Effects of UV-B radiation and temperature drop on morphology and content of phenolic compounds in pea (pisum sativum).

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Master Thesis

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Abstract

For production of high-quality plants in greenhouses control of morphology is essential. Plant growth retardants are efficient, but due to their negative impacts on human health and the environment their utilization should be limited. In northern areas temperature drop treatments are commonly used to reduce shoot elongation, but is not sufficient in warmer periods and areas. Combined effects of different environmental factors thus appear highly interesting. In several plant species UV-B radiation is well known to modify the morphology as well as to stimulate production of protecting phenolic compounds (flavonoids). However, the interaction between UV-B-radiation and temperature drop has been less studied.

In *Arabidopsis thaliana* HY5, which is required for photomorphogenic development, acts in UV-B signalling. The E3 ubiquitin ligase COP1 regulates the HY5 turnover, resulting in high HY5 contents in light and degradation in the dark. In pea (*Pisum sativum*) the HY5 and COP ortologs LONG1 and LIP1 have been shown to play similar roles. Recent (unpublished) studies in our laboratory of *A. thaliana* and pea have suggested that HY5/LONG1 is an important signalling component also in thermoperiodic control of shoot elongation.

The aim of the present study was to investigate the effects of the interaction between UV-B and temperature drop on the morphology and content of phenolic compounds in pea (*Pisum sativum*). We also aimed at shedding light on the roles of LONG1 and LIP1 as well as gibberellin (GA) in responses to UV-B-temperature drop interaction. We used a wild type of pea and three mutants; the *lip1* and *long1* mutants as well as the gibberellin biosynthesis mutant *le*. The dwarf-mutants *lip1* and *le* were less sensitive to damage by UV-B radiation compared to WT, while the tall mutant *long1* was far more sensitive. This might be due to phenolic compounds; both the *lip1* and *le* mutant had higher levels of some flavonols, while the *long1* mutant had lower content of phenolic compounds compared to WT.
This thesis shows that a combination of UV-B radiation and temperature drop can potentially give effects as reduced shoot elongation, although it is important to find the optimum combination for each genotype. In general, less UV-B-related damage was observed when UV-B was provided together with the temperature drop. Although yet not verified in this specific experiment, this might be explained by that low temperature might decrease the formation of thymidine dimers and 6-4-photoproducts in DNA, which are induced by UV-B radiation and that DNA repair might been enhanced during the warmer period.

In conclusion, the present results demonstrate that a combination of UV-B and temperature drop is efficient in inhibiting shoot elongation in pea. Also, a role of LONG1 and LIP1 in UV-B-temperature-signalling associated with control of shoot elongation and flavonoid biosynthesis in pea is suggested.

Acknowledgement

First I would like to thank my supervisors, Prof. Jorunn Elisabeth Olsen and Associate Prof. Line Nybakken, for all the knowledge they have taught me, for their attention and dedication to this research and for all the guidance that they have given me during the writing of this thesis. I thank Marit Siira for her help to take care of my nearly thousand pea plants and Annie Aasen for all practical help when I was analyzing the phenolic compounds by HPLC. Sincere thanks also to Prof. Knut Asbjørn Solhaug for valuable advices and help with setting up the UV-B experiments.

Thanks to a generous scholarship of YARA and the reimbursements of the COST-Action on UV-B radiation, thereby giving me the opportunity to travel to different conferences. I did get a lot up-to-date and practical information about the use of light in the greenhouse industry on the Light Symposium in Wageningen in the Netherlands, I followed the Training School on Phytochemical Analysis in Thessaloniki in Greece and attended the 2nd Annual Network Meeting of COST-Action on UV-B radiation in Mikulov in the Czech Republic. For me all this has contributed to a greater understanding on both UV-B radiation and on phenolic compounds. Also my network has expended quite extensive and it was very exciting to see our research presented by Jorunn E. Olsen at the Annual Network meeting of the COST-Action on UV-B radiation in Mikulov.

I will dedicate this thesis to my children, Mathias and Jesper.

Abbreviations

COP1	**Constituitive Photomorphogenesis 1**
GA	**gibberellic acid**
GA2ox2	**GA 2-oxidase 2**
GA3ox	**GA 3-oxidase**
DIF	**difference between day temperature and night temperature**
DT	**day temperature**
FR	**far-red light**
HPLC	**High Performance Liquid Chromatography**
HY5	**Long HYpocotyl 5**
LIP1	**Light Insensitive Period1, COP1 orthologous protein in pea**
LONG1	**New Elongated pea mutant, HY5 orthologous protein in pea**
NT	**night temperature**
PAR	**photosynthetically active radiation**
R:FR ratio	**ratio of red light to far-red light**
RH	**relative humidity**
UV	**Ultraviolet radiation**
WT	**wild type**

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Introduction

UV-B radiation as a growth regulator

Ultraviolet (UV) radiation is radiation of wavelengths (200- 400 nm) shorter than those of visible light (400-700 nm). It is subdivided into UV-A radiation (315-400 nm), UV-B radiation (280-315 nm) and UV-C radiation (200-280 nm). UV radiation is emitted by the sun.

UV-C radiation is blocked by dioxygen or ozone in the atmosphere and will therefore rarely reach the ground on earth. UV-B radiation is to a large extent blocked by ozone but the levels are very variable and several factors are affecting the amount of UV-B radiation reaching the ground. Some of these factors are the latitude, season, time of the day, the cloud cover and the amount of pollutants. UV-A is hardly affected by ozone and about 95% reaches the ground.

UV radiation has the highest energy per photon of any part of the solar spectrum and therefore has it the potential to damage DNA, proteins and membrane lipids, as well as to inhibit protein synthesis and the photosynthetic apparatus in plants (Jenkins, 2009). Over a long time period the focus of researchers has been on the negative consequences of UV radiation, especially UV-B radiation, due to the concerns about the depletion of the ozone layer.

The latest decade the research focus has changed into a more positive aspect of UV-B radiation, namely the importance of UV-B radiation in coordinating plant growth and development. UV-B radiation, given in small fluence rates, can induce changes in the morphology of the plant and can result in accumulation of compounds which provide protection against potential UV-B damage, like anthocyanins, flavonoids and anti-oxidants like ascorbate (vitamin C).

UV-B radiation can change the quantity and the quality of a crop production by changing the morphology of plants. In the greenhouse industry small, compact plants are preferred and by using UV-B radiation, either by using UV-transparent cladding material or by providing UV-B radiation by UV-B radiating fluorescence tubes in suitable fluence rates, this might be achieved. Through accumulation of UV-B protective compounds, exposure to UV-B can also change the sensitivity for pest or pathogens. This might also be a great advantage in the greenhouse industry due to the possible reduction of pests or pathogens. Many of these compounds are also of nutritional importance and can thus affect the food quality, color, smell, firmness and taste (COST-Action FA0906 UV4Growth, 2009).

Morphological effects of UV-B radiation

Acclimation to UV-B radiation is a complex process in plants and can prevent and repair damage to DNA and the photosynthetic apparatus (especially photosystem II). Under normal outdoor conditions most wild plants are in a sufficient way able to protect themselves from damages caused by UV-B radiation, and little or no damage occurs (Hectors et al. 2012). This is important to keep in mind when discussing the effects of UV-B radiation.

Responses to UV-B radiation can be divided in two main responses. The first is the visible response of changes in the plants morphology. Plants exposed to UV-B radiation commonly show reduced height, decreased leaf area, reduced number of stomata, curling of the leaves edges, short petioles and increased axillary branching (Figure 1) (Janssen et al. 1998). To which extent those visible changes happens is dependent on the intensity of UV-B radiation, the amount of photosynthetically active radiation (PAR), which enhances the repair system of UV-related damage and other environmental parameters like temperature and the genotype (Hectors et al. 2012).

