7, young leaves})$  vs.  $22/\text{y}(\text{day 7, young leaves})$ , where  $P = 0.028^*$  and  $22/\text{y}(\text{day 7, same leaves})$  vs.  $22/\text{y}(\text{day 7, young leaves})$ , where  $P = 0.001^{**}$ .

As it can be seen in figure 3.13 the deletion mutant *phot1phot2* of *Arabidopsis thaliana* reacted like the Col WT. In *Arabidopsis* Ws plants, which is the background line for *phot1phot2*, both effects on epidermal UV-A transmittance, the temperature and the light effect were missing after treatment with yellow light and  $9 \text{ }^\circ\text{C}$  (figure 3.12). However, in the mutant *phot1phot2* a temperature effect was still present. In addition, this effect was as high as observed in other low temperature experiments with

*Arabidopsis* Ws plants. For a comparison please refer to figure 1.4 in part one of this work. The figure shows that low temperature led to a decrease in transmittance from 79 to 63 %. Thus, it seems as if the vanished low temperature effect under yellow light in Ws plants displayed in figure 3.12 must be considered cautiously. Figure 3.13 clearly shows that the double mutant for phototropin displayed a clear temperature effect at white and yellow light.

#### d) Results for *A. thaliana* mutant *cry1cry2* (BG: Col)

Another deletion mutant defective in blue light sensing was also investigated. The double mutant *cry1cry2* of *Arabidopsis* with background Col was lacking the blue light sensors cryptochrome 1 and cryptochrome 2. The figure 3.14 presents the results of an experiment in which plants were treated with the four different experimental conditions. The most striking difference to WT Col plants was the finding that firstly, the light effect of 270  $\mu\text{mol}/\text{m}^2\text{s}$  white light at 22 °C and 9 °C was reduced and that secondly, the temperature effect was markedly reduced in this mutant. These two observations will now be explained in detail. When transferred to an irradiance of 270  $\mu\text{mol}/\text{m}^2\text{s}$ , the increased irradiance led to a 20 to 30 % decrease in epidermal transmittance of WT Col plants. In *cry1cry2* mutants this decrease was reduced by 15 % (figure 3.14). At 9 °C and white light an additional temperature effect led to minimum transmittance values in WT and *phot1phot2* plants. Transmittance was reduced to only 15 % in *Arabidopsis* Col and to 38 and 36 % in WT Ws and *phot1phot2* mutants, respectively. In contrast, *cry1cry2* mutants had clearly higher transmittance values of 63 %. Thus, it can be stated, that in *cry1cry2* the temperature effect on epidermal UV-A transmittance was weakened.

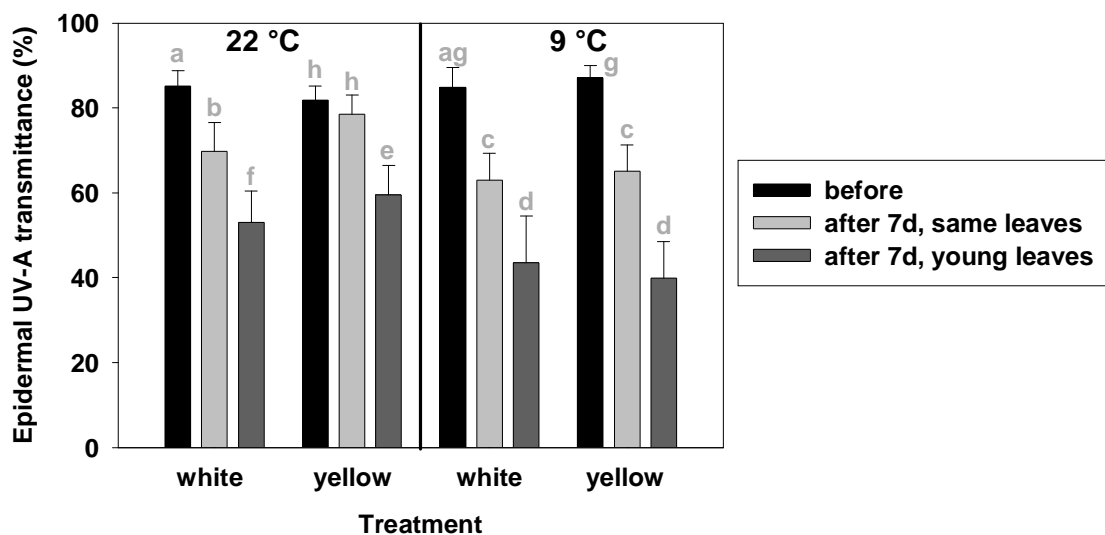


Figure 3.14: Epidermal UV-A transmittance in leaves of *A. thaliana* mutant *cry1cry2* before (dark columns) and after a seven day treatment (grey columns) with either white or yellow light

of same intensity ( $270 \mu\text{mol}/\text{m}^2\text{s}$ ) at  $22^\circ\text{C}$  and  $9^\circ\text{C}$  ( $n = 18$ ). In order to find potential statistically significant differences in the results, a one-way ANOVA and a tukey-test as post-hoc test were performed. Significantly different mean values are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$  with the following exceptions: 22/w(before) vs. 22/y(before),  $P = 0.012^*$ , 22/w(day 7, same leaves) vs. 9/w(day 7, same leaves),  $P = 0.002^{**}$ , 22/w(day 7, young leaves) vs. 22/y(day 7, young leaves),  $P = 0.034^*$  and 22/w(day 7, young leaves) vs. 9/w(day 7, young leaves),  $P = 0.002^{**}$ .

#### e) Comparison of results for WT Col, Ws and mutants *cry1cry2* and *phot1phot2*

When analysing epidermal transmittance values one has always to consider start and end values. In order to facilitate the observation of all start and end values of all varieties transmittance values of end measurements were set in relation to start values. For this purpose only same leaves were analysed. Figure 3.15 therefore presents changes in epidermal transmittance in comparison to start values for all varieties.

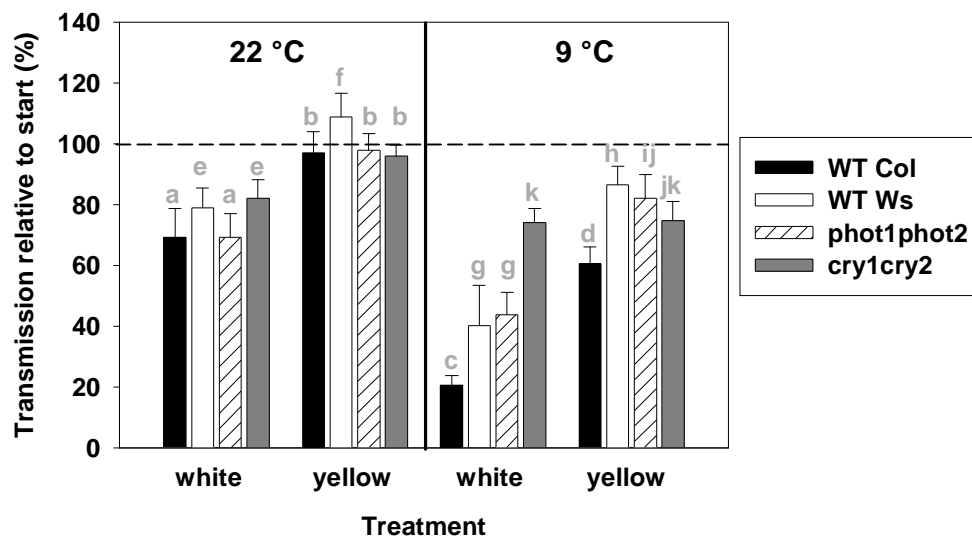


Figure 3.15: Relative epidermal UV-A transmittance in leaves of different varieties of *A. thaliana* after seven days at either  $22^\circ\text{C}$  or  $9^\circ\text{C}$  (22 or 9) under white or yellow (w or y) light of  $270 \mu\text{mol}/\text{m}^2\text{s}$  intensity in percent of start values. For WT Col (black columns)  $n = 18$ , for WT Ws (white columns)  $n = 15$  with the exception of 22/w, where  $n = 14$ , for *phot1phot2* with background Ws (white striped columns)  $n = 8$  for 9/y,  $n=13$  for 22/w and  $n= 15$  for 22/y and 9/w and for *cry1cry2* with background Col (grey columns)  $n = 18$ . Statistically significant differences in the results were discovered by a one-way ANOVA with a tukey-test as post-hoc test. Significantly different mean values are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$  with the following exceptions: 22/w(Col) vs. 9/y(Col),  $P = 0.005^{**}$ , 22/w(Ws) vs. 9/y(Ws),  $P = 0.038^*$ , 22/w(*cry1cry2*) vs. 9/w(*cry1cry2*),  $P = 0.014^*$ , 22/w(*cry1cry2*) vs. 9/y(*cry1cry2*),  $P = 0.025^*$ .



Figure 3.15 demonstrates that at 22 °C the light effect on epidermal UV-A transmittance could be entirely explained by blue light, i.e. when blue light was missing at 22 °C no light effect was observable any more. At low temperature the result was somewhat different. Transmittance was less reduced when blue light was missing of the light spectrum in leaves of WT Col, WT Ws and *phot1phot2*. However, all transmittance values at low temperature were reduced in comparison to start values, indicating that regardless of the light source a temperature effect was still always present. In the mutant *cry1cry2* a light effect was not detected any more, indicating a clear role of cryptochrome in this reaction. Furthermore, the temperature effect in *cry1cry2* was in comparison to the background Col significantly reduced because the change in transmittance to start was less pronounced. This weak temperature effect was similar to results obtained with Ws and *phot1phot2* plants.

#### f) Results for *A. thaliana* mutant *cry2* (BG: Col)

A logical question was then to investigate which of the cryptochromes or if perhaps both were responsible for this observation. Thus, single mutants of *A. thaliana* defective in either *cry1* or *cry2* both with background Col were tested under the conditions described above. Figure 3.16 presents results obtained from experiments with the mutant *cry2*, which lacked cryptochrome 2. Although start values were relatively small, namely about 60 % epidermal UV-A transmittance (in other experiments Col plants of *Arabidopsis* generally displayed values around 80 %), a reducing effect on transmittance of white light was observable for both temperatures. An additional reduction was detected when low temperature was applied, so that altogether this mutant reacted very similar to the WT shown in figure 3.11.

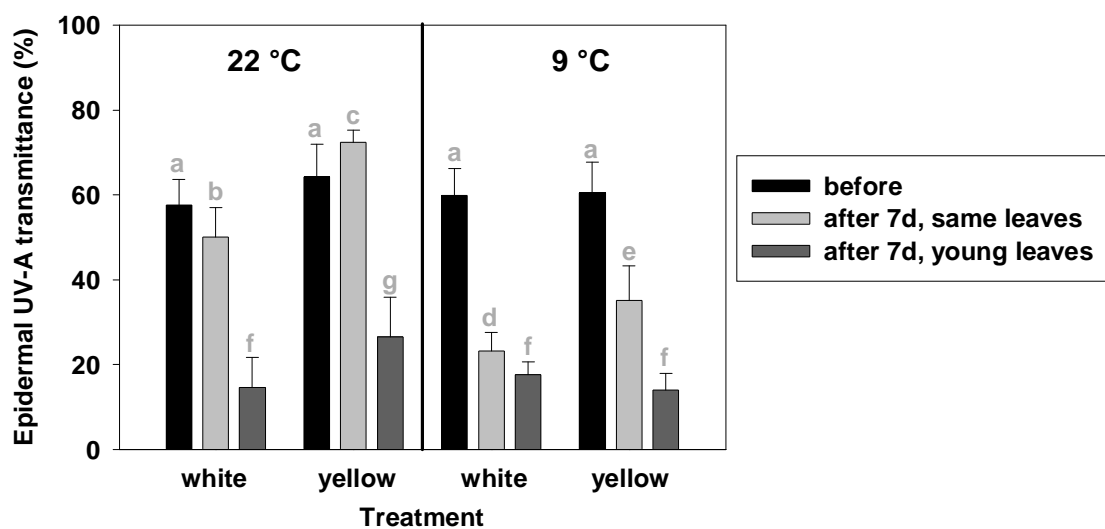


Figure 3.16: Epidermal UV-A transmittance in rosette leaves of *A. thaliana cry2* (background Col) before and after seven days of four different treatments with either white or yellow light of

270  $\mu\text{mol}/\text{m}^2\text{s}$  at 22 or 9 °C (n = 15). In order to reveal potential statistically significant differences in the results, a one-way ANOVA with a tukey-test as post-hoc test was performed. Significantly different mean values are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$  with the following exceptions: 22/w(before) vs. 22/w(day 7, same leaves),  $P = 0.002^{**}$ , 22/y(before) vs. 22/y(day 7, same leaves),  $P = 0.001^{**}$  and 9/w(day 7, same leaves) vs. 9/w(day 7, young leaves),  $P = 0.018^*$ .

#### g) Results for *A. thaliana* mutant *cry1* (BG: Col)

The second single mutant *cry1* was also tested together with *cry2* for its potential to reduce epidermal UV-A transmittance upon high light and low temperature. As it can be seen in figure 3.17 this diagram resembles very much figure 3.14 where results of *cry1cry2* were presented. In figure 3.17 *cry1* mutants were not capable to accumulate much UV-A absorbing compounds even when illuminated with high intense white light in comparison to yellow light of same intensity. At 9 °C transmittance was only lightly reduced and was equal for both light qualities. Statistical analysis revealed that low temperature was not able to reduce epidermal transmittance significantly when compared with values at 22 °C and white light. In summary, a small effect of white light on epidermal UV-A transmittance was observed at 22 °C, which was not present at 9 °C but which was as high as the observed temperature effect at 9 °C (letters c and e in figure 3.17). These results prove that cryptochrome 1 was the candidate in question and was responsible for the light effect on epidermal flavonoid accumulation.

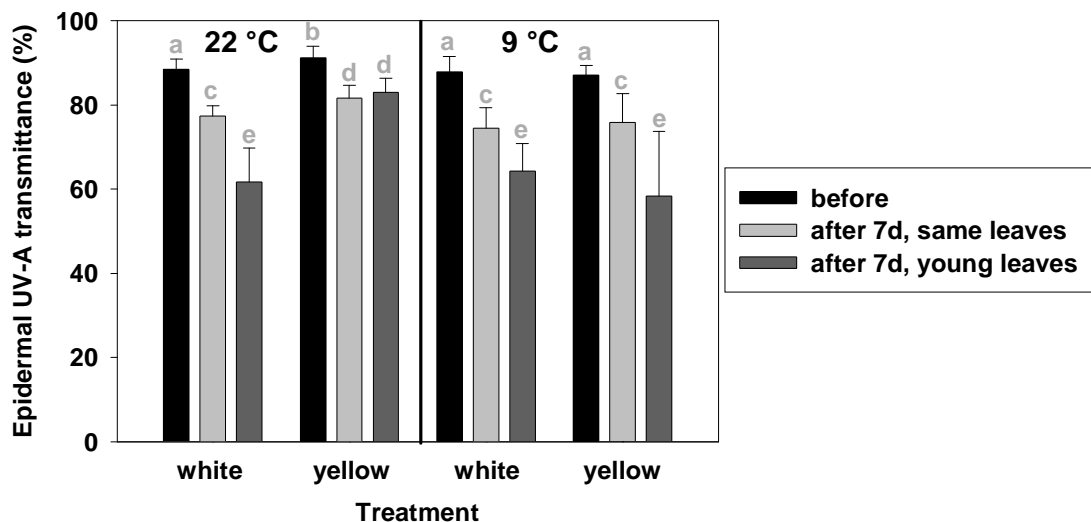


Figure 3.17: Epidermal UV-A transmittance in rosette leaves of *A. thaliana cry1* (background Col) before and after seven days of four different treatments with either white or yellow (w or y) light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  at 22 or 9 °C (n = 15 with the exception of after 7 d, same leaves, 9/w, where n = 14 and after 7 d, young leaves, 22/w with n = 14, 22/y and 9/w with n = 12 and 9/y with n = 6). In order to reveal potential statistically significant differences in the results, a one-way ANOVA with a tukey-test as post-hoc test was performed. Significantly different mean

values are indicated by different letters. Level of significance was always high with  $P < 0.001^{***}$  with the following exceptions: 22/w(before) vs. 22/y(before),  $P = 0.014^*$  and 22/w(day7, same leaves) vs. 22/y(day 7, same leaves),  $P = 0.018^{**}$ .

#### h) Comparison of results for WT Col and mutants *cry1* and *cry2*

When the mutants *cry1* and *cry2* were investigated, of course, WT Col plants were also tested in the same experiment. Results were very similar to other Col results so that transmittance data from this experiment were not shown again. However, in figure 3.18, where the change in transmittance in comparison to start values were presented, these data were included for comparison reasons. In WT Col and *cry2* plants an obvious effect of white light in comparison to yellow light at 22 °C was measured. In *cry1* mutants transmittance was equally reduced at 22 °C after treatment with white or yellow light in comparison to start values and 87 and 90 % of start transmittance was reached. A treatment with 9 °C led in WT Col and *cry2* to strongly reduced transmittance values in comparison to start measurements and this reduction was extreme when white light was applied. In contrast *cry1* plants reacted only very weakly, because 84 and 87 % of start values were measured. Additionally no effect was observable when blue light was missing. These results were very much identical to results obtained from the double mutant *cry1cry2* and it became clear that cryptochrome 1 was the blue light sensor responsible for these observations.

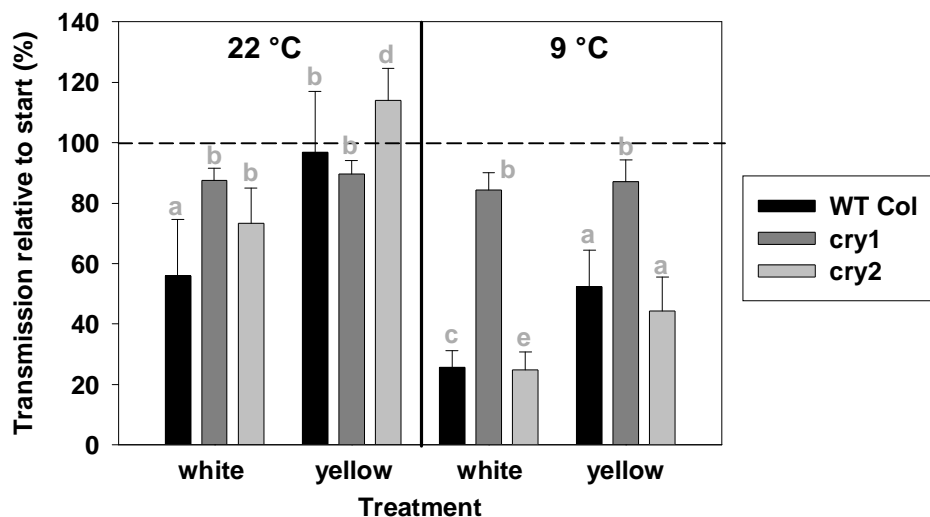


Figure 3.18: Relative epidermal UV-A transmittance in leaves of different varieties of *A. thaliana* after seven days at either 22 or 9 °C (22 or 9) under white or yellow (w or y) light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  intensity in comparison to start values. For WT Col (black columns)  $n = 15$ , for the blue light receptor mutant *cry2* (grey columns)  $n = 15$  and for the mutant *cry1* (dark grey columns)  $n = 15$  with the exception of 9/w, where  $n = 14$ . Potential statistically significant differences in the results were discovered by a one-way ANOVA and a tukey-test as post-hoc test. Significantly different mean values are indicated by different letters. Level of significance

was always high with  $P < 0.001^{***}$  with the following exception: 9/w(Col) vs. 9/w(*cry2*),  $P = 0.005^{**}$ .