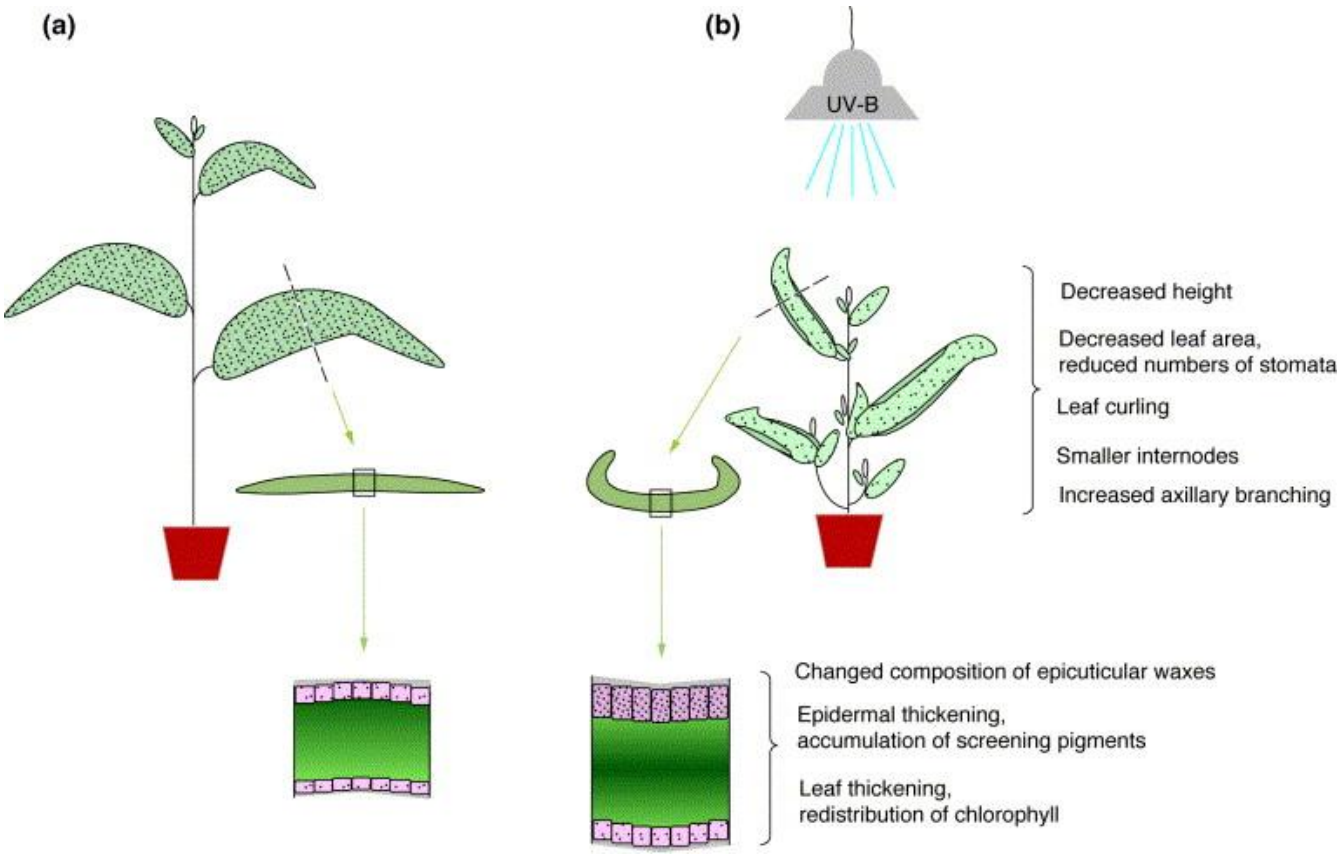

Figure 1. Visible effects of UV-B radiation on plants. Diagram showing UV-B-induced changes in leaf and plant morphology. (a) Control plants, (b) a plant exposed to UV-B radiation. Adapted from Jansen et al. (1998).

Figure 2 shows the morphological traits known to be affected by UV-B radiation as demonstrated by a meta-analysis of 62 individual studies to elevated UV-B radiation compared to control treatments (Caldwell, 2003). Those experiments were done outdoors using special UV lamp systems. In this meta-analysis ten morphological and physiological traits were examined, but overall significant effects of the elevated UV-B could only be found for shoot biomass (dry weight), plant height (shoot elongation), leaf area and increased contents of UV-B absorbing pigments like flavonoids and other phenolic compounds. The other traits studied; changes in the levels of chlorophyll and carotenoid pigments, yield, leaf mass per unit leaf area, net photosynthesis and the activity of photosystem II (PSII) did not show any significant changes in this meta-analysis (Caldwell, 2003).

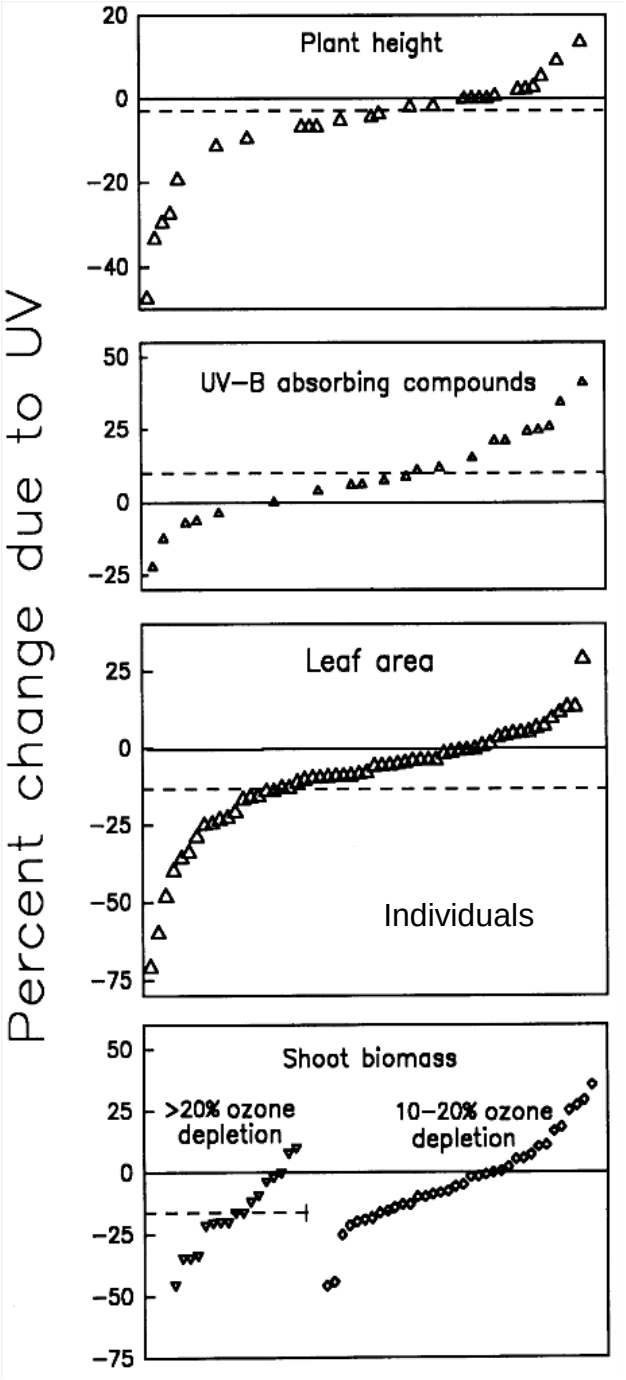

Figure 2. The response of four plant characteristics in field experiments employing supplemental UV-B from lamp systems. Each symbol represents a different study. The dashed lines represent the average response of the 62 studies included in the meta-analysis. The average responses shown were significant at $p \leq 0.05$. For shoot mass, the studies are grouped into two arrays corresponding to studies in which the level of simulated stratospheric ozone reduction was between 10 and 20%, and those in which the simulated ozone reduction was greater than 20%. After Caldwell (2003).