#### 3.1.3.4 HPLC results to *A. thaliana* WT Col and mutant *cry1cry2* with respect to the role of blue light and low temperature in flavonoid accumulation

In the following paragraphs some results of HPLC analysis of WT Col and *cry1cry2* plants are presented in order to check for potential differences in pigment composition. These analyses should help to understand and elucidate the role of cryptochrome in light- and low temperature-induced flavonoid accumulation.

##### a) Results for *A. thaliana* WT Col

First of all, experiments with WT *Arabidopsis* Col were analysed in which blue light was excluded from the light spectrum. HPLC analysis revealed that the light effect on flavonoid biosynthesis at 22 °C (“high light” condition with 270  $\mu\text{mol}/\text{m}^2\text{s}$ ) could completely be eliminated in WT *Arabidopsis* Col plants by exclusion of blue light by a yellow foil: An irradiance of 270  $\mu\text{mol}/\text{m}^2\text{s}$ , which led to a strong accumulation of flavonoids at 22 °C air temperature if given as white light, did not result in any accumulation if given as yellow light (figure 3.19). The amount of kaempferol glycosides observed after treatment with high yellow light at 22 °C was as high as observed generally under control conditions (for example compare with control treatment displayed in figure 2.13 of the UV-B experiment which was also conducted in the growth cabinet). Low temperature led to an additional effect on flavonoid accumulation and this was true for white and yellow light. In comparison to illumination with high white light, yellow light at 9 °C led to reduced amounts of quercetin glycosides and kaempferol glycosides. However, in comparison to control conditions these amounts were still increased.

The figure 3.20 informs on flavonol composition in *A. thaliana* Col after the four different treatments (figure 3.20). HPLC results showed that composition of polyphenols at 22 °C and yellow light was identical to the observed composition at control conditions. Composition of HCAs was not different after the four treatments (data not shown), but increased values of total amount were measured at white light in comparison to yellow light and these values were further increased by low temperature (figure 3.19).

Plants, which were treated with 9 °C and 270  $\mu\text{mol}/\text{m}^2\text{s}$  yellow light had markedly increased amounts of kaempferol glycosides in comparison to 22 °C-samples which were also treated with yellow light (figure 3.20). Thus, this result was a temperature effect at yellow light. It was striking, that only with a combination of low temperature and strong white light significantly more quercetin glycosides were accumulated. This amount was also extremely higher than observed in the light curve experiment at 9 °C

(figure 2.23). This difference could possibly be the result of the increased fraction of blue light in the white light spectrum in the growth cabinet (where the blue light experiments were carried out), in comparison to the growth chamber (where the light curve experiments were implemented) (table III.1 and figure III.1).

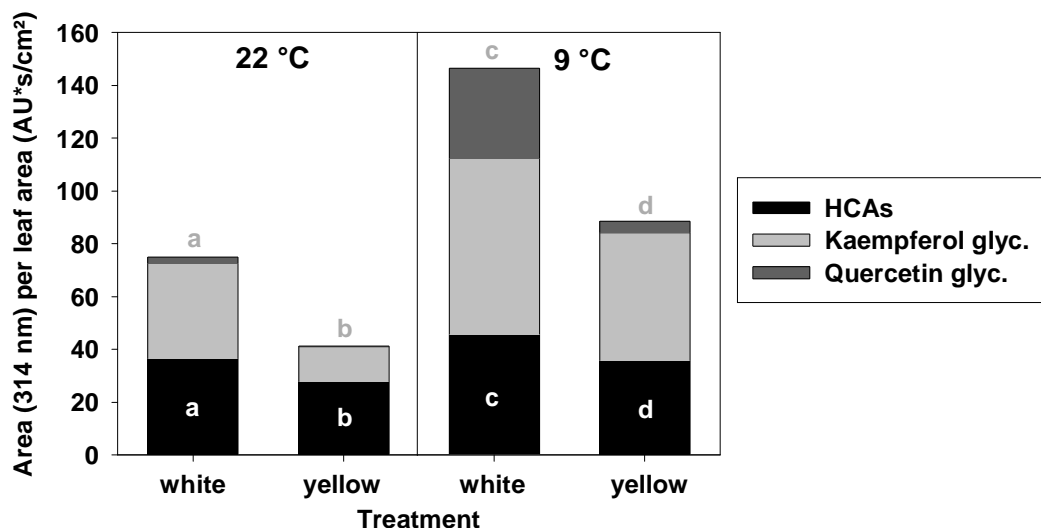


Figure 3.19: Pigment composition in leaves of *A. thaliana* WT Col after illumination with either white (w) or yellow (y) light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  intensity at 22 and 9 °C for seven days ( $n = 5$  for all treatments with the exception of 22/w, where  $n = 7$ ). Differences in total pigment contents were tested with a two-way ANOVA and a tukey-test as post-hoc-test for significance. All total pigments and the amount of HCAs were statistically significant different. P-values were always  $P < 0.001^{***}$  for total pigments (grey letters) and variable for HCAs (white letters): 22/w(a) vs. 22/y(b),  $P = 0.012^*$ , 9/w(c) vs. 9/y(d),  $P = 0.009^{**}$ , 22/w(a) vs. 9/w(c),  $P = 0.009^{**}$  and 22/y(b) vs. 9/y(d),  $P = 0.030^*$ .

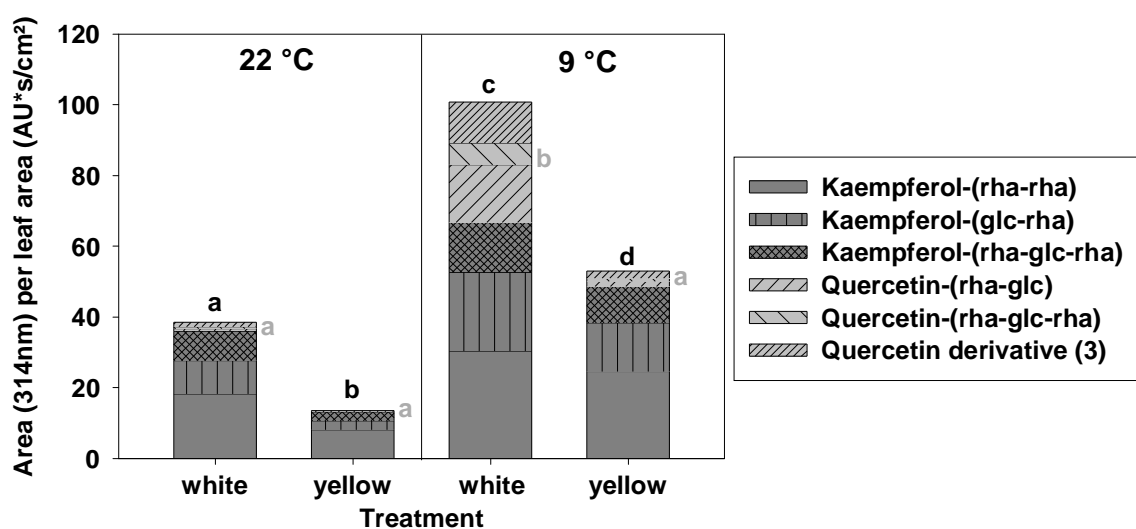


Figure 3.20: Composition of flavonol glycosides in leaves of *A. thaliana* Col which were treated with either white (w) or yellow (y) light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  intensity at 22 or 9 °C for seven days ( $n = 5$  for all treatments with the exception of 22/w, where  $n = 7$ ). The amount of total

flavonoids and of kaempferol glycosides as well as of quercetin glycosides was tested for significant differences using a two-way ANOVA with a following tukey-test. Means of total flavonoids and kaempferol glycosides were significantly different in all cases with  $P < 0.001^{***}$  (black letters). Among total quercetin glycosides only the amount of 9/w was significantly increased ( $P < 0.001^{***}$ ) to all other means (grey letters).

b) Results for *A. thaliana* mutant *cry1cry2* (BG: Col)

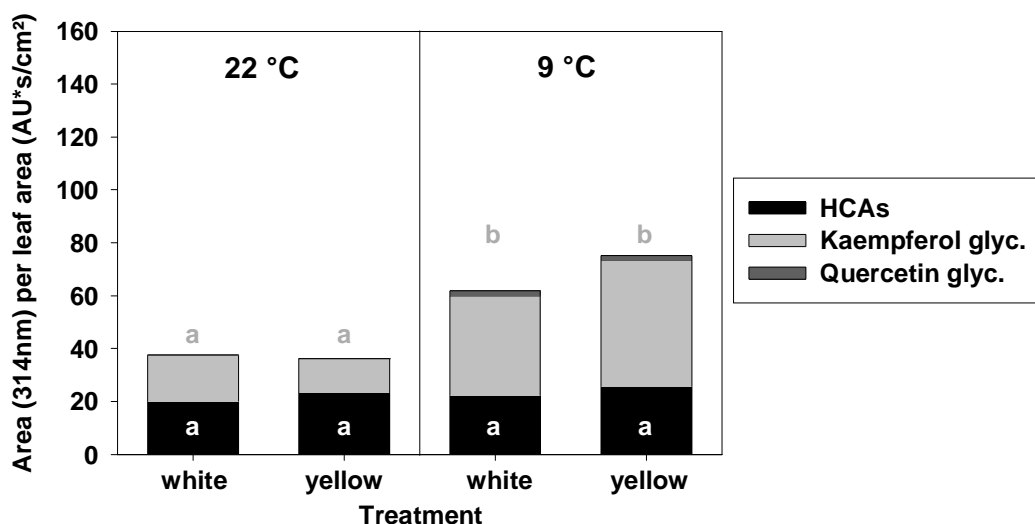


Figure 3.21: Pigment composition in leaves of the double mutant of *A. thaliana* Col, *cry1cry2*, which lacks cryptochrom 1 and cryptochrome 2. Presented is the composition after illumination with either white (w) or yellow (y) light of  $270 \mu\text{mol}/\text{m}^2\text{s}$  irradiance at 22 or 9 °C for seven days ( $n = 5$  for 22/w and  $n = 6$  for 9/w and  $n = 7$  for 22/y and 9/y). Differences in total pigment and total flavonoid contents were tested with a two-way ANOVA and a tukey-test as post-hoc-test (when required) for significance. Total pigments and flavonoids (kaempferol glycosides + quercetin glycosides) were statistically significantly different at 22 °C, independently of light quality, in comparison to total pigments or flavonoids at 9 °C (grey letters). The amount of HCAs was not different in any treatment (white letters). P-values were  $P < 0.001^{***}$  with the following exceptions:  $P = 0.023^*$  and  $P = 0.009^{**}$  for 22/w vs. 9/w of total pigments and total flavonoids, respectively (grey letters).

The double mutant of *A. thaliana* Col, *cry1cry2*, was also investigated under the conditions just described and leaf samples were analysed with HPLC (figure 3.21 and 3.22). This mutant missed all light-induced flavonoids also at 22 °C and  $270 \mu\text{mol}/\text{m}^2\text{s}$  white light (for example compare with control treatment displayed in figure 2.13 of the UV-B experiment which was also conducted in the growth cabinet). Accordingly, the light effect on flavonoid accumulation was not present any more at low temperature and strong white light. Interestingly, the low temperature effect at white light, though still observable and highly significant, was reduced in the mutant in comparison to WT plants (figure 3.19 for Col and 3.21 for *cry1cry2*). About  $67 \text{ AU*s}/\text{cm}^2$  kaempferol

glycosides in Col were in strong contrast to 38 AU\*s/cm<sup>2</sup> kaempferol glycosides in *cry1cry2* plants. Similar, 34 AU\*s/cm<sup>2</sup> quercetin derivatives were found in WT Col plants, whereas in *cry1cry2* mutants only 2 AU\*s/cm<sup>2</sup> were detected. In addition, the amount of HCAs was obviously at least partly controlled by blue light and low temperature via cryptochrome. The double mutant did not show any increased amounts of HCAs in strong white light or under low temperature, but displayed always control amounts of HCAs independently of the treatment in comparison to the WT (figures 3.23 and 3.25). With the exception of values at 22 °C and yellow light, where the amounts of HCAs were not statistically significantly different to each other, all others were tested to be highly significantly different ( $P < 0.001^{***}$ , table 3.1).

For further information on flavonoid composition figure 3.22 displays the flavonol composition in this mutant after the four treatments. As only few amounts of quercetin glycosides were detected, it can be stated that the low temperature-induced accumulation of quercetin glycosides did virtually not take place, though still significantly increased values were detected at 9 °C in comparison to treatments at 22 °C. Kaempferol biosynthesis was only partly affected: At 22 °C the light effect of high intense white light was missing. The amount of kaempferol glycosides was as small as under 22 °C and yellow light and virtually no quercetin glycosides were accumulated. At 9 °C the light effect was also missing. However, kaempferol glycosides were doubled at low temperature, so that a temperature effect was still observed. It was very striking that quasi no quercetin glycosides were found in leaf samples. Thus, the reduced temperature effect in *cry1cry2* plants observed in epidermal UV-A transmittance measurements could be explained by the strongly reduced amounts of quercetin glycosides. This final result is very interesting as it shows, that a light sensor could “function” in a different manner dependent on ambient temperature.

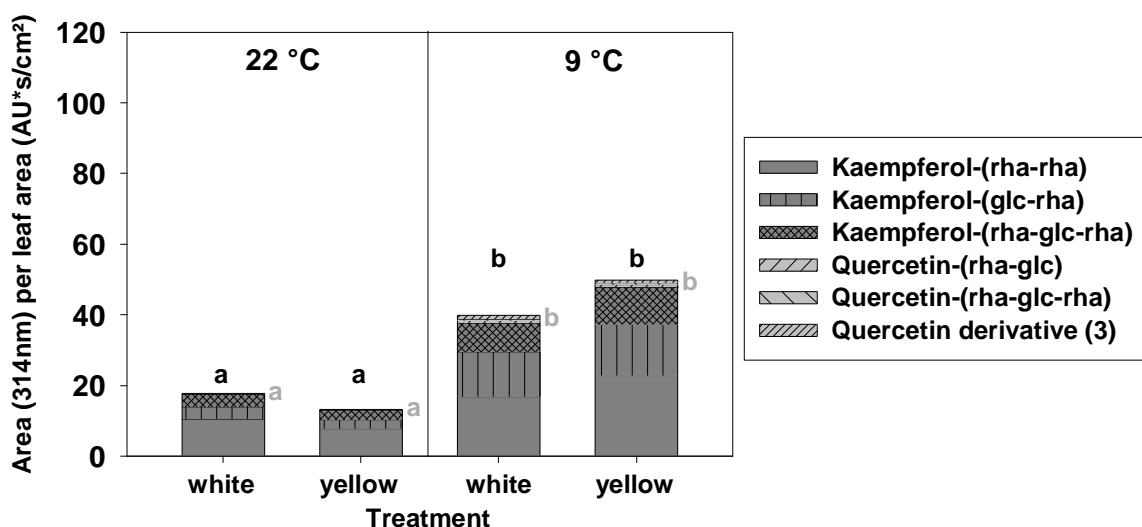


Figure 3.22: Flavonol glycoside composition in leaves of *cry1cry2* mutant of *A. thaliana* Col after a seven day treatment with white (w) or yellow (y) light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  at 22 or 9 °C ( $n = 5$  for 22/w and  $n = 6$  for 9/w and  $n = 7$  for 22/y and 9/y). The amounts of kaempferol glycosides and quercetin glycosides were tested for significant differences using a two-way

ANOVA with a following tukey-test. Means of kaempferol glycosides and quercetin glycosides were significantly different with respect to different temperatures only and independently of different light qualities. Level of significance was always high with  $P < 0.001^{***}$  with the exception of 22/w vs. 9/w, where  $P = 0.013^*$  for kaempferol glycosides.

A direct comparison of HPLC results of WT Col and *cry1cry2* is presented in table 3.1. No statistical difference between Col and *cry1cry2* for the amount of HCAs, kaempferol glycosides and quercetin glycosides were detected after treatment with 22 °C and yellow light. At 9 °C and yellow light similar kaempferol contents were observed which indicates an equal temperature effect on kaempferol accumulation in both varieties at yellow light. The amount of quercetin glycosides was also tested to be not significantly different, however absolute numbers were halved in the double mutant, indicating a reduced temperature effect. Also, whereas the amount of kaempferol glycosides was only halved at 9 °C and white light, quercetin glycosides were reduced by a factor 16 in the mutant. In all residual cases *cry1cry2* mutants displayed reduced pigment amounts indicating a reduced (missing) high light effect and a reduced temperature effect.

Table 3.1: Comparison of HPLC results for HCAs, kaempferol glycosides and quercetin glycosides of WT and *cry1cry2* mutants of *A. thaliana* Col treated with either white (w) or yellow (y, i.e. no blue light) at 22 or 9 °C air temperature for seven days. Amount of pigment is given as peak area at 314 nm absorption in relation to leaf area. Different letters indicate statistically significant differences with  $P < 0.001^{***}$  with the following exception: <sup>1</sup>  $P = 0.004^{**}$ . Significant differences were resolved by applying a one-way ANOVA with a tukey-test as post-hoc-test. Non-different values within treatments are highlighted by bold letters.

Treatment	22/w		22/y		9/w		9/y	
Variety	Col	<i>cry1cry2</i>	Col	<i>cry1cry2</i>	Col	<i>cry1cry2</i>	Col	<i>cry1cry2</i>
<b>HCAs</b> AU*s/cm <sup>2</sup>	a	<b>b</b>	<b>b</b>	<b>b</b>	c	<b>b</b>	d	<b>b</b>
	36.464	19.861	27.691	23.083	45.657	22.131	35.659	25.502
<b>Kaempferol glycosides</b> AU*s/cm <sup>2</sup>	a <sup>1</sup>	<b>b<sup>1</sup></b>	<b>b</b>	<b>b</b>	c	d	<b>d</b>	<b>d</b>
	35.937	17.604	13.268	13.055	66.719	37.688	48.608	47.888
<b>Quercetin glycosides</b> AU*s/cm <sup>2</sup>	<b>a</b>	<b>a</b>	<b>a</b>	<b>a</b>	b	c	<b>ac</b>	<b>c</b>
	2.551	0.166	0.199	0.191	34.108	2.160	4.370	1.874



### 3.2 Discussion (Third part)

This third part aimed to discover one or more signals which were responsible for the induction of flavonoid accumulation at low temperature conditions. In the second part of this work it was already shown that the pattern of flavonoid accumulation at low temperature and high light was similar to each other whereas a UV-B-induced accumulation differed with respect to flavonoid composition. A strong interaction between irradiance and temperature was also demonstrated. It must be admitted that beyond the potential signals which were investigated here, several other potential signals for induction of flavonoid accumulation and gene expression of *PAL* and *CHS* might exist and are described to a certain degree in the literature. For example, the red/far-red sensor phytochrome A (only in seedlings; Bowler et al. 1994, Wade et al. 2003), senescence-related cytokinins (Deikmann & Hammer 1995, Wade et al. 2003), pathogen-derived elicitor plus nitric oxide producing nitric oxide synthase (NOS; Modolo et al. 2002) or elicitor plus salicylic acid (Mauch-Mani & Slusarenko 1996), wounding (Vogt et al. 1994, Gould et al. 2002), cGMP (cyclic guanosine 3', 5'-monophosphate; Bowler et al. 1994, Durner et al. 1998) and sugars (mostly anthocyanin accumulation affected; Tsukaya et al. 1991, McKown et al. 1996, Mita et al. 1997, Wade et al. 2003, Solfanelli et al. 2006) are reported to be involved in induction of gene expression of the key biosynthetic enzymes of flavonoid biosynthesis *PAL* and/ or *CHS*. However, reactive oxygen species (ROS), the redox state of the photosynthetic electron transport chain or light sensors were regarded as most potent signal carriers for flavonoid accumulation at low temperature and were investigated in detail.

*Reactive oxygen species are most likely not involved in epidermal flavonoid accumulation after treatment with low temperature, high light or UV-B*

Epidermal flavonoid accumulation in *A. thaliana* was induced by high irradiances, supplemental UV-B radiation and low temperature. The question was posed which signal could be responsible for flavonoid accumulation under these conditions. One likely signal was found to be oxidative stress derived reactive oxygen species (ROS).

High irradiances are known to generate oxidative stress accompanied by a production of ROS (Dat et al. 2000). At increased light conditions the enzymatic processes for CO<sub>2</sub> fixation become rate limiting which results in a photosynthetic accumulation of more redox and energy equivalents than needed and an accumulation of ROS. The first reactive oxygen species that develops is the superoxide anion radical (O<sub>2</sub><sup>-</sup>), which has a half-life of only 2- 4 μs. Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) can dismutate spontaneously from superoxide or by the action of the antioxidative enzyme SOD (superoxide dismutase). Hydrogen peroxide is considerably more stable than superoxide with a half time of 1 ms, which allows it to diffuse some distance from its site of production. Superoxide and hydrogen peroxide may also be generated at PS I (Karpinsky et al. 2001) but will be scavenged most likely by photoreduction to water with electrons from PS II in the

water-water cycle (Asada 1999). It is also reported that energy transfer from excited triplet chlorophyll to ground state oxygen ( $O_2$ ) produces singlet oxygen ( $^1O_2$ ) under conditions of photoinhibition due to excess PAR (Hideg et al. 2000). However, it is not clear, if the applied irradiance of  $350 \mu\text{mol}/\text{m}^2\text{s}$  white light could be termed as “excess PAR” and was effective in leading to oxidative stress.

UV-B radiation may destroy the D1 and D2 proteins in PS II (Jansen et al. 1996, Booiij-James et al. 2000) and generate through this effect ROS at PS II. However, oxidative damage to the chloroplast appears not to be involved in UV-B induced gene expression (Jordan et al. 1998). Other possible sources for ROS under UV-B radiation are the decreased activity of catalase and increased activity of a plasma membrane located multi-component enzyme system, NADPH oxidase, analogous to the mammalian phagocyte oxidase (Torres et al. 1998, Rao et al. 1996). Other origins of UV-B induced ROS are assumed to be peroxidases and lipoxygenases (A.-H.-Mackerness et al. 2001).

Low temperature slows down many enzymatic processes, especially the activity of the slowest enzyme in the Calvin cycle, Rubisco. This could result in a blockage of the electron transport chain and increased ROS formation as the Calvin cycle runs slower (Feierabend et al. 1992, Dat et al. 2000). However, a decline in variable chlorophyll fluorescence (Fv/Fm) indicating photoinhibition of PS II was not detected for *A. thaliana* exposed to  $9^\circ\text{C}$  (data not shown). Other compartments like mitochondria and peroxisomes may also be important sources for hydrogen peroxide due to photo-inactivation of the antioxidative enzyme catalase at low temperatures (Feierabend et al. 1992). However, it is proposed that other, additional ROS generating pathways must exist at low temperature (Hideg et al. 2000). In summary, under all three treatments a more or less severe accumulation of ROS was likely.

Some indications in the literature exist about an involvement of ROS in flavonoid accumulation. Nagata et al. (2003) observed an accumulation of anthocyanins induced by a ROS generating agent in *Arabidopsis*. The *sac 9-1* mutant of *Arabidopsis* which lacks phosphoinositide phosphatase involved in phosphoinositol signaling displayed a constitutive accumulation of anthocyanin in cells of the lower leaf epidermis adjacent to guard cells (Williams et al. 2005). The presence of fluorescence signals from the NO-radical sensitive dye DAF (2', 7'-dichlorofluorescein diacetate) in cells which accumulate anthocyanins was observed, which points to a NO $\cdot$ -induced accumulation of anthocyanins. However, *sac 9-1* plants were selected based on anthocyanin accumulation prior treatment with DAF and it was also shown that mutants exhibited very reduced roots in comparison to WT. Thus, this last aspect could also result in a reduced nutrient uptake which also induces anthocyanin accumulation in *A. thaliana* (Dixon & Paiva 1995).

In contrast, two other studies concluded that UV-B or fungal elicitor derived ROS were not involved in the induction of gene expression of *CHS* or *PAL*, respectively (A.-H.-Mackerness 2000, Wendehenne et al. 2002). However, the oxygen radical nitric oxide

(NO<sup>•</sup>) and several chemical NO<sup>•</sup> donors can increase mRNA level of *PAL* and *CHS* (Delledonne et al. 1998, Durner 1998, Delledonne 2003, Wendehenne 2004). A.-H.-Mackerness (2001) also revealed that UV-B radiation leads to a NOS-derived NO<sup>•</sup> radical production which induces transcription of *CHS*. Delledonne and co-workers (2003) demonstrated only recently that NOS derived NO<sup>•</sup> might induce gene expression *PAL* and *CHS* and that also synergistic effects of ROS and nitric oxide might play a role in ROS-induced signal transduction processes during the hypersensitive response. The bacterial elicitor *Pseudomonas syringae* was identified as a source of NOS derived NO<sup>•</sup> accumulation (Delledonne et al. 1998). However, no information exists in the current literature whether nitric oxide accumulates at low temperature or high light conditions.

Visualizing oxidative stress products was performed on low temperature treated leaves of *A. thaliana* with the ROS-sensitive dyes DAB and NBT. The location and the extent of superoxide anion radical accumulation can be visualized in leaf tissue by NBT which is reduced by the radical to form a blue coloured formazan precipitate (Ogawa et al. 1997, Gould et al. 2002). NBT stained vascular tissue, especially xylem parenchyma and lignin-containing cells, in cross sections of hypocotyls from spinach seedlings was explained by superoxide production of NADPH oxidase (Ogawa et al. 1997). Ozone-exposed *Arabidopsis* leaves also displayed accumulation of superoxide which was made visible by infiltrated NBT and was detected as scattered blue spots (Schraudner et al. 1998, Overmyer et al. 2000). NBT derived coloured leaf veins were visible in this work in low temperature treated rosette leaves of *A. thaliana* Col (image 3.1 and 3.2). This is generally accepted to be a proof of the uptake of tracer dye via the transpiration stream. Superoxide produced during the process of lignification by cell wall oxidases generally leads to this characteristic staining of leaf veins and other vascular tissues. Hydrogen peroxide in leaves of tobacco cultivars was made visible by infiltrated DAB which is peroxidase-dependent polymerized by hydrogen peroxide to form a brown precipitate (Schraudner et al. 1998). H<sub>2</sub>O<sub>2</sub> release after bacterial infection with *Pseudomonas syringae* in leaves from *A. thaliana* Col was successfully demonstrated using vacuum infiltrated DAB (Torres et al. 2000). Supplemental UV-B radiation also led to an accumulation of hydrogen peroxide in *Arabidopsis* plants made visible by vacuum infiltrated DAB (Kalbina & Strid 2006). In conclusion it can be stated that both DAB and NBT were common dyes for visualizing hydrogen peroxide and superoxide anion radicals in leaves of *A. thaliana*. However, most studies cited applied the dye solutions via vacuum infiltration and after the stress treatment. In this work, dark adapted leaves were loaded with tracer dye in darkness and 22 °C air temperature via the leaf's transpiration stream prior to treatment with low temperature. This was done in order to start with a low level of ROS in the leaf and to trap all reactive oxygen molecules which accumulate during the treatment. In this work, hydrogen peroxide and superoxide accumulation were detected at low temperature in light and darkness. Sources for ROS in light and low temperature were described above and are numerous. However, chloroplast derived ROS appears unlikely as conditions were not severe enough in order to result in photoinhibition of PS II. In contrast, at low temperature and darkness respiration in mitochondria and especially the enzyme alternative oxidase (AOX)

therein could be sources for superoxide and hydrogen peroxide production (Dat et al. 2000). Thus, NBT and DAB staining at low temperature could be explained by ROS production from similar and light-independent sources (results for DAB were not shown).

However, no increase in UV-A absorption in darkness and low temperature was detected. These results suggest that ROS formation might not induce flavonoid accumulation and a role of low temperature alone (i.e. without light) in the induction of flavonoid accumulation could be denied. Unfortunately, experiments with the NO<sup>-</sup> sensitive fluorophore DAF (2', 7'-dichlorofluorescein diacetate) and a NO<sup>-</sup> sensitive electrode (Apollo 2000 with NO<sup>-</sup> electrode ISO-NOP, WPI Europe, Berlin, Germany) and low temperature treated rosette leaves of *A. thaliana* did not produce any consistent results (data not shown). Thus, it can not be excluded that nitric oxide would accumulate at low temperature and light and that this would be involved in low temperature-induced flavonoid accumulation.

Several chemical ROS donors and NO<sup>-</sup> donors are available and were successfully used by several scientists. Beside hydrogen peroxide and superoxide producing riboflavin (Beauchamp & Fridovich 1971), NO<sup>-</sup> generating SNAP, SNP and SIN-1 were applied in this work. SIN-1 produces NO<sup>-</sup> and superoxide, which may react to form highly reactive peroxynitrite (ONOO<sup>-</sup>, de Groot et al. 1993, Durner et al. 1998, Jasid et al. 2006). SNAP and SNP are known to be strong NO<sup>-</sup> donors and may increase mRNA levels of *PAL* in tobacco (Durner et al. 1998) and in *Arabidopsis* (Delledonne et al. 1998). Transcript levels of *CHS* were also found to be increased by SNAP in mature leaves of *A. thaliana* (Mackerness et al. 2001).

ROS generating solutions were applied by two different manners: brushing of adaxial leaf sides or application via the transpiration stream. The first way was very similar to spraying until runoff which was done by Surplus et al. (1998) and A.-H.-Mackerness et al. (2001) who applied salicylic acid and ascorbic acid one hour before beginning the UV-B treatment, and the ROS donor 3-AT (3-amino-1,2,4-triazole) on rosette leaves of *A. thaliana*, respectively. No comparable study exists which applied ROS generating solutions (or tracer dye like NBT or DAB) via the transpiration stream. It must be mentioned that also cut leaves if held turgescient were still capable to accumulate flavonoids. A similar observation was made for leaves of *Fagopyrum tataricum*, which accumulated rutin and displayed increased activity of rutin glucosidase in half cut leaves after treatment with UV-B or sub-zero temperatures (Suzuki et al. 2005). In addition, NBT loaded leaves of *A. thaliana* Col displayed no reduction in variable chlorophyll fluorescence (Fv/Fm; data not shown) indicating that NBT had no influence on the photosynthetic activity of the leaves.

Experiments in which ROS generating solutions were applied did not promote the hypothesis, that ROS might be involved in the induction of flavonoid accumulation as no decrease in epidermal UV-A transmission was observed. Again, a potential role of

nitric oxide in flavonoid accumulation is documented by the the work of A.-H.-Mackerness (2001) who demonstrated a SNAP-induced increase in mRNA level of the gene coding for the flavonoid biosynthetic enzyme CHS. In studies where *PAL* transcription was induced, flavonoid accumulation was not investigated and must not be essential as this early enzyme of the phenylpropanoid biosynthetic pathway is also involved in the synthesis of several plant defense metabolites including salicylic acid (Durner et al. 1998). Thus, further work is needed to elucidate the role of NO<sup>-</sup> in flavonoid accumulation at abiotic adverse conditions.

*Increased excitation pressure of PS II in the chloroplast is not the sensor for epidermal flavonoid accumulation at low temperature conditions*

Light absorbed by the photosystems drives the electron transport chain. At conditions of increased irradiance or reduced temperature (or both) the excitation pressure on the electron transport chain rises with a resulting higher fraction of reduced plastoquinone (Q<sub>A</sub>) (Gray et al. 1997, Huner et al. 1998). The reduction state of Q<sub>A</sub> can be determined by chlorophyll fluorescence measurements and is indicated by the parameter 1-q<sub>L</sub>, (Schreiber et al. 1986, Kramer et al. 2004). The slowing down of enzymatic processes in the Calvin cycle at low temperature may also result in an increased reduction state of the plastoquinone pool. The applied UV-B radiation was too little to have any effect on the reduction state of the electron transport chain.

The redox state of the plastoquinone pool functions as a sensor for several signal transduction pathways which regulate gene expression of chloroplast-encoded, photosynthetic *cab* genes (encode LHCI proteins) (Escoubas et al. 1995) and *psaAB* (encode reaction-center subunits of PS I) and *psbA* (encodes D1 reaction-center protein in PS II) (Pfannschmidt et al. 1999) but also of nuclear-encoded, antioxidative genes like cytosolic ascorbate peroxidases (APXs) (Karpinski et al. 1997) or cold-induced genes like *Wcs19* (Gray et al. 1997). Conclusively, an observed strong light dependency of epidermal UV-A transmission coupled with an increased reduction state of Q<sub>A</sub> with increasing light intensities as well as the interaction of reduction state with low temperature were additional reasons to hypothesize a redox controlled induction of flavonoid biosynthesis.

However, no correlation of the redox state of Q<sub>A</sub> with epidermal UV-A transmittance was detected at low temperature conditions. An irradiance of 120 μmol/m<sup>2</sup>s and a temperature of 9 °C was not enough to strongly increase the reduction state of Q<sub>A</sub> in *A. thaliana* Col but was very well enough to induce a strong accumulation of epidermal flavonoids. At 9 °C and 200 μmol/m<sup>2</sup>s the reduction state was increased in WT *S. tuberosum* and epidermal UV-A transmittance was significantly reduced. However, ferredoxin-related increased reduction states of plastoquinone at control conditions in antisense *S. tuberosum* were not coupled with reduced transmittance values when compared with the WT. A low temperature treatment at 200 μmol/m<sup>2</sup>s PAR resulted in

increased reduction states in WT and *Fda7* plants, but all varieties displayed homogeneously reduced levels of epidermal UV-A transmittance. These results indicated clearly, that epidermal UV-A transmittance was reduced at low temperature independently of the reduction state of  $Q_A$ .

Further arguments point against a role of  $1-q_L$  as sensor for flavonoid biosynthesis.  $1-q_L$  would be a signal of photosynthesis in the chloroplasts. This signal would have to travel through chloroplasts in the mesophyll to the upper epidermal layer, where it had to be sensed by the nucleus of epidermal cells. This relatively long way of a signal transduction pathway appeared to be very unlikely. However, transcription of nuclear-encoded genes like *AOX* were shown to be regulated by the redox state of the plastoquinone pool, which demonstrates at least a signal transduction pathway from the chloroplast to the nucleus of the same cell. In addition, the redox state of  $Q_A$  was shown to be sensed or transduced by remote tissues (Karpinski et al. 1999) so that the idea of remote signaling must be considered. Karpinski et al. (1999) measured an increased reduction state of  $Q_A$  at high light conditions and increased levels of hydrogen peroxide in leaves of *A. thaliana* which were not exposed to the increased irradiance. However, as the experiments with the leaves turned upside down showed, remote signaling seems rather improbable with respect to epidermal flavonoid accumulation.

It is concluded, that the redox state of plastoquinone A is most likely not the sensor for flavonoid accumulation at low temperature in *A. thaliana*. Nevertheless, a role of other redox signals coming from the chloroplast like ascorbate, ferredoxin/ thioredoxin system and others under conditions of increased excitation pressure is still possible but was not investigated (Foyer & Noctor 2003).

*The blue light sensor cryptochrome 1 is responsible for the high light-induced accumulation of flavonoids at 9 and 22 °C air temperature in a temperature-dependent manner*

The third part of this work aimed to discover a signal which was responsible for the low temperature-induced flavonoid accumulation in epidermal cells of *A. thaliana*. Two experiments were keystones for the discovery of this signal carrier. Firstly, it was discovered, that a low temperature-induced reduction in epidermal UV-A transmittance only occurred in illuminated areas of epidermis with the abaxial epidermis displaying a reduced potential to do this. This observation eventually excluded a relatively unspecific signal derived from ROS or from the redox state of the chloroplastic electron transfer chain and it pointed to an epidermis-located sensor. The second experiment was a treatment of plants with low temperature in complete darkness which proved that no flavonoids were accumulated under these conditions (figure 2.30). HPLC analysis revealed that leaves had control levels of kaempferol glycosides and quercetin glycosides, and slightly but significantly reduced amounts of HCAs. The fact, that flavonoid accumulation at low temperature only occurred when light was present, led to

the consideration that the role of low temperature alone could be excluded and that rather photoreceptors might be involved. Also, the influx of calcium ions into the cells as a very fast reaction on decreasing temperatures stands in contrast to a slow accumulation of flavonoids only under prolonged (i.e. more than 12 hours) low temperature conditions.

Perception of light occurs in higher plants via several photoreceptors which can specifically sense different light qualities. Phytochromes perceive red and far-red light and are responsible for physiological responses to light. Phototropins and cryptochromes can sense UV-A/ blue light and mediate photomovement and photomorphogenic responses, respectively. The sensor for UV-B light perception is not known until date (see reviews of Fankhauser & Staiger 2002 and Lin & Shalitin 2003).

A UV-A/ blue light-induced gene expression of *CHS*, which is mediated by cryptochrome 1, was reported earlier (Jackson & Jenkins 1995, Batschauer et al. 1996, Fuglevand et al. 1996, Neff & Chory 1998). *CHS* mRNA levels and anthocyanin content were also increased by the action of far-red light via phytochrome A (Lange et al. 1970, Bowler et al. 1994, Christie & Jenkins 1996, Neff & Chory 1998). A positive regulator of phytochrome A and B mediated *CHS* gene expression was discovered recently by Kim and colleagues (2003) and is called PIF3 (phytochrome interacting factor 3). Also, a synergistic effect of phytochromes A and/ or B and cryptochrome 1 on *CHS* gene expression was reported first by Ahmad and Cashmore (1997) and also by Wade and co-workers (2001). A significant contribution of a blue light sensor to the fluence rate dependence of anthocyanin accumulation under blue light was shown for seedlings of cabbage (*Brassica oleracea*) and tomato (*Lycopersicon esculentum*) (Mancinelli et al. 1991).

The role of blue light perception in the induction of flavonoid biosynthesis was investigated by excluding the blue light fraction of the light spectrum by a yellow foil. UV-A transmittance data and HPLC analysis clearly proved that blue light was responsible to 100 % for the high white light-induced accumulation of kaempferol glycosides, quercetin glycosides and HCAs at 22 °C. This means, 22 °C and yellow light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  resulted in levels as under control conditions (22 °C, 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light) for all UV-absorbing compounds. Similarly, blue light was responsible for the additional effect of high intense white light on these parameters at 9 °C. Thus, at 9 °C and yellow light of 270  $\mu\text{mol}/\text{m}^2\text{s}$  pigment levels as under low temperature conditions (9 °C, 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light) were measured. However, a strong low temperature effect, independently of light quality was also observed, resulting in high contents of kaempferol glycosides and quercetin glycosides and increased values for HCAs at 270  $\mu\text{mol}/\text{m}^2\text{s}$  yellow light which were similar to values observed at 9 °C and 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light.