Phenolic compounds and their accumulation in UV-B

Another main response to UV-B radiation is the accumulation of phenolic compounds, also called phenols. Many phenols have both antioxidant and UV-B screening properties (Jansen et al. 2008). Phenols are a class of chemical compounds consisting of a hydroxyl group bound directly to at least one aromatic ring (C_6). Most phenols are of plant origin.

Phenols have many important roles in plants. A main function is to help the plant through periods of biotic and abiotic stress. Phenols can increase the resistance to pests, they can reduce or inhibit grazing by animals, and mechanical or environmental damage can be repaired by phenol-based polymers, like lignin, suberin or condensed tannins (Vogt, 2010). Another role of phenols is to protect plants against stress caused by light and UV-radiation as well as other stressors, like lowered temperature.

Three different biogenetic pathways lead to plant phenols. The majority of plant phenols are formed by the shikimate/arogenate pathway which leads to the phenylpropane (C6-C3) derivates. Some plant quinones are formed by another pathway, the acetate/malonate pathway. The third pathway is the acetate/melalonate pathway which leads to monoterpenes (Dey et al, 1997).

The shikimate pathway (Figure 3) is found only in microorganisms and plants, and not in animals. In microorganisms the shikimate pathway is regulated by feedback inhibition and by the repression of the first enzyme involved. No such feedback inhibition has been found in plants and this suggests that in plants the regulation of this pathway occurs at the genetic level (Herrmann et al, 1999).

In seven metabolic steps the shikimate pathway leads from phosphoenolpyruvate and erythrose 4-phosphate to the amino acids L-phenylalanine, L-tyrosine and L-tryptophane (Figure 4) These are the precursors of various secondary compounds including phenylpropanoids. In this pathway 11 different enzymes are required. In the following description of the pathway the enzymes are omitted to simplify.

The first reaction is the condensation of erythrose 4-phosphate with phosphoenolpyruvate (PEP). The product is an open-chain C_7 sugar denoted 2-dehydro-3-deoxyarabinopheptulosonate-7-phosphate (DAHP). The second step is the conversion of DAHP into 3-hydroquinate. This is a complex sequence of reactions resulting in a cyclic structure. In the third step 3-dehydroquinate is *cis*-dehydrated to 3-dehydroquinase/shikimate dehydrogenase. Thereafter this is reduced to shikimate. In the next step a reaction with PEP gives 5-enolpyruvylshikimate 3-phosphate (EPSP). The next step in the pathway is the elimination of phosphate from EPSP, which results in chorismate (Dey et al, 1997).

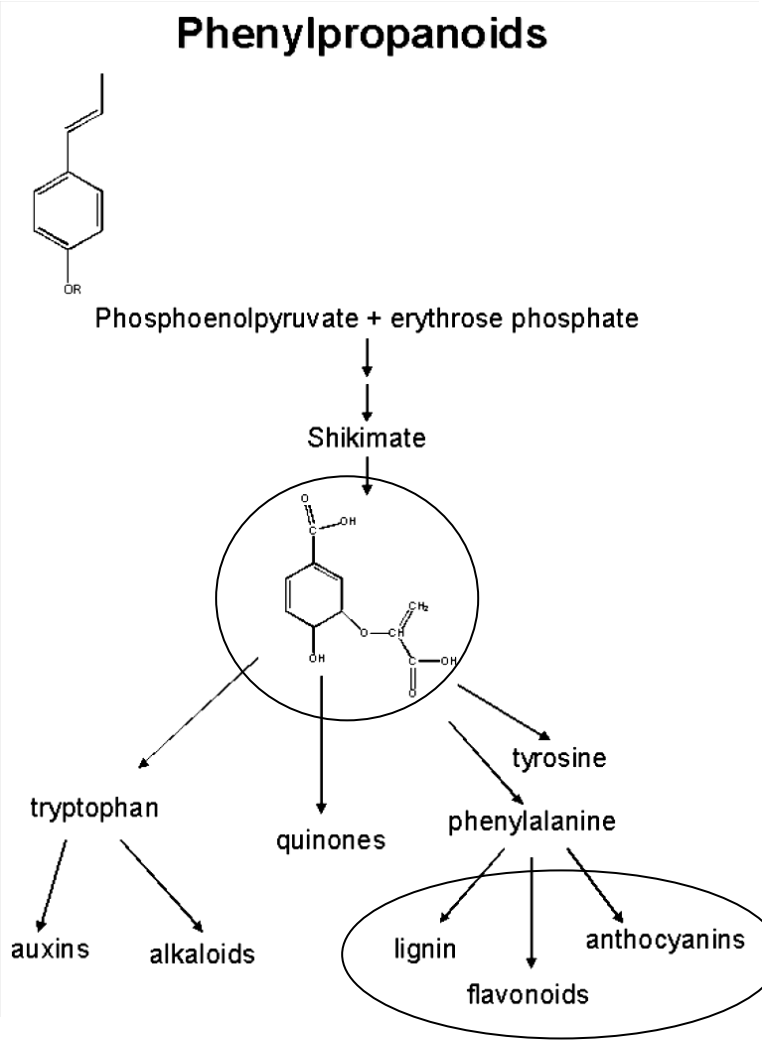

Figure 3. Overview of the shikimate pathway for biosynthesis of a range of aromatic metabolites including phenols (in the ellipse) from phosphoenolpyrovate (PEP) and erythrose phosphate via chorismate (encircled) and aromatic amino acids in higher plants. Figure adapted from www.uky.edu/~dhild/biochem/17/lect17.htm.

From chorismate the amino acids phenylaline, tyrosine and tryptophan can be formed. Those amino acids together with other aromatic amino acids similar in structure are part of the so-called *secondary metabolism*, although this is as relevant to plant survival as *primary metabolism* such as photosynthesis (Vogt, 2010).

In *Arabidopsis thaliana* the phenylpropanoid pathway is simple and yields only a few classes of compounds. Those compounds are the hydroxicinnamic esters, flavonoids such as quercetin and kaempferol and related derivatives, anthocyanins, proanthocyanindins and lignin percursors. Mutants, which are not able to make these compounds, are usually more susceptible to UV-B radiation than wild type (WT) plants (Hectors et al., 2012).