The effect of blue light was further investigated using double mutants of *A. thaliana* of phototropin (*phot1phot2*) and cryptochrome (*cry1cry2*). Whereas the partially blue

light-blind *phot1phot2* mutant still was able to accumulate UV-A absorbing compounds at low temperature conditions similar to WT plants, the double mutation in both cryptochromes as realised in the mutant *cry1cry2* was found to be responsible for the observed blue light effect. Control levels of kaempferol and quercetin glycosides as well as HCA derivatives were detected at 22 °C and yellow or white light. At 9 °C air temperature characteristic low temperature levels of kaempferol glycosides were detected at white and yellow light, indicating a low temperature effect on kaempferol glycoside accumulation which was similar to the effect in WT Col. However, the accumulation of quercetin glycosides at low temperature in the mutant was strikingly small, indicating a reduced low temperature effect on quercetin glycosides in the mutant. In summary, these observations reveal that cryptochrome was involved in flavonoid and HCA accumulation at 22 and 9 °C. Additionally, epidermal UV-A transmittance measurements on treated *cry1* and *cry2* single mutants revealed, that at 22 °C flavonoid accumulation could completely be inhibited by the lack of either blue light or cryptochrome 1. Surprisingly, control levels of UV-A absorbing compounds and HCAs were sustained in the mutants *cry1cry2* and *cry1* also when blue light was missing. These control levels were also observed in low temperature and dark treated *Arabidopsis* WT plants. Thus, this level must be constitutive and might be sustained by a different sensor or a different regulatory mechanism. At low temperature it became clear that cryptochrome must interact with reduced temperature, because an additional effect of 9 °C on flavonoid accumulation was still observable in the mutant which was reduced for quercetin glycosides. In addition, no effect at all was visible on HCAs.

In conclusion, results point to the fact, that cryptochrome 1 might be involved in the light-dependent flavonoid accumulation and might act in a temperature-dependent manner (figure 3.23). This last aspect is very surprising as it implies that low temperature might change signal transduction processes following cryptochrome 1 perception. With respect to regulation of the amount of total HCAs, both the high white light and the low temperature-induced accumulation of HCAs was completely inhibited indicating that without any cryptochrome the whole inductive biosynthetic branch of HCAs was stopped. On the other hand, this observation also suggests that cryptochrome must either interact with a temperature-sensitive partner or be itself somehow temperature-sensitive, because it is clearly involved in a pure low temperature sensing signal transduction pathway where blue light sensing is not included (no low temperature effect on HCAs at yellow light).

Consequently, the question arises how the low temperature effect on flavonoid accumulation is established in the double mutant at low temperature. An interaction of cryptochrome 1 (Cry1) with a temperature sensitive partner must be assumed, because the high white light effect is explained totally by Cry1 at 22 °C, but is only partly explained at low temperature. On the other hand, a complete different pathway responsible for the additive low temperature effect must also be considered which would act in parallel to a high white light-induced flavonoid accumulation through the action of cryptochrome 1. However, this last assumption is not favoured as it would not



explain the reduced effect on quercetin glycosides and the missing effect on HCAs in the mutant.

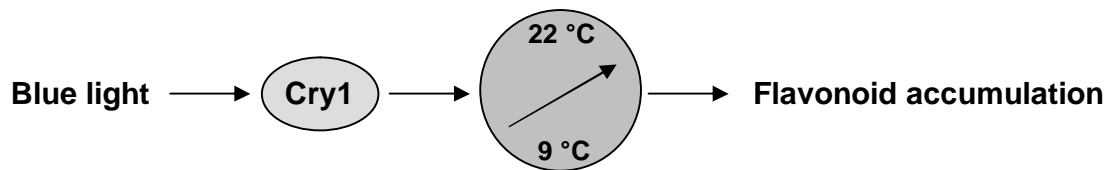


Figure 3.23: Schematic signal transduction via blue light mediated cryptochrome 1 (Cry1) and a second temperature sensor that modulates flavonoid accumulation.

From the literature it is known that anthocyanin accumulation in few days old seedlings of *A. thaliana* is induced at low intense white light (50  $\mu\text{mol}/\text{m}^2\text{s}$ ) and warm temperatures (21 °C) via the action of cryptochrome 1 (Cry1) and phytochrome A (PhyA) or B (PhyB) (Neff & Chory 1998). When Cry1 was missing by mutation, anthocyanin accumulation was not abolished but only reduced. Only when two of three light sensors (Cry1, PhyA, PhyB) were missing, no anthocyanin accumulation was observed, indicating redundancy among light sensors at white light. In contrast, under blue light of same intensity, PhyA and Cry1 were both necessary for a full anthocyanin accumulation. Conversely, if one of them or both were missing, no anthocyanin accumulation was detected. Thus, at white and yellow light conditions, the double mutant *cry1cry2* still could fall back upon PhyA and PhyB and establish a certain level of flavonoids. However, this hypothesis would not explain the low temperature effect.

Cryptochromes are low abundant nuclear proteins with a nuclear/ cytosol distribution pattern (see review of Lin & Shalitin 2003). This means, in the darkness cryptochromes may be imported to the nucleus and in light they are exported or remain in the cytosol. Cryptochromes interact with several proteins like phytochromes (PhyA), COP1, circadian clock proteins and/ or chromatin and DNA (Lin & Shalitin 2003). The interaction with phytochromes is tight as they act concurrently to mediate photomorphogenic responses like stimulation of leaf expansion, entrainment of the circadian clock, regulation of gene expression and others. A significant convergence between signal transduction pathway of cryptochromes and phytochromes makes a dissection of the cryptochrome signal transduction extremely difficult. Cry1 interaction with the key repressor of photomorphogenesis in the dark, COP1 or other COP/DET proteins may be light-dependent and suppresses the activity of these proteins. Cry1 also interacts via COP1 with the activators of light-regulated genes HY5, a bZIP transcription factor (Ang et al. 1998, Fankhauser & Staiger 2002, Lin & Shalitin 2003) or with CIP7 (COP1 interactive protein), which both activate light-induced gene expression and can activate *CHS* gene expression (Yamamoto et al. 1998). COP1 repression of HY5 is achieved by targeting HY5 to the proteasome. Thus, blue light-induced cryptochrome signal transduction appears complex with several interacting partners of which only few are known like phytochromes or COP1.

The tight interaction between Cry1 and PhyA (Wang & Deng 2002) and a convergence in signaling plus an assumed redundancy points to a possible role of PhyA or PhyB in the low temperature-induced flavonoid accumulation. It would be interesting to investigate a *cry1phyA* mutant at low temperature and high light conditions in order to test whether this mutant would be still able to accumulate flavonoids under these conditions. It is also known that PhyA regulates several far-red light responses which include anthocyanin accumulation (Bowler et al. 1994). A phytochrome A mediated induction of gene expression of *CHS* via another phytochrome interacting protein named PAP1 and HY5 was also reported from Yang et al. (2002). However, an involvement of COP1 or other proteins in the low temperature-induced accumulation must also be considered. It is already known that low temperature changes the phosphorylation status of proteins via the action of calcium-dependent protein kinases (Monroy et al. 1998). For example, it can be imagined that a physical interaction between Cry1 and COP1 or other proteins could be changed at low temperature which could result in a less severe suppression of CIP7 or HY5 which could in turn accumulate more and lead to a more intense activation of light-induced gene expression like flavonoid biosynthesis (see model in table 3.2). If Cry1 would be absent through mutation, either a phytochrome could replace somehow Cry1 leading to an incomplete interaction between the photoreceptor and COP1 or suppression of CIP 7 or HY5 would be raised by a different perturbation at low temperature, which also could result in an increased gene expression of flavonoid biosynthetic genes as observed in the double mutant. This model could also explain a light-dependent flavonoid accumulation at warm and low temperatures. On the contrary, HCA accumulation at high intense white light and low temperature appears to be mediated also by cryptochrome, but the mechanisms must be different as also the *cry1cry2* mutation was sufficient to eliminate the low temperature effect. Thus, a rather short signal transduction pathway is proposed which lacks a key repressor like COP1. Such short signal transduction pathways with direct contact of the light receptor with the transcription factor were reported for phytochromes (Fankhauser & Staiger 2002, Quail 2002). In this model, Cry1 itself must sense the reduced temperature, for example, by a change of its phosphorylation status, which then would be transduced as a strong signal directly to the transcription factor binding to the key biosynthetic gene for HCA accumulation, which is *PAL*. Similar, the use of different signal pathways from the same photoreceptor was reported for phytochromes (Quail 2002). In short, an additional activation status of cryptochrome by low temperature is proposed. If cryptochrome is missing as realized in the double mutant *cry1cry2*, activation is lost with no induction of HCA accumulation at high white (blue) light and/or low temperature. However, no indications about these hypothetical mechanisms exist in the current literature, so that these considerations must remain speculative and are exciting starting-points for future research.

Table 3.2: Hypothetical model explaining the low temperature-induced accumulation of kaempferol and quercetin glycosides in *A. thaliana* WT Col and the double mutant *cry1cry2* (shaded rows) (Cry1 = cryptochrome 1; y = yellow light; BL = blue light; cc = *cry1cry2*; w = white light; Phy = phytochrome, P-status = phosphorylation status).

Treatment	Blue light	Cry1 status	COP1 status	CIP 7/ HY5 level	gene expression
WT: 22°C/y	no BL	inactive	repression of HY5	low	no
cc: 22°C/y	no BL	-	repression of HY5	low	no
WT: 22°C/w	BL	active	interaction with Cry1	high	high
Cc: 22°C/w	BL	-	no interaction with Cry1, repression of HY5	low	no
WT: 9°C/w	BL	active	interaction with Cry1, change in P- status, no repression of HY5	extremely high	extremely high
cc: 9°C/w	BL	-	Phy replaces Cry1, incomplete interaction with COP1 + change in P-status, loose repression of HY5	high	high
WT: 9°C/y	no BL	inactive	change in P-status in COP1, incomplete repression of HY5	high	high
cc: 9°C/y	no BL	-	change in P-status in COP1, incomplete repression of HY5	high	high

In contrast to the cryptochrome mediated photomorphogenic responses, phototropins are involved in photomovement responses including phototropism, chloroplast relocation or stomatal opening (see review of Lin & Shalitin 2003). Thus, it was eventually not surprising that phototropins were not involved in flavonoid accumulation at high light and low temperature but rather cryptochromes which clearly are involved in morphological changes to which epidermal flavonoid accumulation could be counted.

Finally, it can be concluded that the low temperature-induced flavonoid accumulation is in part regulated by the blue light sensor cryptochrome 1 which involves an unknown, temperature-sensitive interacting partner. In contrast, HCA accumulation, which was not in focus of this work, was shown to be completely controlled by Cry1. This was an unexpected result as it implies that Cry1 can sense somehow temperature. However, it could not be answered how the temperature effect on quercetin glycoside accumulation was reduced in the double mutant, whereas it was equal to WT for kaempferol glycosides. Speculatively, different sensitivities for blue light of promoters of the genes

encoding flavanone-3-hydroxylase (*F3H*, for kaempferol biosynthesis) and flavonol-3'-hydroxylase (*F3'H*, for quercetin biosynthesis) could possibly be the reason for this observed difference.

#### 4 Fourth part: Comparative gene expression studies in leaves of *A. thaliana* WT (Col) exposed to low temperature, UV-B and high irradiances

##### 4.1 Results

Epidermal flavonoid accumulation was observed after treatment with low temperature, high irradiances or supplemental UV-B radiation. This was shown by epidermal UV-A transmittance measurements as well as by HPLC analysis. Thus, merging signal transduction pathways under these conditions were suggested (figure 4.1). Flavonoid accumulation under the conditions described could be the result of a *de novo* synthesis of the corresponding biosynthetic enzymes like PAL, CHS, F3'H and others. A *de novo* synthesis would be reflected in an increased gene expression of these enzymes and could be detected by microarray analysis or northern blotting.

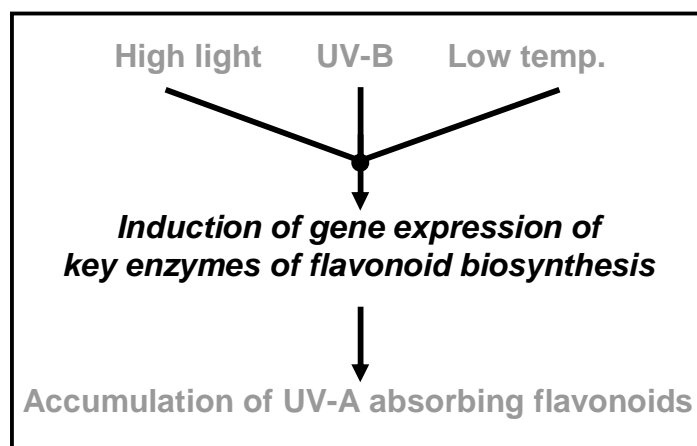


Figure 4.1: Flowchart demonstrating the working hypothesis of this chapter. It was assumed that an induction of gene expression of essential genes coding for enzymes of flavonoid biosynthesis was the reason for the uniform accumulation of flavonoids at high light, supplemental UV-B radiation and low temperature.

##### 4.1.1 Results of microarray experiments: overview

In order to investigate this assumption, gene expression studies were performed on InCyte microarray chips which displayed about 25% of the *Arabidopsis* genome, i.e. about 6,500 genes. Each chip was a glass slide containing nucleotide sequences of about 8,000 *A. thaliana* ESTs (expressed sequence tags), which were dotted in a threefold copy in order to guarantee three technical replicates. Some genes were represented by two or more ESTs. Image 4.1 shows a fluorescence image of one hybridized microarray.

A sampling time-point of four hours after transfer to new conditions was chosen. Treated samples were always compared with control samples. Only genes which were

expressed by a factor of more than 1.5 or less than 0.75 in comparison to expression level in control sample were analysed further. These genes were quoted then as “up-“ or “down-regulated”.

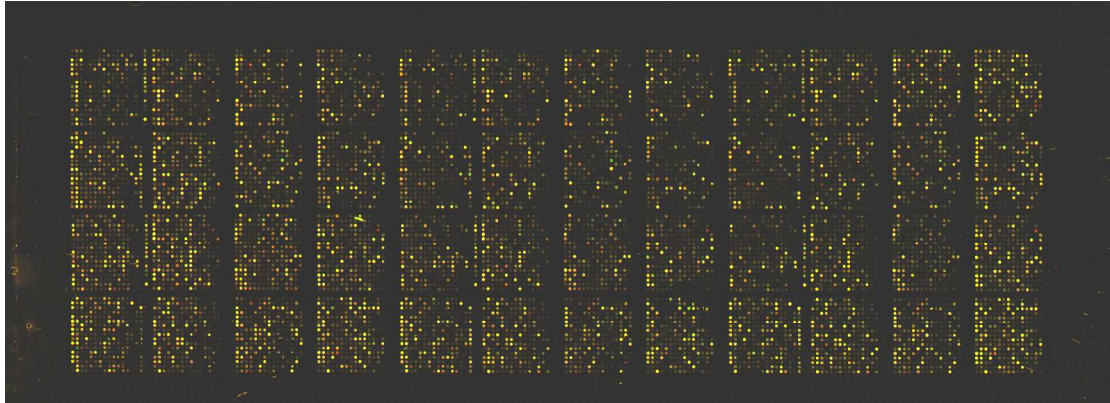


Image 4.1: Image of one InCyte microarray hybridized with Cy3-labelled control mRNA (green) and Cy5-labelled treated mRNA (red). Each dot represents one single EST with a specific annotation number for a gene of *A. thaliana*. Red dots indicate up-regulated ESTs or genes, green dots were down-regulated genes. A yellow dot indicates that expression level of this EST was equal in control and treated samples, i.e. this gene was not differentially expressed.

In comparison to control plants, which were grown at 22 °C and 120  $\mu\text{mol}/\text{m}^2\text{s}$  white light, only 24 genes were up-regulated and 8 genes were down-regulated by a factor of more than 1.5 or less than 0.75, respectively, in all separate replicates and in all treatments (figure 4.2). These 32 genes counted for only 0.5 % of all spotted genes. According to figure 4.2, it becomes clear that low temperature had the biggest and UV-B radiation the lowest influence on gene expression if one considers numbers of genes which were differentially expressed.

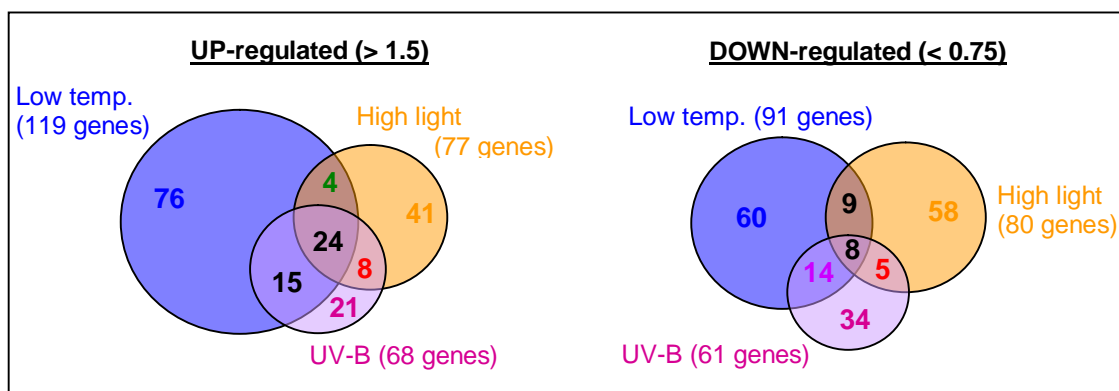


Figure 4.2: Results of microarray analysis. The scheme shows numbers of genes, which were up- or down-regulated by a factor of more than 1.5 or less than 0.75 in all three replicates of each treatment.

#### 4.1.2 Genes, which were up-regulated by a factor of more than 1.5 in all treatments

Table 4.1 shows that 18 of the up-regulated 24 genes could be grouped to either stress-related processes (eleven genes) or signal transduction-related and translational processes (six genes). According to the information found on the tair homepage (<http://www.arabidopsis.org>), many of the up-regulated stress-related genes could be induced by several stress treatments, displaying a universal stress response. It was surprising though, to find the gene coding for the heat shock protein hsp20 (At1g06460, no. 13) in this list, as this indicates that low temperature also led to an up-regulation of heat-induced genes. It was also unexpected, that the gene of *NPQ1* (At1g08550, no. 12) was also up-regulated after treatments with low temperature or supplemental UV-B radiation. NPQ1 or violaxanthin-deepoxidase is an enzyme of the xanthophyll cycle and deepoxidizes violaxanthin to zeaxanthin at high light stress in order to dissipate excess (light) energy as heat (Demmig et al. 1987, Hager & Holocher 1994)).

Surprisingly, no genes related to flavonoid biosynthesis were up-regulated by a factor of more than 1.5, although all treatments led to flavonoid accumulation in all plants. However, one gene, At3g18080 (no. 5), was a protein from the glycosyl hydrolase family. As all flavonoids in *A. thaliana* are flavonol glycosides, it could be possible that at least this enzyme might be involved in glycosylation processes of flavonoids.

Table 4.1: Table listing all 24 genes, which were up-regulated by a factor of more than 1.5 in all technical and biological replicates and in all treatments. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed and bold At-codes indicate that cDNA of these ESTs was validated by sequencing (c = chloroplast, cyt b<sub>6</sub>/f = cytochrome b<sub>6</sub>/f complex, cytos = cytoskeleton, m = mitochondria, mem = membrane, n = nucleus, rib = ribosome, \* = discussed in the text).