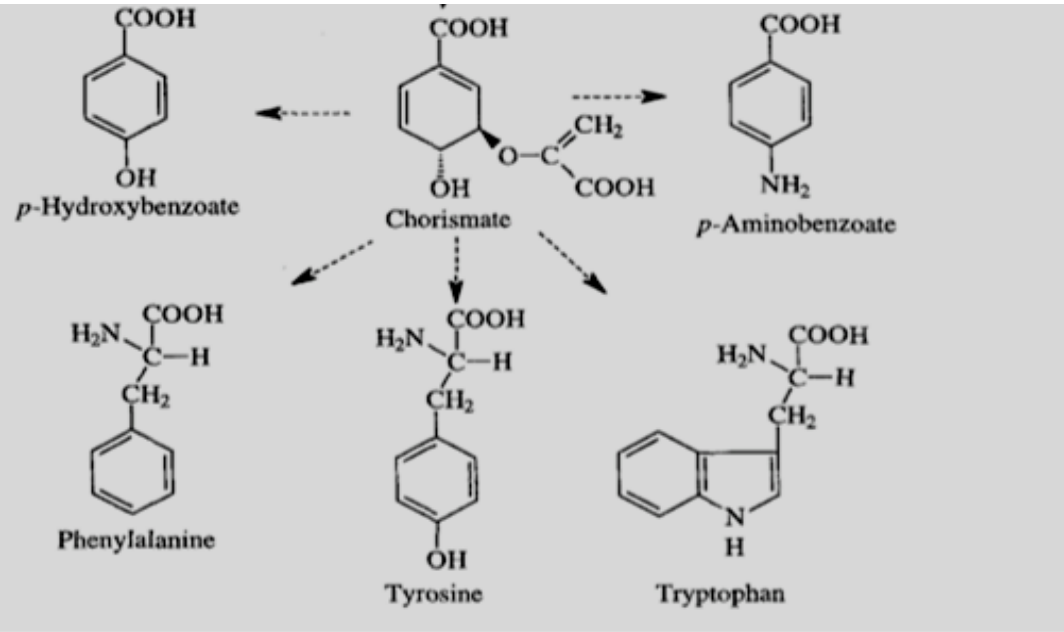

Figure 4. The part of the shikimate pathway leading from chorismate to the amino acids phenylaline, tyrosine and tryptophan. Figure from Herrmann and Weaver (1999)

Flavonoids are water soluble molecules containing 15 carbon atoms. Flavonoids consist of a benzene ring condensed with a six-membered phenyl ring in the 2-position. More than 5000 different flavonoids are known. The various structures are modified by hydroxylation and methoxylation. Many flavonoids are glycosylated and many are also acylated with aliphatic and aromatic acids (Dey, 1997). In plants, the water-soluble glycosides are most common, although the presence of aglycones also has been reported in non-woody tissues (Wollenweber et al., 1980)

From the amino acid phenylalanine, which is formed by the shikimate pathway via chorismate, 4-coumaroyl-CoA is produced (Figure 5). 4-coumaroyl-CoA together with malonyl-CoA forms the backbone of flavonoids

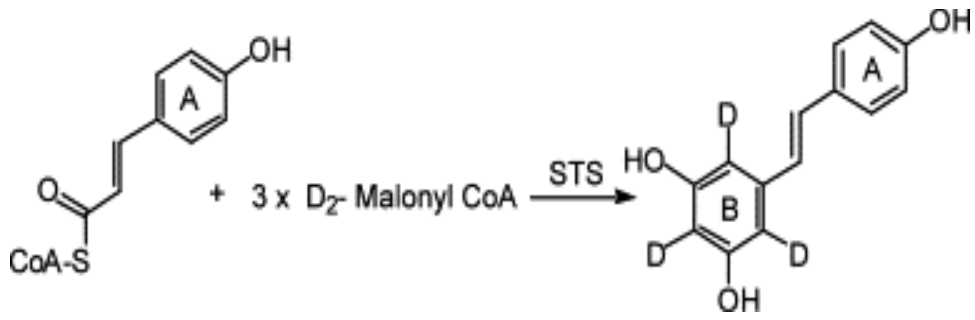

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Figure 5. Formation of the flavonoid naringenin chalcone (right) by stilbene syntase (STS) from coumaroyl-CoA and 3 malonyl-CoA. Figure adapted from www.science.direct.com.

Chalcones contain two phenyl rings. From chalcones the three-ringed structure of a flavonoid is formed by use of CoA esters as immediate substrates (Figure 6). Flavonoids are classified according to the oxidation state of ring C (pyran ring) which connects the two benzene rings A and B. Flavonoids can be directly converted to isoflavones, flavones or dihydroflavonols, and thereafter to a variety of polyphenols (Dey, 1997).

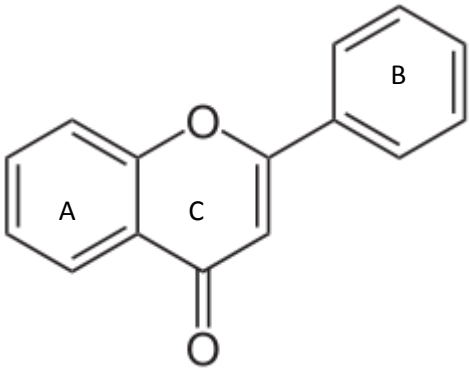
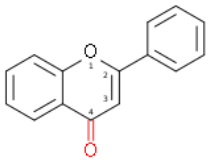
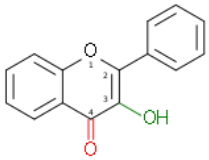
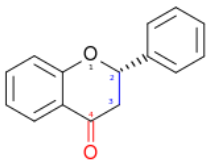
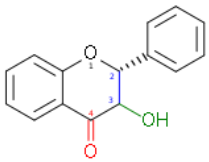
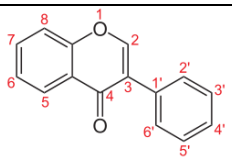
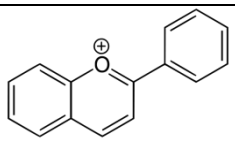


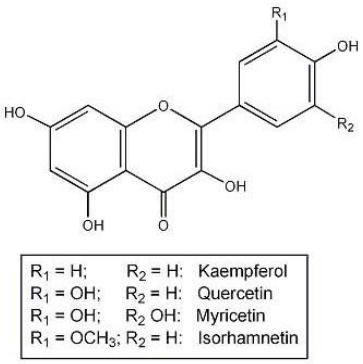
Figure 6: Structure of flavonoid (2-Phenyl-1-benzopyran-4-one). Figure adapted from http://en.wikipedia.org/wiki/File:Flavon.svg.

Flavonoids can be divided in 6 major subgroups, based on their molecular structures; flavone (e.g. luteolin, apigenin) flavonol (e.g. quercitin, kaempferol), flavanone, flavanonol, anthocyanins and isoflavonoids (Table 1)

In vegetables, like broccoli (*Brassica oleracea*), French bean (*Phaeseolus vulgaris*), broad bean (*Vicia faba*) and pea (*Pisum sativum*), five food flavonoids have been widely investigated in different studies; three major flavonols; quercetin, kaempferol and myricetin as well as two major flavones; luteolin and apigenin. The major flavonoids found in such vegetables are quercetin followed by kaempferol. In pea the presence of quercetin and kaempferol in their glycosylated forms have been demonstrated, but myricetin, luteolin and apigenin were not found (Hertog, 1994).

Table 1. Major classes of flavonoids divided in 6 major groups with description, structural form and examples. Formulas from http://en.wikipedia.org/wiki/Flavonoids.

Group	Description	Structural form	Example
Flavone	2-phenylchromen-4-one		Luteolin, Apigenin
Flavonol	3-hydroxy-2-phenylchromen-4-one		Quercetin, Kaempferol, Myricetin
Flavanone	2,3-dihydro-2-phenylchromen-4-one		Hesperetin, Naringenin
Flavanonol	3-hydroxy-2,3-dihydro-2-phenylchromen-4-one		Taxifolin, Dihydroquercetin
Isoflavone	3-phenylchromen-4-one		Genistein, Glycitein
Anthocyanidin	2-phenylchromenylium		Cyanidin, Malvidin



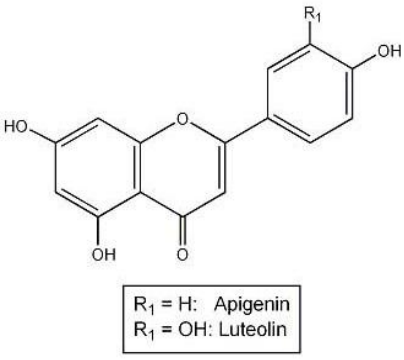


Figure 7. Left: Chemical structures of some flavonols. Right: Chemical structures of some flavones. Figures from http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids.html