No	At-code	Gene name	Biological process (localisation)	General function
1	At2g28900	Mitochondrial import inner membrane translocase subunit (Tim17/Tim22/Tim23) family protein	Intracellular protein transport (m)	Transport (channel or pore class transporter activity)
2	At5g23575	Transmembrane protein	?	?
3	At4g27440	Protochlorophyllide reductase B (PCR-B) precursor; NADPH-protochlorophyllide oxidoreductase B (POR B)	Chlorophyll biosynthesis (c)	Chlorophyll biosynthesis
4	At1g69830	Alpha-amylase, putative	Carbohydrate metabolism (c)	Carbohydrate metabolism
5*	At3g18080	Glycosyl hydrolase family protein	Carbohydrate metabolism	Carbohydrate metabolism
6*	<b>At4g34950</b>	Nodulin-family protein	G-protein coupled receptor protein (cyt b <sub>6</sub> /f)	Signal transduction
7*	At5g26570	Calmodulin-binding protein (CaM-binding protein)	Calmodulin binding	Signal transduction

8*	At1g75800	Pathogenesis-related thaumatin family protein (PR5K), similar to receptor serine/threonine kinase	Response to pathogen	Signal transduction/ Stress
9	At3g62550	Universal stress protein (USP), early response protein (ER6)	Response to stress	Stress
10	At3g53990	Universal stress protein (USP), early response protein (ER6)	Response to stress	Stress
11	At4g19120	early responsive to dehydration stress (ERD3)	Response to stress	Stress
12*	<b>At1g08550</b>	Non-photochemical quenching (NPQ1), violaxanthin de-epoxidase (AVDE1)	Xanthophyll cycle (c)	Stress
13*	At1g06460	Heat shock protein (hsp20)	Response to heat (heat shock, m)	Stress
14	At5g15960	Stress induced protein (KIN1)	Cold acclimation, response to ABA	Stress
15* /16	At4g39260 (2 X ESTs)	Cold-circadian rhythm (CCR1), glycine-rich RNA-binding protein (GRP8)	Response to cold, circadian rhythm	Stress ?
17	At4g30650	Low temperature and salt responsive protein (LT16A), response to cold (RC12A)	Response to cold, salt stress (endo-mem)	Stress
18	At2g15970	Cold acclimation protein (W)COR413-PM1	Response to cold, ABA, desiccation (plasma mem)	Stress
19*	At5g52310	Cold regulated protein (COR78), low temperature-induced protein (LTI140), also: LTI78 or RD29A	Response to cold, ABA, desiccation, salt stress; promotor is ABA- and stress responsive	Stress/ Transcription
20	At1g13930	Weakly similar to drought-induced protein Sdi-6 common sunflower	Translational elongation (rib)	Transcription/ Stress ?
21	<b>At3g19910</b>	Zinc finger, C3HC4-type RING finger, ubiquitin ligase	Regulation of transcription, protein degradation	Transcription
22	At5g14170	SWIB complex BAF60b domain-containing protein	?	Transcription ?
23	At2g28720	Histone H2B	Chromosome organization (n)	?
24	At1g20010	Beta tubulin TUB5	Microtubule-based process (cytos)	Cytoskeleton

The gene At5g52310 named *COR78* (no. 19) is also known to be induced upon various stressors and its protein has an additional, potential role in transcriptional processes (Takahashi et al. 2001). The promotor is ABA- and stress-responsive and *COR78* is controlled by DRE/CRT *cis*-acting elements in the promotor region via the gene *AtPLC2*. Interestingly, *AtPLC2* (At3g08510), a phospholipase C, was up-regulated by a



factor of more than 1.5 in this experiment after treatment with low temperature or high light (table 4.3, no. 4). Another up-regulated gene, which was presented by two different ESTs on the chip was *CCR1* (At4g39260, no. 15/16). The transcript undergoes circadian oscillations, which are known to be delayed after low temperature treatment (Kreps & Simon 1997). Finally, three genes were identified whose proteins were directly related to signal transduction processes. One gene coded for a G-coupled receptor (At4g34950, no. 6), another coded for a CaM-binding protein (At5g26570, no. 7) and the last gene encoded a putative receptor serine/ threonine kinase (At1g75800, no. 8). However, no information was found, that could link any of these genes to induction of gene expression of flavonoid biosynthetic genes.

It was mentioned earlier that each EST/ gene on the chip was present in three (technical) replicates and that additionally each treatment was investigated by analysing three chips, containing RNA of three individual control and treated plants. Thus, until a gene was included in table 4.1 and in all other tables below, its expression levels had to be increased (or decreased) by a factor of more than 1.5 (or less than 0.75) in all technical and biological replicates. I.e. the expression level had to be higher than 1.5 (or less than 0.75) altogether nine times. In order to demonstrate the variation in expression level among biological replicates, which depends on expression levels in control and in the treated leaves, values for ten randomly chosen genes which were up-regulated after all treatments are presented in figure 4.3.

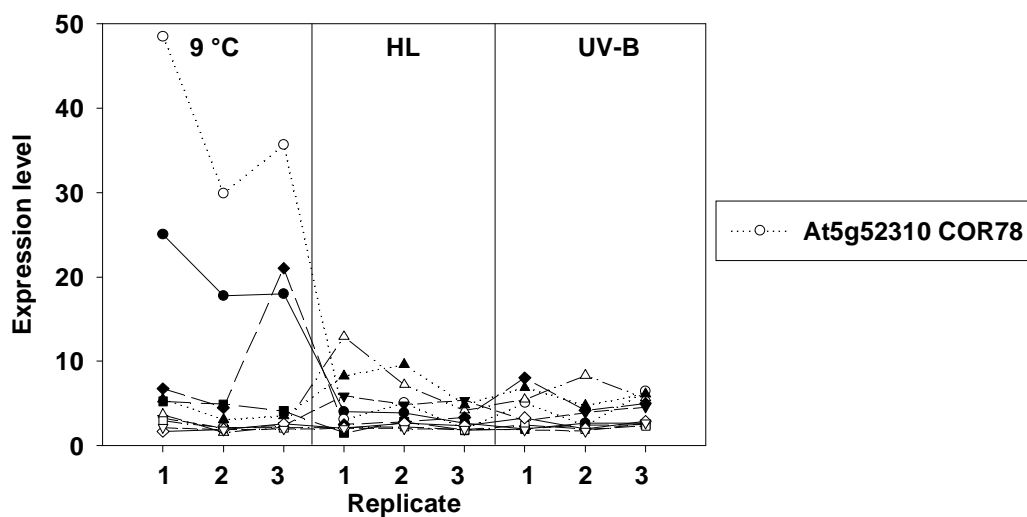


Figure 4.3: Exemplary expression levels for the first ten genes (when sorted by systematic no.) of genes which were up-regulated by a factor of more than 1.5 after a four hour treatment with low temperature, high light and UV-B in all three biological replicates, which were rosette leaves of three *A. thaliana* Col plants (n = 3 for each data point, i.e. three technical replicates).

#### 4.1.3 Genes, which were down-regulated by a factor of less than 0.75 in all treatments

Among the eight genes, which were down-regulated in all treatments in comparison to control conditions (table 4.2), four genes could be related to carbohydrate metabolism. Similar to gene At3g18080 (no. 5 in table 4.1) of up-regulated genes, one of these four down-regulated genes coded for a protein from the glycosyl hydrolase family 3 (At5g49360, no. 2), which hydrolyzes O-glycosyl compounds. As flavonoids generally occur as flavonol glycosides in *A. thaliana*, this protein might also be involved in a glycosylation processes. Another gene in this list is a myb-related transcription factor named *CCA1* (circadian clock associated 1, At2g46830, no.5). According to annotation details this transcription factor has repressive activity and is believed to be involved in the circadian clock of *Arabidopsis*. It also has overlapping functions with LHY (late elongated hypocotyl) in a regulatory feedback loop that is closely associated with the circadian oscillator in *Arabidopsis* (Oda et al. 2004). Northern blot analysis (will be shown in image 4.4) could prove a strong down-regulation in all treatments. Of three other down-regulated genes only very few information was available and it was not possible to assign any biological process nor general function to any of them.

Table 4.2: Table listing all 8 genes, which were down-regulated by a factor of less than 0.75 in all technical and biological replicates and in all treatments. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed (c = chloroplast, mem = membrane, n = nucleus, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1	At1g03870	Fasciclin-like arabinogalactan-protein 9 (FLA9)	Cell adhesion (endo-mem system)	Carbohydrate metabolism
2*	At5g49360	Glycosyl hydrolase family 3 protein, beta-xylosidase (BXL1)	Hydrolyzing O-glycosyl compounds (endo-mem system, extracellular matrix)	Carbohydrate metabolism
3	At2g20670	Expressed protein	Sugar porter activity	Carbohydrate transport
4	At1g64500	Glutaredoxin family protein	?	Carbohydrate metabolism
5*	At2g46830	Myb-related transcription factor CCA1, transcriptional repressor	Circadian rhythm (n)	Transcription
6	At4g36850	PQ-loop family protein, transmembrane family protein	? (extracellular)	?
7	At3g54500	Expressed protein	?	?
8	At1g55960	Expressed protein	? (c)	?

#### 4.1.4 Genes, which were up-regulated only after treatment with two of three conditions

Some genes were up-regulated in only two of three conditions. These genes are shown in the following three tables.

##### 4.1.4.1 Genes, which were up-regulated only after treatment with low temperature or high irradiances

Firstly, four genes were up-regulated after treatment with low temperature and high light but not after UV-B radiation (table 4.3). One of them was mentioned earlier, a phospholipase C (At3g08510, no. 4) involved in COR78-related signal transduction processes. Another stress-related gene was *RD19a* (At4g39090, no. 3), coding for a cysteine proteinase responsive to desiccation and salt stress.

Table 4.3: Table listing all 4 genes, which were up-regulated by a factor of more than 1.5 in all technical and biological replicates after treatment with low temperature or high light but not with UV-B. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). A bold At-code indicates that cDNA of this EST was validated by sequencing (c = chloroplast, m = mitochondria, mem = membrane, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1	At2g16660	Nodulin-like protein	Carbohydrate, protein sodium transport (endo-mem system)	Transport
2	At1g20020	Ferredoxin-NADP(+) reductase (FNR), putative, oxidoreductase activity	Electron/ sodium transport (c, inner mem)	(Electron)-Transport
3*	At4g39090	Cysteine proteinase RD19a, thiol protease	Induced by desiccation, salt stress, proteolysis and peptidolysis not by ABA (endo-mem system)	Stress/ protein degradation ?
4*	<b>At3g08510</b>	Phosphoinositide specific phospholipase C, PI-PLC2	Intracellular signal cascade (m)	Signal transduction

##### 4.1.4.2 Genes, which were up-regulated only after treatment with low temperature or supplemental UV-B radiation

Expression level of 15 genes was increased by a factor of more than 1.5 after treatment with low temperature and supplemental UV-B but not with high light (table 4.4).

Among them were five genes whose proteins could be related to transcription and splicing, three genes coded for proteins which were stress-related and another three were involved in protein degradation. One of the transcription-related genes was *AGL20* (At2g45660, no. 1) and its protein is a positive regulator of flowering according to annotation details from the *tair*-homepage. Additionally, one further glycosyl transferase (At4g27560, no. 10) and a chloroplastic ABC transporter (At1g59870, no. 9) were found to be up-regulated.

Table 4.4: List of 15 genes, which were up-regulated by a factor of more than 1.5 in all technical and biological replicates after treatment with low temperature or UV-B but not with high irradiances. Information to At-codes were mainly derived from the *tair*-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed and bold At-codes indicate that cDNA of these ESTs was validated by sequencing (c = chloroplast, cp = cytoplasm, m = mitochondria, mem = membrane, n = nucleus, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1*	At2g45660	AGL20, positive regulator of flowering, transcription factor activity, protein containing MADS-box	Flowering (n, m)	Transcription
2	At3g13180	NOL1/NOP2/sun family protein	Regulation of transcription, DNA dependent (c)	Transcription
3	At1g20693 (2 X ESTs)	High mobility group B protein HMGB2, having a distinctive DNA-binding motif, the HMG-box domain; can be phosphorylated by CK2alpha	Assembly or disassembly of chromatin, transcription factor activity	Transcription
4	<b>At2g47890</b>	Zinc finger family protein, B-box type, zinc ion binding	Regulation of transcription, transcription factor activity (n)	Transcription
5	At4g25500	Argine/ serine-rich splicing factor AT-SRP40, RSP40; transcript is alternatively spliced	Nuclear mRNA splicing via spliceosome	Splicing
6	At2g15450	Expressed protein, similar to auxin-down-regulated protein ARG10 of <i>Vigna radiata</i>	?	?/ Stress?
7	At3g05880	Rare-cold-inducible 2A (RCI2A), LTI16A	Low temperature-, dehydration- and salt-responsive (integral to mem)	Stress
8	At5g15970	Stress-responsive protein (KIN2), COR6.6	Cold- and ABA-responsive; cold	Stress

			acclimation and salt tolerance (cp)	
9*	At1g59870	ATP-binding cassette transporter (ABC transporter) family protein	ABC transporter activity (c, inner mem)	Transport
10*	<b>At4g27560</b>	Glycosyltransferase family protein with Pfam-domain, UDP-glucuronosyl and UDP-glycosyl transferase	Transfer of glycosyl- and glucuronosyl groups	Carbohydrate metabolism
11	At2g28840	Ankyrin repeat family protein, zinc ion binding	Protein ubiquitination	Protein degradation
12	At1g71020	Armadillo/ beta-catenin repeat family protein containing U-box and Pfam domain	Protein ubiquitination	Protein degradation
13	At3g47160	Expressed protein, similar to MTD2 of <i>Medicago truncatula</i>	Protein ubiquitination	Protein degradation
14	At2g15890	Expressed protein	? (c)	?
15	At3g04550	Expressed protein	? (c)	?

#### 4.1.4.3 Genes, which were up-regulated only after treatment with high irradiances or supplemental UV-B radiation

The third table 4.5 presents genes, whose expression level was up-regulated after treatment with high light or UV-B radiation, but not after low temperature. Half of the eight genes could be related to transcriptional or signal transduction processes. One of them was calmodulin (*CaM*, At1g66410, no.4), coding for a frequent calcium ion binding signal transducer. Residual three other genes encoded transcription factors with different domains: Proteins of At2g23340 (no. 5) and At1g68840 (no. 6) have one AP2 domain, the protein of At5g53980 (no. 7) has a homeobox domain. The gene coding for the enzymatic antioxidant “catalase 3” (At1g20620, no.3) was also up-regulated. The gene *DHS1* (At4g39980, no. 1) was also found to be up-regulated. Its protein is also called aldolase and is part of the shikimate pathway involved in the biosynthesis of shikimate which is the precursor for HCA-, anthocyanin- (flavonoid) and phenolic amino acid biosynthesis (Entus et al. 2002).

Table 4.5: Table listing all 8 genes, which were up-regulated by a factor of more than 1.5 in all technical and biological replicates and after treatment with high light or UV-B but not with low temperature. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed and a bold

At-code indicates that cDNA of this EST was validated by sequencing (c = chloroplast, n = nucleus, p = peroxisome, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1*	At4g39980	3-Deoxy-D-arabino-heptulosonate 7-phosphate synthase 1, DAHP synthetase 1 (DHS1)	Response to pathogenic bacteria and wounding, aromatic AA biosynthesis, shikimate pathway (c)	Shikimate and aromatic AA biosynthesis
2	At4g27440	(NADPH-) Protochlorophyllide (oxido-) reductase B (PCR B, POR B)	Chlorophyll biosynthesis (c)	Chlorophyll biosynthesis/ Stress?
3*	At1g20620	Catalase 3 (CAT3), senescence 2 (SEN2)	Hydrogen peroxide metabolism, senescence (p)	Antioxidant/ Stress
4*	At1g66410	Calmodulin-1/4 (CAM4, CaM)	Calcium ion binding, signal transducer activity	Signal transduction
5*	At2g23340	Protein of DREB subfamily A-5 of ERF/ AP2 transcription factor family with one AP2 domain	Regulation of transcription, DNA dependent (n)	Transcription
6*	At1g68840	DNA-binding protein with AP2 domain (RAV2, RAP2.8); transcription factor	Regulation of transcription, DNA dependent (c, n)	Transcription
7*	At5g53980	Homeobox-leucine zipper family protein with Pfam homeobox domain; transcription factor	Regulation of transcription, DNA dependent (n)	Transcription
8	At3g26580	Expressed protein	? (c)	?

#### 4.1.5 Genes, which were down-regulated only after treatment with two of three conditions

Corresponding to up-regulated genes, down-regulation of genes after treatment with two of three conditions also happened. The following three tables will present these genes.

##### 4.1.5.1 Genes, which were down-regulated only after treatment with low temperature or high irradiances

Table 4.6 contains nine genes which were down-regulated by a factor of less than 0.75 in comparison to control conditions after treatment with low temperature or high light.

Altogether five genes were involved in signal transduction (one gene) and transcriptional processes (four genes). At1g72930 (no. 5) encodes a receptor-like protein located in the membrane with function in the defense response signaling pathway. The residual four genes coded for transcription factors with various domains. The proteins of two further genes could be assigned to carbohydrate metabolism, and both were involved in either transferring glycosyl groups (At1g70290, no. 1) or hydrolyzing glycosyl compounds (At5g49360, no.2), suggesting again a potential role in flavonol glycosidation processes. The locus At5g49360 was already found to be down-regulated after treatment with all three conditions but as a different EST (compare table 4.2).

Table 4.6: Table listing 9 genes, which were down-regulated by a factor of less than 0.75 in all technical and biological replicates and after treatment with low temperature or high light but not with UV-B. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>; n = nucleus, m = mitochondria, mem = membrane, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1*	At1g70290	Enzyme with trehalose-6-phosphate synthase-like (TPS) and trehalose-6-phosphate phosphatase-like (TPP) domains; but no activity has been detected yet; putative TPS with profile of glycosyltransferase family 20	Trehalose biosynthesis (putative), transfer of glycosyl groups (endo mem system)	Carbohydrate metabolism
2*	At5g49360 (+ 1 EST in table 4.2)	Glycosyl hydrolase family 3 protein, beta-xylosidase (BXL1)	Hydrolyzing O-glycosyl compounds (endo-mem system, extracellular matrix)	Carbohydrate metabolism
3	At2g26080	Glycine dehydrogenase/ decarboxylase, putative	Glycine decarboxylation via glycine cleavage system (m, n)	Photorespiration
4	At5g49740	Protein similar to ferric-chelate reductase (FRO1) with oxidoreductase activity	Electron transport (mem)	Electron transport
5*	At1g72930	Toll-interleukin-resistance (TIR) domain containing protein, disease resistance protein, receptor-like protein	Defense response signaling pathway (mem)	Signal transduction
6	At5g28400	Myb family transcription factor with myb-like DNA binding domain	Regulation of transcription, DNA dependent (n)	Transcription
7	At2g25900	CCCH-type zinc finger family protein, AtCTH, transcription	Regulation of transcription	Transcription

		factor	(extracellular)	
8	At5g28770	bZIP transcription factor family protein	Regulation of transcription, DNA dependent (n)	Transcription
9	At5g15850	Zinc finger protein CONSTANS-Like 1(COL1), transcription factor	Circadian rhythm, transcription factor activity, zinc ion binding (intracellular)	Transcription

#### 4.1.5.2 Genes, which were down-regulated only after treatment with low temperature or supplemental UV-B radiation

Table 4.7 lists all 14 genes, which were down-regulated after treatment with low temperature or UV-B. Three genes encoded transcription factors with one of them, ABF3 (At3g3400, no.9) being responsive to ABA as it may bind to ABA-responsive elements of promoter regions. The proteins of five genes were related to metabolism or chlorophyll biosynthesis and two genes were found to encode aquaporins with water channel activity (At2g37170, no.1 and At3g16240 no.2). The bHLH transcription factor (At3g59060, *PAP3*, no. 8) was probably the most important factor to be found differentially expressed as it is clearly involved in phytochrome signaling.