Temperature drop as a growth regulator

For many species produced in greenhouses an important quality trait is the compactness of the plants and this is commonly achieved by applying chemical growth retardants. However, chemical growth retardants have negative impacts on both the environment and human health. Therefore it is desirable to reduce their use and use other methods to reduce shoot elongation in plants. It has been shown that a negative DIF, which means that the day temperature is lower than the night temperature (negative temperature difference between day and night), will reduce shoot elongation in many plant species compared to the opposite and constant temperature regimes at the same average daily temperature (Myster and Moe, 1995). Such treatment of plants is commonly difficult to obtain without energy-demanding cooling in a greenhouse in warmer areas and periods. However, it has also been shown that a daily temperature drop of 7-8 ˚C for some hours early in the morning or later in the light period gives reduced shoot elongation in a variety of species (Myster and Moe, 1995). Therefore, temperature drop in the morning obtained by opening vents is a commonly used tool to inhibit stem elongation in the greenhouse industry in the Northern countries. In pea the mechanism behind this inhibited stem elongation in response to negative DIF or a temperature drop in light has been shown to be associated with increased inactivation of the active gibberelin, GA_1 (Grindal et al. 1998; Stavang et al, 2005; 2007; 2010).

The role of the plant hormon gibberelin

Gibberelins (GA) are plant hormones which control growth and development through the life cycle. In control of elongation growth GA acts in stimulation of cell division in the subapical meristem by affecting the transcription of cell cycle regulating genes (Sachs, 1965; Hansen et al.1999). Furthermore, GA acts through stimulation of cell elongation by influencing the orientation of microtubuli, and thus cellulose microfibrilles in the cell wall, and possibly by influencing enzymes that soften the cell wall (Taiz and Zeiger, 2010). All gibberellins are derived from the *ent*-gibberellane skeleton and they all have 19 or 20 carbon units grouped into either four or five ring systems (Figure 8). Gibberelins are known to be synthesized in young tissues of the shoot and in the developing seed.

The transcript level of *GA 2-oxidase2* (*GA2ox2*) in pea was shown to be affected by temperature during day-time (Stavang et al. 2005; 2007). Also, in *A. thaliana* an effect of increased temperature on a *GA2ox* (*GA2ox1*) was demonstrated (Stavang et al. 2009).

However, a mutant in *GA2ox1*, denoted *slender* (*sln*), has been identified in pea, but this mutant responded like the WT to a temperature drop in light and lower day than night temperature, indicating that *GA2ox1* is not involved in the response (Stavang et al., 2005). This is supported by the notion that there was no significant effect of such treatments on the *GA2ox1* transcript level (Stavang et al. 2005).

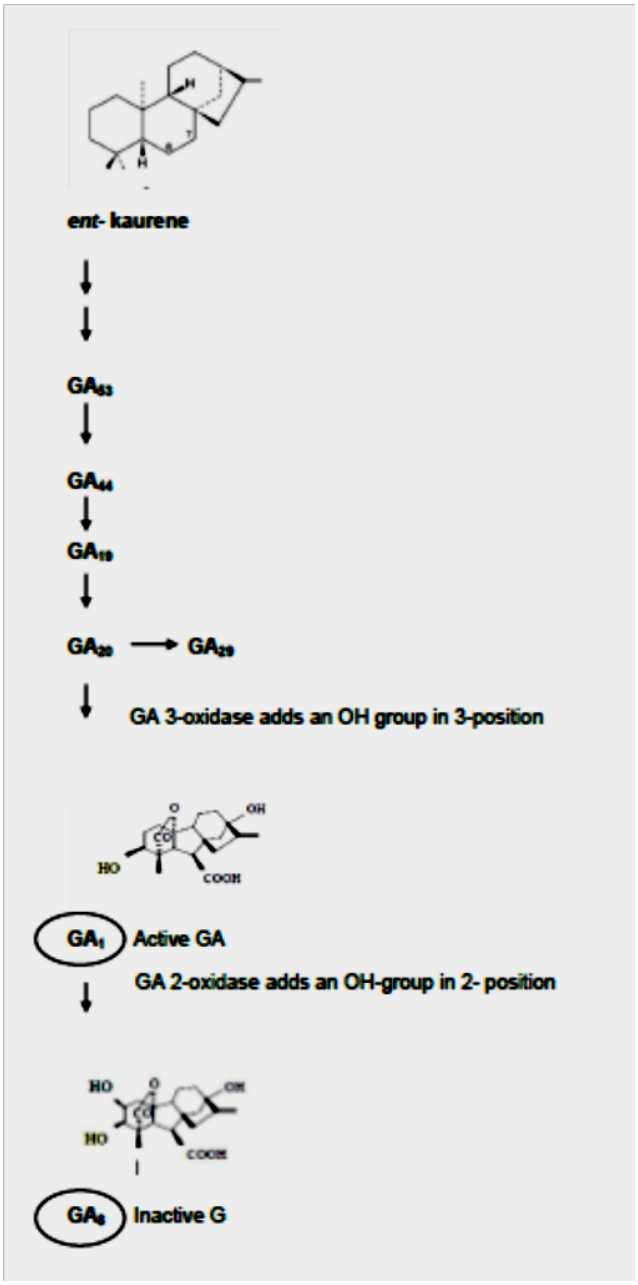


Figure 8. Simplified pathway of GA biosynthesis in vegetative tissue of pea.

HY5 in *Arabidopsis thaliana* and LONG1 in *Pisum sativum*

The way how plants react to light is strictly regulated. The photomorphogenesis-related CONSTITUTIVE MORPHOGENESIS 1 (COP1) protein and the transcription factor LONG HYPOCOTYL 5 (HY5) in *A. thaliana* play central roles in this aspect. Homologs to HY5 and COP1 were recently identified and denoted NEW ELONGATED PEA mutant (LONG1) and LIGHT INSENSITIVE PERIOD1 (LIP1) in pea (Weller et al, 2009). HY5 and LONG1 are not totally identical, LONG1 is structural different from HY5 by having an additional N-terminal domain RING-type Zn-finger domain of the cellulose synthase A subunit (Nishimura et al, 2002; Song et al, 2008). Despite this difference, they are believed to have similar functions in the regulation of photomorphogenesis (Weller et al. 2009).

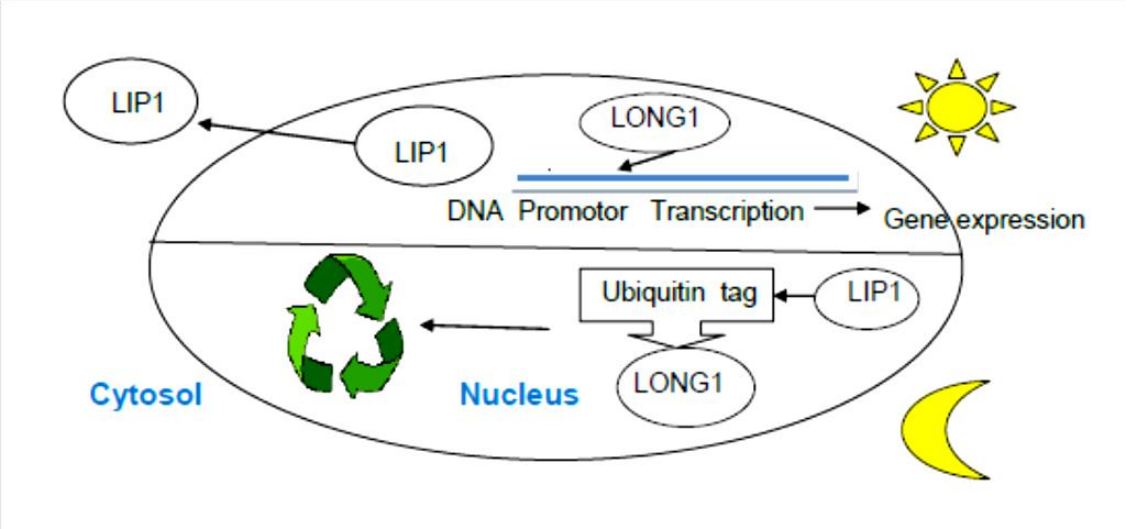


Figure 9. Hypothesized functions of LIP1 and LONG1 in pea. LIP1 protein regulates the turnover of proteins required for photomorphogenic development. During the night, LIP1 adds ubiquitin tags to LONG1. LONG1 is then degraded. During the day, LIP1 exits the nucleus, allowing LONG1 to accumulate and bind, directly or indirectly, to promoter elements in its target genes.