Table 4.7: Table listing all 14 genes, which were down-regulated by a factor of less than 0.75 in all technical and biological replicates after treatment with low temperature or UV-B but not with high light. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed (c = chloroplast, er = E.R., endoplasmatic reticulum, m = mitochondria, mem = membrane, n = nucleus, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1*	At2g37170	Plasma membrane intrinsic protein 2B, member of subfamily PIP2, aquaporin, PIP2.2 or PIP2B, water channel activity	Water release from vascular bundles into surroundings (plasma mem)	Water uptake
2*	At3g16240	Delta tonoplast integral protein (delta-TIP), water channel, aquaporin	Water transport (vacuolar mem)	Water transport
3	At1g13080	Cytochrome P450 monooxygenase, member of cytochrome P450 family, CYP71B2	Electron transport, monooxygenase activity (m, er)	Metabolism
4	At1g60590	Similar to polygalacturonase, member of glycosyl hydrolase family 28	Carbohydrate metabolism (m)	Carbohydrate metabolism



5	At2g42080	DNAJ heat shock N-terminal domain –containing protein with chaperone activity	Protein folding (c)	Protein metabolism
6	At4g22570	Adenine phosphoribosyltransferase, putative	Nucleoside metabolism	Nucleoside metabolism
7	At1g58290	Glutamyl-tRNA reductase 1 (GluTR; HEMA1)	Response to light, heme/ chlorophyll biosynthesis (c)	Chlorophyll biosynthesis
8*	At3g59060	Basic helix-loop-helix family protein (bHLH) with HLH DNA-binding domain; phytochrome-associated protein 3	Transcription factor activity, DNA binding	Transcription
9*	At4g34000	ABA responsive elements-binding factor (ABF3), transcription factor	Response to stress and ABA, binds to ABA response elements (ABRE): regulation of transcription, DNA dependent (n)	Transcription
10	At5g62430	Similar to H-protein promotor binding factor-2a with dof-type zinc finger domain	Regulation of transcription	Transcription
11	At3g52470	Harpin-induced family protein (HIN1) with enzyme regulator activity	Harpin-responsive (mem, endo mem system)	Stress ?
12	At3g26460 (2 X ESTs)	Similar to major latex protein type 1 (MLP)	?	?
13	At5g64940	ABC1 family protein	? (c, extracellular region)	?
14	At5g64130	Expressed protein	?	?

#### 4.1.5.3 Genes, which were down-regulated only after treatment with high irradiances or supplemental UV-B radiation

Finally, five genes which were down-regulated after treatment with UV-B and high light but not after low temperature treatment are listed in table 4.8. One down-regulated gene (At1g72430, no. 4) was auxin-responsive and the proteins of two others were directly involved in the TCA cycle.

Table 4.8: Table listing all 5 genes, which were down-regulated by a factor of less than 0.75 in all technical and biological replicates after treatment with high light or UV-B but not with low temperature. Information to At-codes were mainly derived from the tair-homepage (<http://www.arabidopsis.org>). Where no information was available, a “?” was placed (c = chloroplast, m = mitochondria, mem = membrane, n = nucleus, \* = discussed in the text).

No.	At-code	Gene name	Biological process (localisation)	General function
1	At1g65930	Isocitrate dehydrogenase, putative	Carbohydrate metabolism, TCA cycle, NAD <sup>+</sup> / NADP <sup>+</sup> -activity, (m, n)	Carbohydrate metabolism, TCA cycle
2	At3g55410	2-Oxoglutarate dehydrogenase E1 subunit	Carbohydrate metabolism, TCA cycle, NAD <sup>+</sup> / NADP <sup>+</sup> -activity (m)	Carbohydrate metabolism, TCA cycle
3	At2g16500	Arginine decarboxylase (ADC1)	Arginine catabolism (c)	AA catabolism
4*	At1g72430	Related to auxin-responsive protein	Auxin-responsive (c)	Stress
5	At2g46540	Expressed protein	? (endo mem system)	?

#### 4.1.6 Results of microarray experiments for genes which are related to flavonoid and HCA biosynthesis

These microarray analyses were performed mainly to discover up- or down-regulated genes with a direct or indirect link to flavonoid or HCA biosynthetic pathways and their corresponding signal transduction pathways. With the exception of one glycosyltransferase (At4g27560) and several glycosyl hydrolases, no other flavonoid biosynthetic genes were detected (table 4.9). These last two enzymes might possibly be involved in flavonoid glycosylation processes as they might be potential candidates for transferring glycosyl groups to and from aglycones. Not even key enzymes of flavonoid biosynthesis, though present on the chips, were up- (or down-) regulated by a factor of more than 1.5 (or less than 0.75) in all technical and biological treatments and in at least two of three treatments. However, one potential component of a flavonoid inductive signal transduction pathway was found to be down-regulated after treatment with low temperature or UV-B: a bHLH transcription factor which is known to be associated to phytochrome (At3g59060, no. 8 in table 4.7).

Table 4.9: Glycosyltransferases and -hydrolases found to be up-regulated by a factor of more than 1.5 (shaded grey) or down-regulated by a factor of less than 0.75 in all technical and biological replicates in two of three or all treatments according to indication. At4g27560, the only transferase found in all lists, was validated by sequencing the cDNA of this EST.

Regulation	At-code	Gene name	Differentially expressed after ...		
			... low temperature treatment	... high light treatment	... treatment with UV-B
Up	At3g18080	Glycosyl hydrolase family	X	X	X
Up	<b>At4g27560</b>	Glycosyltransferase family protein with Pfam-domain, UDP-glucuronosyl and UDP-glycosyl transferase	X	-	X
Down	At5g49360	Glycosyl hydrolase family 3 protein, beta-xylosidase (BXL1)	X	X	X
Down	At5g49360	Glycosyl hydrolase family 3 protein, beta-xylosidase (BXL1)	X	X	-
Down	At1g60590	Similar to polygalacturonase, member of glycosyl hydrolase family 28	X	-	X

Thus, it was directly searched among the results for genes with relation to flavonoid or HCA biosynthesis with the software GeneSpring. Colour codes in figure 4.4 shows expression levels of these genes at different treatments. A strong up-regulation is shown in red, a strong down-regulation in blue and no change in expression level in comparison to control conditions would be presented in yellow.

As can be seen from the pathway in figure 4.4 very few of the presented genes were up-regulated. UV-B had a strong positive effect on *PAL1*, *PAL2* and *CHS*. Among the genes coding for enzymes related to HCA biosynthesis, one cinnamoyl-CoA reductase (gene E, At4g30470) and one O-methyl transferase (gene M, At1g21130, putative *OMT1*) were slightly up-regulated. Low temperature had a weak effect on *PAL* and a more pronounced positive effect on three *OMTs*. Two of these were putative *OMTs 1* (genes M and O, At1g21130 and At1g21110) and one was a *OMT* family 2 gene with similarity to caffeic acid *OMT* (gene N, At1g76790). Finally, four hours of high light had virtually no effect on enzymes of the flavonoid biosynthetic pathway. However, gene M, a putative *OMT1* (At1g21130) was also lightly up-regulated after this treatment.

In summary, no enzymes of the biosynthetic pathway leading to flavonoids and HCAs were markedly up-regulated in the cold or high light treatment, although these conditions led to flavonoid accumulation within a few days. Only UV-B treated plants displayed a strong up-regulation of the key enzymes of flavonoid biosynthesis, phenylalanine ammonia-lyase (*PAL1* and *PAL2*) and chalcone synthase (*CHS*).

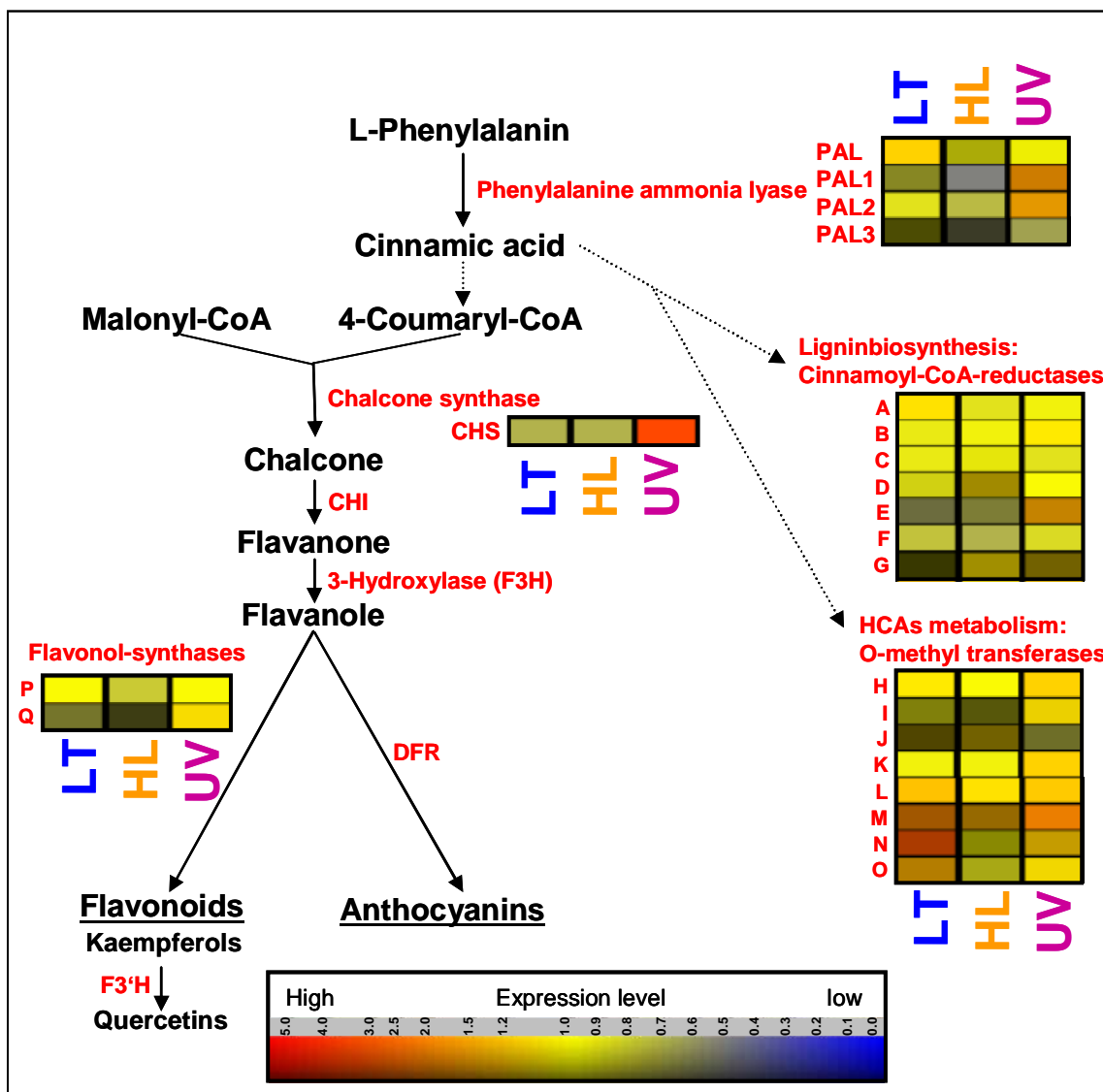


Figure 4.4: Gene expression levels of genes related to flavonoid and HCA biosynthesis which were present on the microarray chip after treatment with low temperature (LT), high white light (HL) and UV-B radiation (UV) for four hours. The expression level is indicated by a colour scheme. Red coloured words indicate genes coding for biosynthetic enzymes. Some main enzymes of flavonoid biosynthesis like chalcone flavanone isomerase (CHI), 3-hydroxylase (F3H), dihydro flavonol reductase (DFR) and flavonol-3'-hydroxylase (F3'H) were not present on the chip.

Table 4.10: Table containing At-codes to biosynthetic genes presented in the figure 4.4 (fam. = enzyme family, FS = flavonol synthase, OMT = O-methyl transferase). Bold At-code indicates that cDNA of this EST was validated by sequencing.

	At-code (name)		At-code (name)
<b>PAL</b>	<b>At3g10340</b>	G	At1g80820
<b>PAL1</b>	<b>At2g37040</b>	H	At1g77530 (fam. 2, caffeic acid OMT)
PAL2	At3g53260	I	At4g35160 (fam. 2, caffeic acid OMT)
PAL3	At5g04230	J	At3g62000 (fam. 3)
<b>CHS</b>	<b>At5g13930</b>	K	At5g54160 (OMT 1)

A	At1g15950	L	At1g21100 (OMT 1, putative)
B	At1g76470	<b>M</b>	<b>At1g21130 (OMT 1, putative)</b>
C	At2g33590	N	At1g76790 (fam. 2, caffeic acid OMT)
D	At5g58490	O	At1g21110 (OMT 1, putative)
E	At4g30470	P	At5g63600 (FS), putative
F	At1g15950	Q	At5g63600 (FS, different EST)

#### 4.1.7 Validation of microarray results by sequencing and northern blotting analysis

So far, only results of gene expression analysis by microarrays were presented, which were to the most part not validated by sequencing and/ or northern blot analysis. However, several genes were examined further and those were marked in the tables by bold letters. With the exception of *PAL* (At3g10340), all marked genes could be validated positively by sequencing (data not shown). It was also not possible to establish a specific probe for northern blotting analysis of *PAL*, so that gene expression of this gene could not be validated (image 4.2). Firstly, it was also tried to use specific primers on material from the array and secondly, to use unspecific and specific primers on cDNA from low temperature treated leaves. However, no success was achieved by any of these procedures (data not shown).

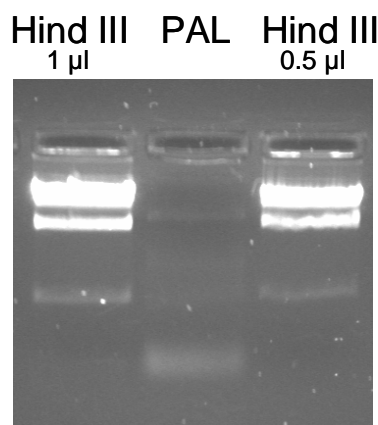


Image 4.2: Image of the gel electrophoresis of the DIG-labelled probe for *PAL* (At3g10340) raised from the according clone from the microarray with unspecific primers SP6 and t7 in a PCR reaction. Hind III indicates loading of a small amount of DNA of HindIII-digested- $\lambda$ -phage and was used for DNA quantification. Several weak bands were detected within the *PAL*-probe. Thus, it was not possible to raise a specific *PAL* probe for northern blot analysis.

The following table lists all genes whose expression levels were validated by northern blotting analysis. In addition to sampling timepoints of zero and four hours treatment, later timepoints, namely eight, 24 and 48 hours were also investigated. Expression levels were determined before the treatments, and 4, 8, 24 and 48 hours after treatments.

Table 4.11: List of genes, which were present on the microarray chips and whose expression level was further analysed by northern blotting analysis.

At-code	Gene name
At2g37040	PAL1
At3g53260	PAL2
At5g13930	CHS
At2g46830	CCA1
At3g19910	RING zinc finger

Microarray experiments could present gene expression levels of several flavonoid biosynthetic enzymes. Gene expression of enzymes which were known to be important for flavonol biosynthesis and whose genes were present on the microarray chip, was markedly increased only after UV-B treatment. This result was in contrast to the observed induction of pigment synthesis at low temperature and high light conditions. Therefore, gene expression of two different isoforms of *PAL* and of *CHS* was investigated at several timepoints up to 24 hours after transfer to inducing conditions with DIG-labelled northernblots (image 4.3).

The first important biosynthetic enzyme for the synthesis of flavonoids is phenylalanine ammonia-lyase (*PAL*). Increased RNA levels of *PAL1* (At2g37040) could be proved after treatment with low temperature or supplemental UV-B radiation. Low temperature led to increased levels after eight hours, whereas UV-B led to strongly increased transcript levels already after four hours. In comparison, microarrays, on which RNA was analysed after four hour treatments, showed increased expression level only for UV-B (figure 4.4). Thus, the microarray result for low temperature was not in direct contradiction to results obtained from northern blot analysis. However, as only samples after two, eight and 24 hours of low temperature were investigated, and the sample after four hour treatment was missing, no final and clear answer can be given to this point. A clear induction of gene expression by UV-B was detected for *PAL2* (At3g53260). Low temperature led to an only weak induction of gene expression of *PAL2*. In contrast, no increased RNA levels of *PAL2* were detected by northern blots in samples which were exposed to high irradiances for different hours. This result is in accordance with microarray results. The expression level of the gene coding for the second important key enzyme of flavonoid biosynthesis, the chalcone synthase (*CHS*) was also investigated with northern blot analysis. A significant increase in RNA copies could be proved already after two hours of UV-B radiation. In low temperature and high light treated samples a distinct increased signal was detected not before a 24 hour treatment. After an exposure of four hours increased expression level were not observed yet. These results were also in good agreement with results from microarray experiments.

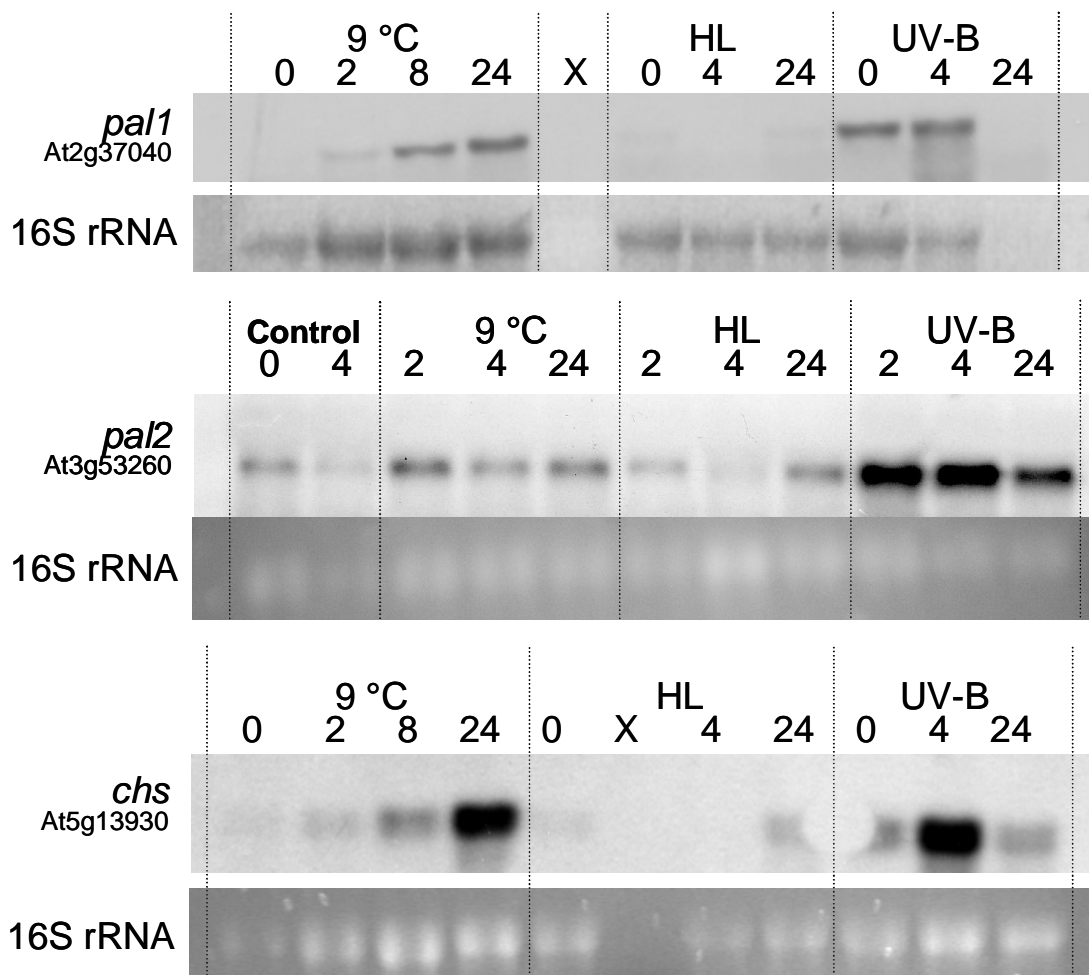


Image 4.3: Image of 16S rRNA bands of total RNA-gels and DIG-labelled northernblots for the genes coding for flavonoid biosynthetic enzymes PAL1, PAL2 and CHS. Plants were treated with low temperature (9°C), high light (HL) or supplemental UV-B radiation for different hours, namely 2 and/ or 4 and/ or 8 and 24 hours prior to sampling. Start samples ("0 hours") were also analysed. A 4-hour control sample was taken for *PAL2* only. An "X" indicates an unloaded slot in the gel.

Finally, it can be concluded that results obtained from microarray analysis for genes *PAL1*, *PAL2* and *CHS* were representative and that UV-B very quickly and effectively activates gene expression. In contrast, low temperature had an only weak influence on gene expression of these genes, which usually was detectable only after eight hours treatment and finally high light did not show clearly increased gene expression of *PAL* isoforms and *CHS*.

Two more non-biosynthetic genes were analysed with northern blotting as listed in table 4.11. The first was *CCA1* (At2g46830) which was down-regulated in all treatments (table 4.2, no. 5). Northern blot analysis could also show that this gene was only expressed in leaves of control plants (image 4.4) at sampling times of 0 and 4 hours and were in good agreement with microarray results for *CCA1*.

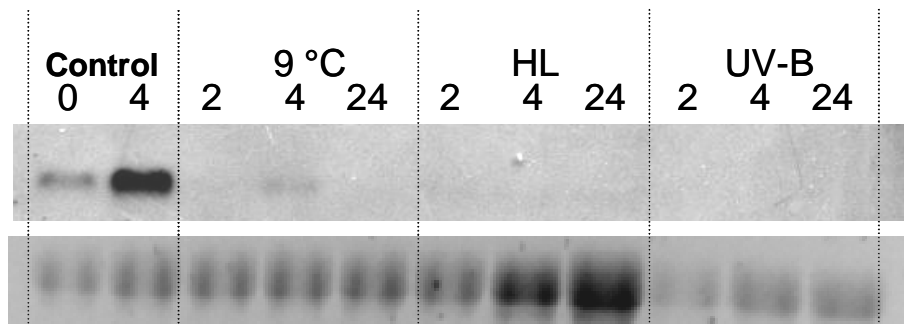


Image 4.4: Images of 16S rRNA bands of total RNA-gels and DIG-labelled northernblots for the gene *CCA1* (At2g46830). Plants were treated with low temperature (9°C), high light (HL) or supplemental UV-B radiation for different periods of time, namely 2, 4 and 24 hours prior to sampling. Start samples (“0 hours”) were also analysed.

The second gene which was analysed further was a RING zinc finger (At3g19910, table 4.1, no. 21). This gene was found to be up-regulated in the microarray experiments after all treatments. Although a specific probe was prepared (image 4.5), no signals were detected in repeated northern blots (not shown). Thus, microarray results for this gene could not be confirmed by northern blot analysis.

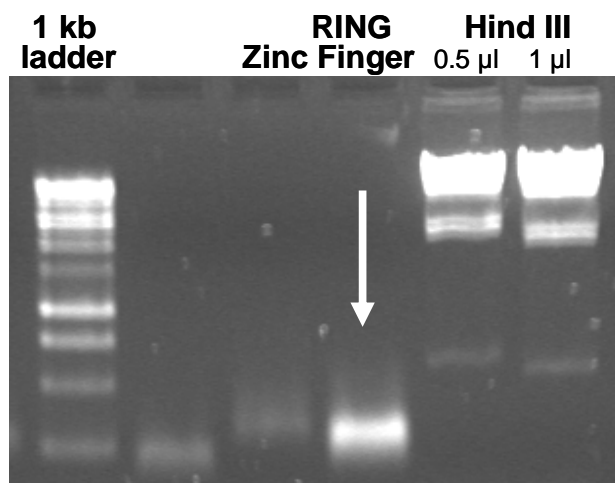


Image 4.5: Image of the gel electrophoresis of the DIG-labelled probe for RING zinc finger (At3g19910) raised from the according clone from the microarray with specific primers in a PCR reaction. Hind III indicates loading of a small amount of DNA of HindIII-digested- $\lambda$ -phage and was used for DNA quantification. One strong band was detected within the probe which was about 300 to 400 bp in length as estimated with the 1 kb ladder as marker. However, it was not possible to get any positive signals with this probe in northern blot analysis.



#### 4.2 Discussion (Fourth part)

*Microarray analysis could not show clearly whether shared or distinct signal transduction pathways leading to flavonoid accumulation were used by low temperature, high irradiance or supplemental UV-B radiation*

The aim of the microarray experiments was to discover signal transduction pathways which were involved in the accumulation of epidermal flavonoids under treatments with low temperature, high light or supplemental UV-B radiation. The timepoint of four hours was chosen in order to discover relevant components of signal transduction pathways leading to the induction of flavonoid biosynthesis. Flavonoid accumulation at the conditions low temperature, high light and supplemental UV-B radiation was shown by epidermal UV-A transmittance measurements as well as by HPLC analysis. Obviously, this exposure time was only sufficient to induce gene expression of genes coding for key biosynthetic enzymes of flavonoid biosynthesis for the treatment with supplemental UV-B radiation. Discussion of the present microarray results has to consider two restrictions. Firstly, it must be kept in mind that the microarray used in this work displayed only one third of the *Arabidopsis*' genome so that some relevant genes were most likely not present on the chip. Secondly, not all of the discussed genes were validated by sequencing and/ or northern blotting analysis due to time limitations.

The pathway of flavonoid biosynthesis is known, and enzymes involved in flavonol biosynthesis are phenylalanin ammonia-lyase (PAL\*, four different isoforms, one of them being putative), chalcone synthase (CHS\*), chalcone flavanone isomerase (CHI\*), flavanone-3-hydroxylase (F3H) and flavonol synthase (FS\*, six different isoforms, one of them being putative). The enzyme responsible for the formation of quercetin from kaempferol is called flavonol-3'-hydroxylase (F3'H). Anthocyanin biosynthesis is mediated by the enzyme dihydroflavonolreductase (DFR\*).

Flavonol glycosides were synthesized by glycosylation of the aglycone with glucose and/ or rhamnose by the action of different UDP-flavonol glycosyl transferases (\*, eight isoforms, three of them being putative). Genes coding for enzymes involved in flavonoid biosynthesis and which were indicated by \* were present on the microarray chip. Unfortunately, only genes coding for one putative isoform of FS (At5g63600), for only two glucosyl transferases (At2g36790 and At5g17050, of which the latter was putative), and no gene coding for F3'H were present on the microarray chip. According to microarray results where samples of four hour treatments were analysed, high light did not lead to any increased expression level of any gene coding for a flavonoid biosynthetic enzyme present on the chip in comparison to control samples (figure 4.4). In contrast, low temperature led to a weak increase in expression levels of two isoforms of PAL (PAL, At3g10340 and PAL2, At3g53260). Only the treatment with supplemental UV-B radiation led to a strong induction of the expression of several biosynthetic genes like PAL, CHS and FS. Gene expression levels of the two glucosyl transferases involved in flavonoid glycosylation were not increased, however a different glucosyl transferase

(At4g27560) was increased by a factor of more than 1.5 after the low temperature and high light treatment (table 4.9).

There are several studies investigating the gene expression level of *chs* in *A. thaliana* after several stimuli. A time course accumulation of mRNAs of *pal* and *chs* at low temperature conditions of 4 °C and a continuous PPFD of 100  $\mu\text{mol}/\text{m}^2\text{s}$  was reported for *A. thaliana* Col by Leyva et al. (1995). mRNA levels increased after 10 hours and peaked after 2 to 4 days of induction. An only weak increase in mRNA levels of *CHS* and no increase in expression level of *PAL* was detected after three hours. This could possibly explain why no increased expression levels were detected in the microarray. Leyva and colleagues (1995) also discovered that light was essential for an induction of gene expression of *PAL* and *CHS* at low temperature, as no transcription was detected in darkness and that low temperature had an additive effect on the light-induced accumulation of mRNAs of *PAL* and *CHS*, which is in agreement with my UV-A transmittance measurements and HPLC results. *CHS* gene expression in leaves of *A. thaliana* Ler is also induced at low temperature of 7 °C, which is close to 9 °C, and at low irradiance of only 20  $\mu\text{mol}/\text{m}^2\text{s}$  after 8 hours (Wade et al. 2003). However, the flavonoid content detected at 9 °C and 20  $\mu\text{mol}/\text{m}^2\text{s}$  was not increased in comparison to control levels (figure 2.25).

Furthermore, *CHS* gene expression is induced by UV-A/ blue light and also by UV-B radiation in mature leaves of *A. thaliana* Ler, with UV-A/ blue light and UV-B leading to a synergistic effect on *CHS* gene expression (Fuglevand et al. 1996, Wade et al. 2001, Wade et al. 2003, Kalbina & Strid 2006). As white light also contains the blue light fraction, the UV-B treatment applied in this work was in fact a treatment with blue and UV-B light, which is a synergistic light combination that leads to a very rapid and strong induction of *CHS* gene expression after only few hours. This rapid and strong induction of *CHS* gene expression and of several other flavonoid biosynthetic genes was also observed in microarray experiments. For comparison and regarding a UV-B induced accumulation of flavonoids, transcript levels of *CHS* and *PAL* were strongly up-regulated after six hours of UV-B treatment (8  $\text{kJ}/\text{m}^2/\text{d}$ , Li et al. 1993). Similarly, a *CHS* mRNA accumulation in leaves of culture cells of *A. thaliana* during treatment with UV-B radiation was detected by Christie & Jenkins (1996) after 4 to 8 hours.

A light-dependent accumulation of *CHS* transcript levels was detected for *A. thaliana* Ler grown at 21 °C (Jackson et al. 1995). No *CHS* mRNA was detected at a PPFD of 20  $\mu\text{mol}/\text{m}^2\text{s}$ , but mRNA level was increased after illumination with 60  $\mu\text{mol}/\text{m}^2\text{s}$  for 48 hours and was further increased after illumination with 150  $\mu\text{mol}/\text{m}^2\text{s}$  for 48 hours, indicating a light/ fluence-dependent regulation of the *CHS* promoter. In addition, a similar observation was made by Jackson and co-workers (1995) for the anthocyanin biosynthetic enzyme DFR together with a 20-fold increase in anthocyanin content (measured as absorption at 530 nm) after transfer from low light (25  $\mu\text{mol}/\text{m}^2\text{s}$ ) to high light of 175  $\mu\text{mol}/\text{m}^2\text{s}$  for 24 hours, suggesting a light-induced accumulation of anthocyanins in *A. thaliana* Ler at warm temperatures.

Similarly, northern blot analysis on *PAL1*, *PAL2* and *CHS* confirmed a very weak induction after the high light treatment, a moderate induction after low temperature and a strong induction after the UV-B treatment (image 4.3). These results indicate again different activation mechanisms for flavonoid accumulation after irradiation with high irradiances or treatment with low air temperature in comparison to radiation with supplemental UV-B radiation. However, the hypothesis of a synergistic effect of low temperature and high irradiances on induction of flavonoid biosynthesis can still be supported. But still, different patterns of flavonoid accumulation as observed by HPLC analysis could not be explained at all with results of gene expression analysis. Measurements of enzyme rates of flavonoid biosynthetic enzymes could have possibly contributed more to explain the observed differences in flavonoid accumulation.

It is concluded, that at four hours after transfer to high light or low temperature, apparently only early stages of the induction process of flavonoid biosynthesis were observed. Alternatively, activity of flavonoid biosynthetic enzymes was post-transcriptionally regulated under these conditions. However, according to current literature it must be assumed that flavonoid biosynthesis is mainly regulated on the transcriptional level (Li et al. 1993, Levya et al. 1995). In contrast to low temperature and high irradiances, UV-B led to a more prompt response, in spite of equal final levels of flavonoids in all treatments. Hence, high light and low temperature showed similar induction characteristics, whereas UV-B appears to have used a different signal transduction pathway.

Northern blot analysis for the genes *PAL1*, *PAL2* and *CHS* were extended over a longer inductive period and allowed an analysis of later stages. These results support the findings of the microarray study and also demonstrate that strong differences between the three conditions must exist. Obviously UV-B activates very quickly and effectively gene expression of essential genes of flavonoid biosynthesis. Low temperature had an only weak influence on gene expression of these genes, which was observed frequently not before eight hours of treatment. No clearly positive effect on gene expression of *PAL* and *CHS* was found in the high light treatment. Hence, all three conditions acted differentially on gene expression. HPLC results could also detect a strong difference between UV-B treatment and low temperature or high light. If both aspects were considered together, it points to a fundamentally different mechanism of flavonoid accumulation at UV-B in comparison to low temperature or high light, which might share a signal transduction pathway. Results presented in the third part of this work on the role of the blue light sensor cryptochrome 1 in the accumulation of flavonoids at low temperature and high white light support this last assumption. A different signal transduction pathway for UV-B with a UV-B specific sensor would then likewise be very probable.

The expression level of two further genes with a possible role in phenylpropanoid metabolism were found to be increased in the microarrays. Firstly, *DHS1* (At4g39980),

DAHP synthetase or aldolase, no. 1 in table 4.5) codes for the first enzyme in the shikimate pathway involved in the biosynthesis of shikimate which is the precursor for HCA-, anthocyanin- (flavonoid) and phenolic amino acid biosynthesis (Richter 1998, Entus et al. 2002). Interestingly, it was shown by Entus et al. (2002) that DHS1 appears to be activated by its reduction via the ferredoxin/ thioredoxin redox control system in the chloroplast. Genexpression of *DHS1* was up-regulated by a factor of more than 1.3 by low temperature, more than 2.1 by high light and more than 2.7 by UV-B (the smallest normalized expression value of three individual biological replicates was given in each case; residual data not shown). An induction of *DHS1* and *PAL* in *Arabidopsis* after physical wounding was observed by Keith and co-workers (1991). The second gene coded for an ABC-transporter (At1g59870, ATP binding cassette transporter, no. 9 in table 4.4). ABC-transporter may be involved in the vacuolar flavonoid deposition (Heldt 1999). However, tair annotation details to this gene described a location in the chloroplast, which clearly contradicts a vacuole localized function.

Finally, a gene which encodes a phytochrome associated transcription factor called PAP3 or PIF5 (At3g59060, *PAP3/ PIF5/ PIF3-like6 (PIL6)*, no.8 in table 4.7) was detected to be down-regulated by a factor of less than 0.4 by low temperature, less than 0.7 by high light and less than 0.5 by UV-B (the highest normalized expression value of three individual biological replicates was given in each case; residual data not shown). According to annotation details *PAP3* codes for a MYC-related bHLH transcription factor which is physically associated with the component of the circadian clock, TOC1 (timing of CAB expression), and is thus a member of PIF3. As mentioned before, the PIF3 protein is a positive regulator of *CHS* gene expression via phytochrome A or B (Kim et al. 2003). However, PIF3 proteins are also involved in the expression of circadian clock genes *CCA1* (circadian clock associated 1) and *LHY* (late and elongated hypocotyl) whose proteins in turn regulate repression of TOC1 (Oda et al. 2004). As flavonoid biosynthesis was discovered to be under circadian clock control by Harmer et al. (2000) and *CHS* gene expression was shown to be induced via phytochrome and PIF3 (Kim et al. 2003), one could imagine that a phytochrome mediated induction of *CHS* gene expression via PIF 5 and the circadian clock might also exist. However, this last assumption must remain speculative because a direct link between *CHS* gene expression and PAP3/ PIF5 is missing in the literature.

*The circadian clock of Arabidopsis might be affected by treatments with low temperature, high light or UV-B*

Many light-induced genes are known to be under control of the circadian clock (see update of Eriksson & Millar 2003). It was also detected that the whole phenylpropanoid biosynthetic pathway is under clock control (Harmer et al. 2000). The promotor of the gene coding for the key enzyme of flavonoid bioynthesis CHS is known to be under circadian clock control with low transcript activity six hours after the light was switched on and high transcript activity at 20 - 22 hours after start of illumination indicating an

advantageous pigment accumulation in advance of the photoperiod (Thain et al. 2002). In accordance to a high light-induced induction of *CHS* gene expression and accumulation of flavonoids, the overall level of transcript activity was raised in seedlings of *Arabidopsis* upon increased irradiance from 150 to 250  $\mu\text{mol}/\text{m}^2\text{s}$  (Thain et al. 2002). In addition, clock affecting mutations like *toc1* shorten the period of *CHS* oscillations which indicates a circadian regulation of *CHS* expression by *TOC1*. According to the present microarray analysis three circadian clock related genes were found to be differentially expressed in all treatments in comparison to control samples. Two were down-regulated, i. e. *CCA1* (At2g46830, circadian clock associated 1, no. 5 in table 4.2) and *COL1* (At5g15850, CONSTANS-like 1, no. 9 in table 4.6) and the third was up-regulated. *CCR1* (At4g39260, cold circadian rhythm 1, no. 15/16 in table 4.1) which was up-regulated. *CCR1* codes for a glycine-rich RNA-binding protein and is also named *GRP8*.

*CCA1* codes for a MYB-related transcription factor in the nucleus which acts as a repressor. Thus, a down-regulation of *CCA1* is truly an activation which was also indicated by northern blotting (image 4.4). The current model of the circadian clock in *Arabidopsis* describes a negative feedback loop between three components: the two redundant genes *CCA1* and *LHY* (late and elongated hypocotyl) and *TOC1* (timing of CAB expression 1) (Eriksson & Millar 2003, Wang & Tobin 1998, Stracke et al. 2001, Oda et al. 2004, Salomé & McClung 2005). Light-induction of *CCA1* and *LHY* occurs by phytochrome via the bHLH transcription factor PIF3 (phytochrome interacting factor 3) (Oda et al. 2004). However, the blue light sensor cryptochrome 1 is also assumed to be involved in the light activation of the central oscillators (see review of Fankhauser & Staiger 2002). *CCA1* and *LHY* are expressed normally early in the day and their proteins decay at the end of the day.

*CCR1/GRP8* was up-regulated in all treatments at least by a factor of more than 1,8 (data not shown). According to annotation details from the Tair homepage (<http://www.arabidopsis.org>) this gene encodes an RNA binding protein structurally similar to other stress-induced proteins. Its gene expression is known to be induced by low temperature and also underlays circadian oscillations which are depressed by *AtGRP7* (Heintzen et al. 1997). Kreps & Simon (1997) reported overall increased mRNA levels of *CCR1* in seven days old seedlings of *A. thaliana* Col together with a delayed or disrupted circadian cycling in the first 36 hours of low temperature treatment (4 °C) in comparison to 20 °C under long-day conditions (16 hours light and eight hours dark). This last observation could at least explain increased gene expression level after the low temperature treatment as observed in the microarray. The phase delay was even more pronounced when the low temperature period was increased under constant illumination (e.g. under free-running conditions): 12 hours at 4 °C resulted in a four-hours delay, 20 hours at 4 °C led to a 12-hours delay. In consequence, a prolonged low temperature treatment could be assumed to lead to a stoppage of circadian cycling as proposed for tomato by Martino-Catt & Ort (1992). However, this was not observed by Kreps & Simon (1997). Finally, *CCR1* appears to be one of several other circadian-

controlled genes which was found to be increased by low temperature, high light and UV-B.