On basis of the known functions of COP1 and HY5 in *A. thaliana* the anticipated functions of LIP1 and LONG1 in pea are illustrated in figure 9. In the dark COP1/ LIP1 adds ubiquitin tags to some photomorphogenesis-related transcriptional activators; HFR1 (LONG HYPOCOTYL IN FAR-RED), LAF1 (LONG AFTER FAR-RED LIGHT) and HY5/LONG1. HY5/ LONG1 is a transcription factor protein which regulates activities of other genes. When tagged by ubiquitin, HY5/ LONG1 and the other transcriptional activators are degraded (Bae et al, 2008).

In the light, COP1/ LIP1 is exported from the nucleus to the cytosol. Then, without being tagged by ubiquitin, the transcriptional activators HY5/LONG1, HFR1 and LAF1 are allowed to accumulate and can directly bind to promoter elements in genes that start photomorphogenic development, or they can cause an indirect effect by acting on other transcription activators which than bind to promoter elements. (Weller et al, 2009).

It is known that in pea the level of *GA2ox2* is far lower in the *long1* mutant than in the WT (Weller et al. 2009). This results in no or little inactivation of the active GA_1 and therefore the *long1*-mutant is tall. It is still unknown how HY5/ LONG1 acts on the *GA2ox2* gene, if there is a direct or indirect interaction. It can be hypothesized that due to the low level of *GA2ox2,* the *long1* mutant will therefore continue shoot elongation also under a daily temperature drop. Indeed, in preliminary studies this mutant was not able to distinguish between a temperature drop during day and night (Todorcevic, 2013) and neither between alternating day and night temperatures (negative or positive DIF) and constant temperature at the same average daily temperature (unpublished results; personal communication J.E. Olsen).

The *lip1* mutant in pea has been mutated in the *LIP1* gene and accordingly lacks the LIP1 protein which is anticipated to add ubiquitin tags resulting in degradation of LONG1. The level of LONG1 should thus always be high, both day and night, therefore also the GA2ox2 level is high and the active GA_1 is inactivated (Weller et al. 2009).The result is that *lip1* mutants are dwarfs. As expected, in a preliminary study the *lip1* mutant showed inhibited shoot elongation both upon a temperature drop during the day and during the night in contrast to the WT, which responded to a temperature drop in light only (Todorcevic, 2013).

The *le* mutant in pea lacks GA 3-oxidase (GA3ox), which means that the active GA_1 is generally not made or made in very low levels only. This mutant is accordingly also a dwarf.

In summary, in pea WT the level of LONG1 is anticipated to be high during light (LIP1 has not added ubiquitin tags to the transcription activators and transcription takes place) and low in the dark (LIP1 has added ubiquitin tags to the transcription activators). The result is that only during light periods photomorphogenic development takes place. In the *long1* mutant the level of LONG1 is always low, which means there is no degradation of the active GA_1 by GA2ox2 and therefore the plants are tall. In *lip1* mutants the level of LONG1 is anticipated always to be high, so degradation takes place all the time and the plants are low. In the *le* mutants the active GA_1 is generally not made and the plants are low as well.

Pea as a model plant

In the experiments of this master thesis the common pea was used as a model plant for several reasons; (1) It has been used as a model plant before in several studies of thermoperiodism and photomorphogensis and many relevant genes have been characterized, like light receptors and the genes involved in the GA metabolism, (2) It grows quickly and is easy to grow, (3) several mutants are available.

The mutants we used were *lip1*, *long1* and *le*. Those were compared with the WT.

The fluorescence excitation ratio method (Multiplex)

The fluorescence excitation ratio method is a non-destructive method to assess compounds in any plant material like the skin of fruits, the epidermis of leaves etc. In this thesis this method was used to assess the amount of flavonoids in the epidermis of the leaves of pea plants by an instrument called Multiplex (Force-A, Orsay, France). The technique is based on the excitation of fluorescence by two wavelengths, one wavelength that is absorbed by the compounds of interest, flavonoids in this case, and one that is not absorbed.

The Multiplex instrument is composed of an optical head which contains four light-emitting diodes in the UV-A (370 nm), the blue (460 nm), the green (515 nm) and the red (637 nm) spectral regions (Figure 10).

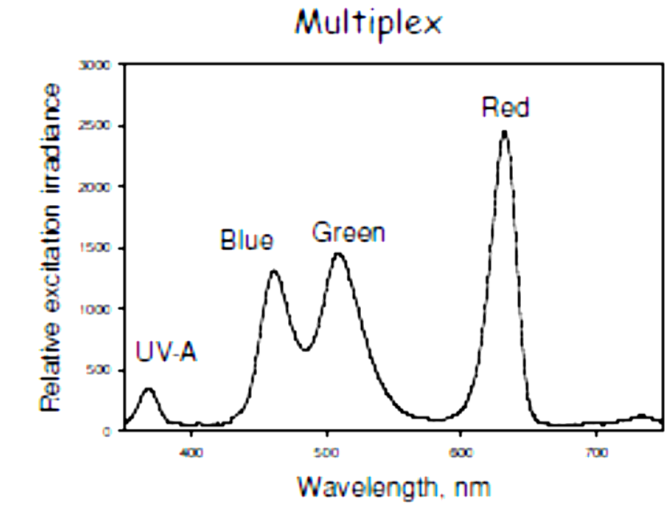


Figure 10. The four spectral regions emitted by the diodes in the Multiplex instrument. Figure is from a presentation by Prof. K.A. Solhaug (2012).

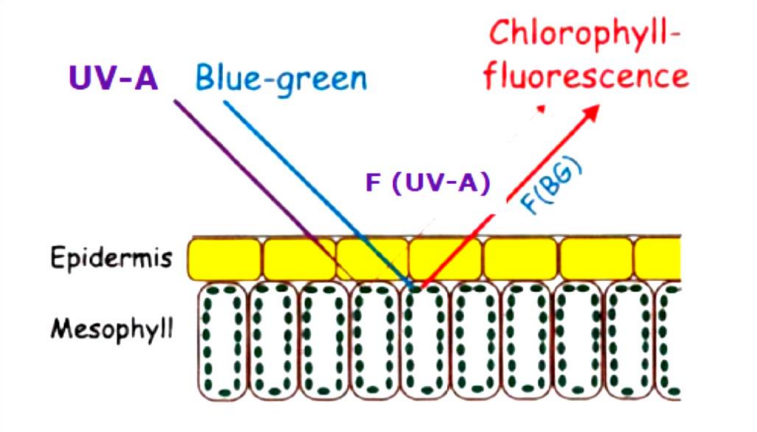


Figure 11: UV-A and blue-green (BG) signals are emitted and together with the fluorescence sent out these will give a chlorophyll fluorescence index. Figure modified from Birger et al. (2001).