*COL1* codes for a zinc finger transcription factor similar to *CONSTANS* which is the flowering-time gene in *Arabidopsis*. *COL1* was down-regulated by a factor of less than 0.6 by low temperature, less than 0.5 by high light and less than 0.7 by UV-B (the highest normalized expression value of three individual biological replicates was given in each case; residual data not shown). *COL1* is known to be circadian clock regulated with peak of transcript levels around dawn (Ledger et al. 2001). A fluence rate dependent shortening of circadian period by either blue or red light was also observed.

In summary it can be concluded that the possibility exists that all three treatments might have an influence on the circadian clock because *CCA1*, the gene coding for the transcriptional repressor was down-regulated significantly. However, it is not clear and in the field of speculation, whether the down-regulation of *CCA1* might be involved in gene expression of *CHS* and flavonoid accumulation. Thus, further scientific work would be necessary to understand the effects of low temperature, high light and UV-B on the circadian clock and to elucidate any potential relationship between *CCA1* and *CHS*.

*Low temperature, high irradiance and supplemental UV-B radiation led to an universal stress response in leaves of A. thaliana Col without activation of the antioxidative machinery*

Altogether 24 of about 6500 genes were discovered to be up-regulated by a factor of more than 1.5 in all separate biological replicates and in all treatments (image 4.2). 50 % of them could be related to stress (table 4.1). Among them were stress-responsive genes like *hsp20*, which encodes a heat shock protein, *COR78*, a cold-regulated gene (Horvath et al. 1993), and *NPQ1*, whose protein is the violaxanthin-deepoxidase which is an enzyme of the xanthophyll cycle and deepoxidates violaxanthin to zeaxanthin at high light stress in order to dissipate excess (light) energy as heat (Demmig et al. 1987, Hager & Holocher 1994). It was not expected that a gene coding for a heat shock protein or for NPQ1 would be induced by low temperature or a cold-regulated gene would be induced by UV-B or high light. Thus, these 12 genes seem to belong to a universal stress response of *Arabidopsis* to treatments with low temperature (LT), high irradiance (HL) and UV-B (UV). Three additional up-regulated stress responsive genes were detected in lists which displayed up-regulated genes found in only two of three conditions: Two cold-induced genes (*LTI16A/RCI2A* and *KIN2/COR6.6* in table 4.4, LT+UV) and one desiccation-responsive gene (*RD19a* in table 4.3, LT+HL). In contrast, 50 % of down-regulated genes in all treatments could be classified to carbohydrate metabolism.

Surprisingly, in contrast to the large number of stress-induced genes, the expression level of only three genes coding for antioxidant enzymes was raised: *catalase 3* (*CAT3* in table 4.5, HL+UV), *NPQ1* and *KINI*, the last owing a potential function as an anti-freeze protein according to annotation details from the tair homepage. *CAT3* detoxifies reactive hydrogen peroxide in the peroxisomes and is associated with senescence. It is also known that its enzyme activity might be inactivated under conditions of increased irradiance or low temperature (Feierabend et al. 1992), which in turn could explain an up-regulation of *CAT3* gene expression. In agreement with the last assumption, microarray results presented in this work point to an induction of *CAT3* in response to oxidative stress because LT led to a weak induction (factor 0.9 to 2.1), HL to a strong induction (factor 4.5 to 13.1) and UV to a moderate induction (factor 1.7 to 4.0) of gene expression of *CAT3* (combined results from two separate ESTs, data not shown).

No stress-responsive genes were discovered to be down-regulated. Genes coding for several antioxidant enzymes of the ascorbate-glutathione cycle (see review of Foyer & Noctor 2000) like glutathion reductase (GR), monodehydroascorbate reductases (MDHARs) and dehydroascorbate reductases (DHARs) and others like superoxide dismutase (SOD) or glutathion peroxidases (GPXs) and were present on the microarray chip but were not detected to be differentially expressed after treatment with at least two of three conditions. However, one putative isoform of GPX was up-regulated by a factor of more than 1,6 after treatment with supplemental UV-B radiation (At4g11600). This gene was also up-regulated weakly after treatment with low temperature (factor was 1.4 to 1.9) or high light (factor was 1.4 to 1.7). A further gene coding for a putative DHAR (At1g19570) was found to be up-regulated by a factor of more than 2.7 by high light but was not uniformly up-regulated by UV-B (factor was 1.1 to 1.7) or low temperature conditions (factor was 0.9 to 1.2). Also, genes involved in vitamin E/ tocopherol biosynthesis such as *VTE1* (At4g32770, tocopherol cyclase) and *VTE3* (At3g63410, methyltransferase), both present on the chips, were not regulated under the conditions investigated.

In summary, experimental conditions applied in this work were severe enough to induce a general stress situation on the one hand, but were not sufficient to induce an effective stress response with up-regulated antioxidative systems to overcome a potential stress-derived cellular damage.

## V. Concluding discussion and future perspectives

The mechanisms underlying the induction of flavonoid biosynthesis at low temperature could be elucidated partly. In addition to the necessity of illumination described by Leyva et al. (1995) it could be shown, that the two factors, low temperature and light, intensified each other in their effect at all investigated irradiances. Thus, first of all the reduction state of the photosynthetic electron transport chain (psETC) appeared to be an attractive candidate for the temperature sensor to look for (Huner et al. 1998). Essentially two findings contradicted to this assumption. Firstly, experiments in which abaxial leaf sides were illuminated, demonstrated, that illumination of epidermal cells was decisive for flavonoid biosynthesis. This result alone did not completely exclude a photosynthesis-related mechanism but made such a mechanism unlikely as a complicate cooperation of an epidermal light sensor with the photosynthetically active mesophyll would have been demanded. Secondly, experiments with ferredoxin-deficient potato plants did not show any effect of the increased reduction state in the mutants on the epidermal screening by flavonoids. Thus, the reduction state of the psETC could be excluded as a low temperature sensor. The role of ROS in the accumulation of flavonoids could also be denied as ROS accumulation at low temperature was independent of illumination and thus in contrast to a strictly light-dependent flavonoid accumulation.

The observed perception of light in the epidermis itself brought light sensors to the discussion. In contrast to phytochromes, blue light sensors appeared as more suitable candidates as they show a reaction within the irradiance range of question (Sakai et al. 2001). The investigation of phototropin- and cryptochrome-deficient double mutants of *A. thaliana* together with HPLC analysis and the identification of the induced substances gave unequivocal results. The biosynthesis of quercetin glycosides, and possibly the enzyme flavonole-3'-hydroxylase, was obviously regulated by low temperature and light via cryptochrome 1. On the other hand biosynthesis of kaempferol glycosides, which are precursors of quercetin biosynthesis, seemed to be induced independently of a blue light sensor by low temperature: Even the cryptochrome-deficient double mutant displayed increased amounts of kaempferol glycosides similar to "normal" low temperature levels after treatment with yellow light and low temperature in comparison to control conditions. Cryptochrome was responsible for the additional white light effect on flavonoid accumulation at 9 and 22 °C but the low temperature effect was still present in the double mutant and was reduced for quercetin glycosides. These findings let the well-known induction of flavonoid biosynthesis by UV-A/ blue light (Fuglevand et al. 1996, Jenkins et al. 2001) appear in a completely different light. Firstly, it was not known before that a cryptochrome 1-mediated signal transduction pathway could be influenced by low temperature. As signal transduction chains are very short between cryptochrome and gene expression (Liscum 2003), now very targeted experiments could be possible. Secondly, the considerable, UV-A- and blue light-independent induction of kaempferol glycosides by high light at low temperature was also not known before. In continuation of this work it would be



necessary to investigate more light-dependent signals and discover a temperature-sensitive cryptochrome 1 interacting partner. It can also not completely be excluded that either phytochrome A or B or the circadian clock, which is also light- and temperature-sensitive would be involved in low temperature-induced kaempferol (and quercetin) accumulation.

Alongside with the analysis of the induction by low temperature and light, results also allow comparisons to the UV-B induced flavonoid accumulation. Although conditions were chosen in a way that the resulting epidermal UV-A screening was about the same after all treatments, UV-B treated plants displayed a considerably faster development of epidermal screening than the two other conditions. This was also observed in the gene expression studies, where only UV-B affected a fast induction of expression of key enzymes of flavonoid biosynthesis, which was delayed at low temperature and stayed not detectable at high light conditions. These results are in good agreement with reports where it was shown that the induction of flavonoid biosynthesis by UV-B and blue light is regulated by different signal transduction pathways (Christie & Jenkins 1996).

Several secondary questions could not be answered in this work and could be the basis for future research. The biosynthesis of flavonoids is subject to intensive research since many years and is probably the best investigated metabolism in plants. However, still large gaps in knowledge of the regulation of flavonoid biosynthesis persist and were also present in this work. First of all, many scientists and most scientific works do not distinguish between anthocyanin- and flavonoid accumulation and often mix both together by analysing mRNA levels of PAL. Secondly, *A. thaliana* seedlings and mature plants are very frequently not separated clearly from each other. This is especially important when investigating an anthocyanin induction by sugars like sucrose (Teng et al. 2005). Thus, it is worth dissecting flavonoid- from anthocyanin accumulation in seedlings and mature plants when investigating control mechanisms for flavonoid accumulation. To my opinion, completely different regulation mechanisms would not be unexpected.

## VI. Summary

The central subject of this work was to find answers to the question of how flavonoid accumulation might be induced at low temperature in *Arabidopsis thaliana* (*A. thaliana*). Specifically, the question was addressed how low temperature is sensed. Several hypotheses were investigated. A reduction of the plastoquinone pool in the photosynthetic electron transport chain by low temperature, which would also involve differential gene expression (Huner et al. 1998, Wilson & Huner 2000) was considered as a potential sensor for the induction of flavonoid biosynthesis as well as a low temperature derived signal of oxidative stress through the accumulation of reactive oxygen species (ROS) (Foyer & Noctor 2003). Two further hypotheses included a low temperature-linked calcium signal (Monroy et al. 1993, Örvar et al. 2000) and the involvement of one or more light receptors in the low temperature-induced flavonoid accumulation.

In order to solve this question, comparative analysis of flavonoid accumulation at high light and supplemental UV-B radiation were also performed. The kinetics of flavonoid accumulation as well as their composition in rosette leaves of *A.thaliana* were comprehensively analysed. A role of ROS, the reduction state of the plastoquinone pool and the photoreceptors phototropin and cryptochrome in the induction of flavonoid accumulation at low temperature was investigated by several different experiments. In addition, the induction of flavonoid biosynthesis at low temperature, high light or UV-B radiation on the transcriptional level was analysed by microarray analysis and northern blotting. A comparative analysis of gene expression of one third of the *Arabidopsis* genome at the three conditions via microarray analysis was also performed in order to discover relevant signal transduction components.

In experiments with *A. thaliana* an accumulation of six flavonols, three kaempferol- and three quercetin glycosides, could be demonstrated after treatment with low temperature, high light or UV-B radiation. Distinct differences in the pattern of accumulation after UV-B treatment were detected which involved a strong accumulation of quercetin glycosides and a rapid induction of several flavonoid biosynthetic genes. Experiments with mutants defective in photoreceptors could prove a light- and low temperature-dependent accumulation of quercetin glycosides which was regulated mainly by the blue light sensor cryptochrom 1. In contrast, most of the kaempferol accumulation at low temperature was blue light independent. This result implies that low temperature might change signal transduction processes following cryptochrome 1 perception. ROS and the reduction state of the photosynthetic electron transport chain (psETC) could be excluded as signal carriers.

Results show that at least two low temperature sensing systems in *A. thaliana* might exist: One, that seems to interact with cryptochrome and seems to control the enzyme flavonol-3'-hydroxylase, and an independent second, which apparently controls kaempferol biosynthesis.

## VII. Zusammenfassung (deutsch)

Die Beantwortung der Frage, wie unter Kühle die Flavonoidbiosynthese in *Arabidopsis thaliana* induziert wird, war zentrale Aufgabe dieser Arbeit. Insbesondere wurde die Frage adressiert, wie Kühle wahrgenommen wird. Verschiedene Hypothesen wurden bearbeitet. Eine Reduktion des Plastochinon-Pools in der photosynthetischen Elektronentransportkette durch geringe Temperaturen und eine damit verbundene differentielle Genexpression (Huner et al. 1998, Wilson und Huner 2000) wurde als möglicher Sensor für eine Induktion der Flavonoidbiosynthese ebenso betrachtet, wie ein durch Kühle hervorgerufenen Signal von oxidativem Stress durch die Akkumulation von reaktiven Sauerstoffspezies (ROS) (Foyer und Noctor 2003). Zwei weitere Hypothesen umfassten ein Kühle-gekoppeltes Calciumsignal (Monroy et al. 1993, Örvar et al. 2000) und die Beteiligung eines oder mehrerer Lichtrezeptoren an der kühleinduzierten Flavonoid-akkumulation.

Zur Klärung dieser Frage wurde vergleichend auch die Flavonoidakkumulation unter hohen Lichtstärken und durch UV-B untersucht. Sowohl die Kinetik der Flavonoidakkumulation als auch ihre Zusammensetzung in Rosettenblättern von *A. thaliana* wurde dabei umfassend analysiert. Die Rolle von ROS, vom Reduktionsgrad des Plastochinon-Pools und von den Lichtrezeptoren Phototropin und Cryptochrom in der Induktion der Flavonoidakkumulation wurde mit verschiedenen Experimenten untersucht. Zusätzlich wurde die Induktion der Flavonoidbiosynthese durch Kühle, hohe Lichtstärken und UV-B Strahlung auf Genexpressionsebene mittels Microarrays und Northernblots analysiert. Eine vergleichende Analyse der Genexpression von einem Drittel des *Arabidopsis*-Genoms unter den drei Bedingungen mittels Microarrays wurde durchgeführt um relevante Komponenten der entsprechenden Signaltransduktionswege zu entdecken.

In Versuchen mit *A. thaliana* konnte eine Akkumulation von sechs Flavonolen (je drei Kämpferol- und Quercetinglykosiden) bei Kühle, unter hohen Lichtbedingungen und nach UV-B –Bestrahlung nachgewiesen werden. Dabei waren deutliche Unterschiede in der Akkumulation nach UV-B Behandlung zu den beiden anderen Behandlungen zu beobachten, die in einer starken Quercetinakkumulation und einer schnellen Induktion verschiedener Biosynthesegene bestanden. In Experimenten mit Photorezeptor-Mutanten konnte nachgewiesen werden, dass eine licht- und kühlebedingte Akkumulation von Quercetinglykosiden vor allem durch den Blaulichtsensor Cryptochrom 1 vermittelt wird. Dagegen war ein großer Teil der Akkumulation von Kämpferolglykosiden Blaulicht-unabhängig. Das bedeutet, dass offenbar Kühle eine Cryptochrom-vermittelte Signaltransduktion verändern kann. ROS und der Reduktionsgrad der photosynthetischen Elektronentransportkette konnten als Signalgeber ausgeschlossen werden.

Die Ergebnisse zeigen, dass mindestens zwei Kühle-rezeptierende Systeme in *A. thaliana* existieren: Eines, das mit Cryptochrom interagiert und die Flavonol-3'-Hydroxylase kontrolliert sowie ein davon unabhängiges System, das die Kämpferolbiosynthese kontrolliert.

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**IX. Erklärung**

**„Hiermit erkläre ich, dass die vorliegende Arbeit – abgesehen von den Beratungen durch meine akademischen Lehrer – nach Inhalt und Form meine eigene Arbeit ist. Die Arbeit wurde bis jetzt weder vollständig noch in Teilen einer anderen Stelle im Rahmen eines Prüfungsverfahrens vorgelegt. Ferner erkläre ich, dass ich noch keine früheren Promotionsversuche unternommen habe.“**

Für die mündliche Prüfung wird die Form der Disputation gewählt.

Der Zulassung von Zuhörerinnen/Zuhörern bei der mündlichen Prüfung wird nicht widersprochen.

**Kiel, den 30. Mai 2007**

.....  
( **Karena Hoffmann-Wülfing** )

## X. Lebenslauf

**Name** Karena Hoffmann-Wülfing, geb. Sprick

**geboren** am 21. Oktober 1974 in Göttingen

**Staatsangehörigkeit** deutsch

**Familienstand** verheiratet, 1 Tochter

**Heimatort** Wisch b. Kiel

### Schul- und Berufsausbildung

bis Juni 1994 Gymnasium Bad Neuenahr-Ahrweiler, Abitur

Okt. 1994 bis Juli 1996 Ausbildung zur Staatlich geprüften Biologisch-Technischen-Assistentin (BTA) in Landau

### Wissenschaftliche Ausbildung

Okt. 1996 bis Juni 2002 Studium der Biologie an der CAU Kiel  
*Schwerpunkte:* Biologische Meereskunde, Zoologie und Physikalische Ozeanographie  
*Diplomarbeit:* Veränderungen photophysiologicaler Parameter antarktischer Diatomeen unter verschiedenen Umweltbedingungen in Laborexperimenten.  
*Abschluss:* Diplom

Sept. 1999 bis Juli 2000 Studium der Marinen Biologie und Küstenökologie an der University of Plymouth als Stipendiat des DAAD  
*Forschungsarbeit:* It is likely that 17 $\beta$ -estradiol, 20-hydroxyecdysone and cadmiumchloride have an effect on the vitellogenin level in haemolymph of the green crab, *Carcinus maenas* (L.)?  
*Abschluss:* Bachelor of Science (BSc) in Marine Biology and Coastal Ecology

seit Okt. 2002 Promotion bei der AG Ökophysiologie der Pflanzen am Botanischen Institut der CAU Kiel

## Beiträge auf Tagungen und Kongressen

### Poster

- September 2003                      ”Imaging of ROS during Cold Stress in Leaves of *A. thaliana*”, Karena Sprick, Christine Desel, Karin Krupinska and Wolfgang Bilger. Conference on ”Plant Stress, Reactive Oxygen and Antioxidants” in Freising-Weihenstephan (Germany). The poster was honoured with a travel grant from the Society for Free Radical Research (SFRR).
- September 2004                      „Contrasting Age Dependence of the Inducibility of Flavonoid Biosynthesis in Leaves of *Arabidopsis thaliana* and *Hedera canariensis*”, Karena Sprick, Matthias Schultze, Wolfgang Bilger. Botanikertagung in Braunschweig (Germany).
- März 2005                              “Comparative Gene Expression Studies in Leaves of *Arabidopsis thaliana* Exposed to Different Abiotic Stressors”, Karena Hoffmann-Wülfing, Sabine Milde, Anne Hoffmann, Arto Soitamo, Mirva Piippo, Eva Mari Aro, Wolfgang Bilger. Expert meeting on Vitamin E organized from the GRK 820 and held in Salza (Germany).

### Vortrag auf Kongress

- August 2004                            „Accumulation of Flavonoids under Moderate Irradiance and Cold Conditions“, Karena Sprick. III. PhD Congress of the Scandinavian Plant Physiology Society (SPPS) in Sem Gjestegard, Asker (Norway)

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