Figure 11 shows how the Multiplex instrument works: UV-A and blue-green (BG) signals are emitted by the Multiplex instrument. The ratio between the signals emitted and the far-red (FR) chlorophyll fluorescence (FRF) excited is measured and will give an index, called FLAV index, which is proportional to the content of the amount of flavonoids, assuming that all UV-A absorbance is due to flavonoids. The relative amounts of UV-A absorbing flavonoids are estimated as log (FRF_R/FRF_{UVA}).

Figure 12 shows that flavonoids (in this figure quercetin glucoside) in the skin of winegrapes absorbs UV-A, but not green and red light.

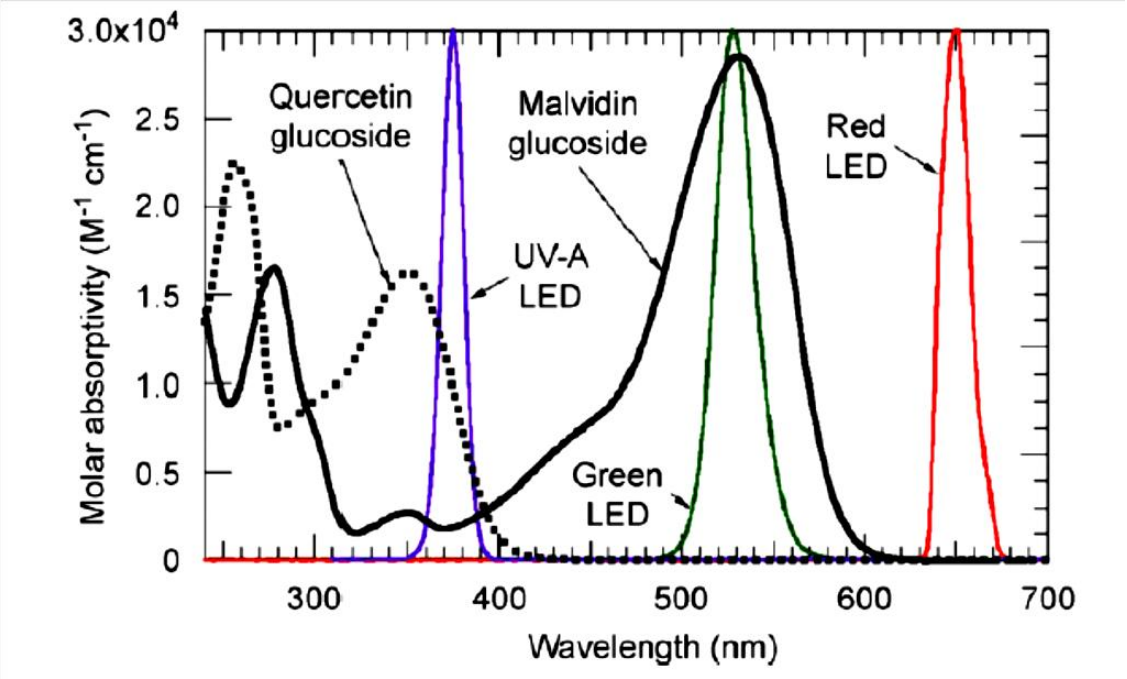


Figure 12. The absorption spectra of the skin in winegrape berries (*Vitis vinifera*). It absorbers malvidin glucoside (anthocyanin) and quercetin glucoside (flavonoid). This was compared to the light emission spectra of the LED source used (UV-A, green and red LED). Figure from Cerovic (2008).

The HPLC method

High performance liquid chromatography (HPLC) is basically a highly improved form of column chromatography. However, instead of gravity-dependent dripping of solvent through a column, the solvent is forced through a column under high pressure commonly of about 200 atmospheres. HPLC is therefore much faster than regular column chromatography. The components of a mixture will be separated since they differ in their strength of interaction with the stationary phase of the column.

There are two variants in use in HPLC; normal phase and reverse phase HPLC. In normal phase HPLC the mobile phase is non-polar and the liquid stationary phase is polar. This is opposite in the reverse phase HPLC. The technique used in this master thesis was reverse phase HPLC.

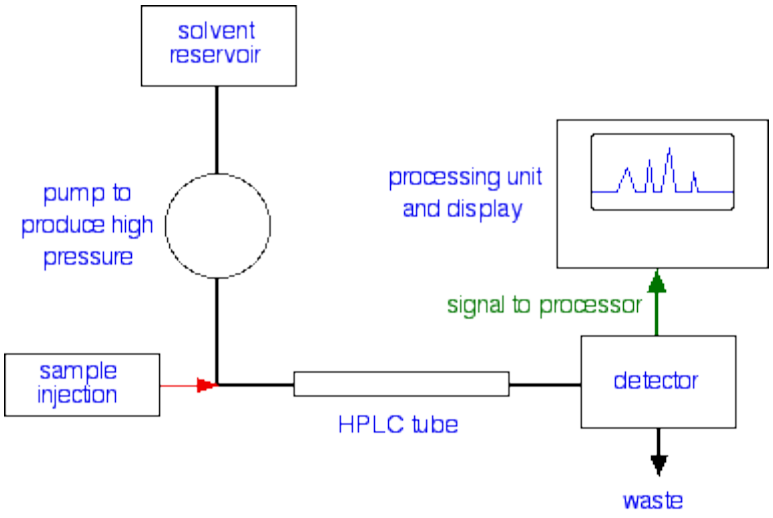


Figure 13. The components of a typical HPLC system. The peaks in the display unit indicate the different, separated compounds. Figure from www.chemguide.co.uk/analysis/chromatography/hplc.html

A sample is injected and is pressed under high pressure through the HPLC column (Figure 13). The time taken for a particular compound to travel through the column to the detector is known as the retention time (Figure 14). Different compounds have different retention times depending on their chemical characteristics including polarity. To identify compounds it is important that the pressure, the temperature and the exact composition of the solvent are carefully controlled.

For UV-absorbing compounds one way of detecting when a substance has passed through the column is to use UV absorption. UV-radiation is then sent through the liquid and a UV detector can read how much of the radiation is absorbed.

The output will be recorded as a series of peaks- each representing a compound in the mixture passing through the detector and absorbing UV radiation. Thereafter the compounds present can be identified by comparing their retention time with those of known samples. After identifying the compounds the quantity of the different

compounds can be calculated. This formula is described under materials and methods.

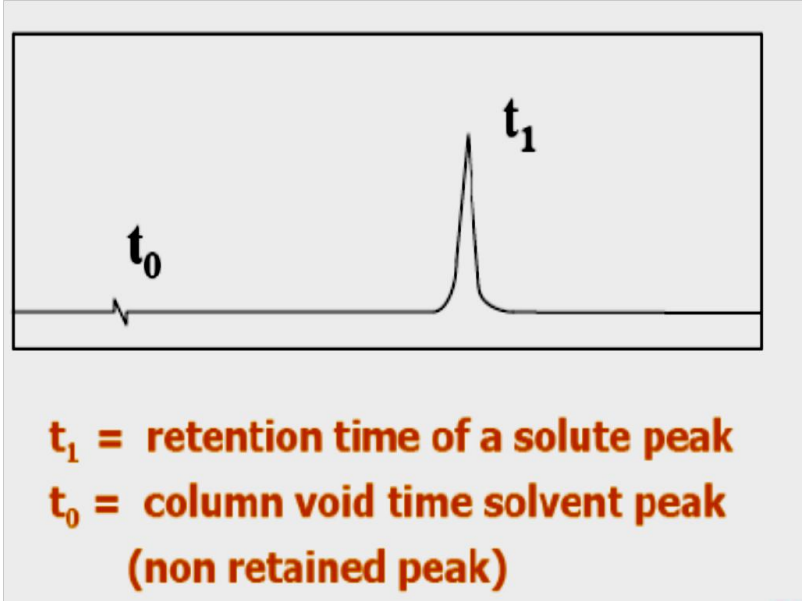


Figure 14. Chromatogram from HPLC. In this figure t_0 shows the peak caused by the solvent and t_1 shows the peak by a compound like a flavonoid. Figure is adapted from the lectures of Victoria Samanidou (2013)

Aims of the study

The aim of this thesis was to develop a better understanding of the mechanisms underlying UV-B-radiation (280 nm-315 nm) regulated growth in pea, and how UV-B interacts with temperature drop on affecting the morphology of pea plants, determined by measuring shoot elongation, leaf area and dry weight, as well as to assess the effect of UV-B on the accumulation of UV-protective flavonoids.

Materials and methods

Plant material and growing conditions

Pea plants (*Pisum sativum L.*) were used in the experiments; a wild-type (WT; cv. Torsdag), three different mutants; denoted *long1*, *lip1*, and *le*. The peas were sown in 3:1 fertilized peat (Tjerbo Torvfabrikk, Rakkestad, Norway): perlite in 11 cm pots. The pots were placed in 75 x 80 x 80 cm growth chambers (manufactured by Norwegian University of Life Sciences) at a constant temperature of 20 ˚C and under fluorescent tubes at photosynthetic photon flux density (PPFD) of 100 µmol m^{-2} s^{-1} at 400-750 nm (MASTER TL-D Super 80 36W/840 Philips, Eindhoven, The Netherlands) and a R/FR- ratio: 1.7, achieved through incandescent lamps (Osram, Munich, Germany). The irradiance was measured at about 15 cm from the bottom of the chamber using a LI-COR Quantum/ Radiometer/ Photometer (Model LI-250, Li-Cor, Lincoln, NE, USA). The plants were exposed to a 12 h photoperiod from 9.00-21.00. The walls of the chambers were covered with aluminium foil to ensure a uniform distribution of the light and UV-B-radiation. The plants were watered daily. The relative air humidity (RH) could not be precisely controlled in these chambers and was around 70%. Trays of water were placed below the bottom plate of the chamber. After a growing period of 6 days, the plants were exposed to UV-B radiation, either at a constant temperature of 20 °C or in combination with a 6 h temperature drop from 21 to 13 °C. 20 °C was used as the constant temperature since this is the average diurnal temperature in the temperature drop treatment. All treatments lasted for 10 days and had a constant irradiance (PPFD) of 100 µmol m^{-2} s^{-1}.

The UV-B radiation fluorescent tubes used had a light spectrum from 290-315 nm (TL 40W/12 RS SLV, Philips, Eindhoven, The Netherlands) (Figure 15). A film of 0,15 mm clear cellulose diacetate foil (Jürgen Rachow, GmbH, Hamburg,Germany) was placed ca. 10 cm under the UV-B lamps to ensure that the plants did not receive any UV-C radiation. UV-spectra were measured with the Optronic model 756 spectroradiometer (Optronic Laboratories, Orlando, FL, USA) and used to calculate biologically effective UV-B (UV-BBE) based on the Green weighting function for DNA damage normalized to 1 at 300 nm (Green et al., 1974). Two different fluence rates of UV-B radiation were used; the high dose was estimated to 0.50 W m^{-2}, the low dose was estimated to 0.35 W m^{-2}. The fluence rate was measured at all sides of a tetrahedron in the middle of the chamber 15 cm above the surface, and the values were summarised (Björn, 1995). The reason why fluence rate was choosen instead of just irradiance was because of the fact that plants in the chambers receive UV-B radiation from all sides, not just from above.

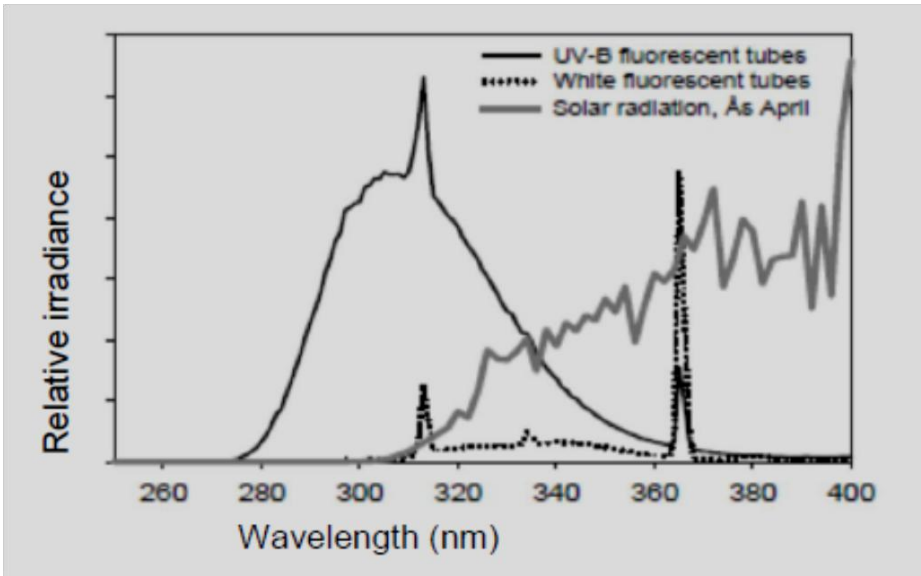


Figure 15. UV radiation from unfiltered UV-B Q-panel UV313, white light tubes (Philips MASTER TL-D Super 80 36W/840) and natural sunlight at Ås, Norway (59˚N) in April. Figure is adapted from Torre et al. 2012.

General experimental procedures

In the experiments the role of different lengths of periods with UV-B radiation, given in a high fluence rate (0,50 W m^{-2}) or a low fluence rate (0,35 W m^{-2}) in combination with a temperature drop on pea plants of wild type, *long1*, *lip1*, *le* mutants were investigated.

Pre-experiment 1

The aim of this pre-experiment was to evaluate the sensitivity of the *long1* mutant to UV-B radiation.

15 plants of each of the WT and the *long1* mutant per treatment were exposed to 4 different treatments. A constant temperature of 20 ˚C was used. The **first treatment** was the control treatment. In the **second, third and fourth treatment** 2 h, 4 h or 6 h of UV-B radiation was given in high fluence rate (0,50 W m^{-2}) (Table 2).

On basis of knowledge that *A. thaliana* HY5 is important in UV-B signaling (Jenkins, 2009) higher susceptibility to UV-B of the genotype *long1* compared to the WT was anticipated. Therefore *long1* mutants were exposed to UV-B only for up to 4 h. Since the *lip1* mutant was anticipated to contain higher levels of LONG1 due to lack of degradation in darkness (as discussed above in the introduction), a perliminary experiment 6 h UV-B treatment of the *lip1* mutant was included.

Table 2. Experimental conditions during the first pre-experiment with pea. UV-B radiation was provided at a fluence rate of 0.50 W m^{-2}.

Genotype	Treatment	Light period	UV-B radiation period	Temperature
WT ***long1***	Control	9.00-21.00	-	20 ˚C
WT ***long1***	2 h UV-B	9.00-21.00	12.00-14.00	20 ˚C
WT ***long1***	4 h UV-B	9.00-21.00	12.00-16.00	20 ˚C
WT ***lip1***	6 h UV-B	9.00-21.00	12.00-18.00	20 ˚C

During this experiment we observed that the genotype *long1* showed a lot of damage. The observed damage was curled leaf edges, yellow spots on the leaves and yellow stems. This happened already after the treatment with UV-B radiation for 2 hours. Therefore we changed the conditions **for the next pre-experiment**:

Pre-experiment 2

In this pre-experiment we exposed plants to UV-B radiation at a fluence rate of 0,35 W m^{-2} and shortened the period in which the plants were exposed to UV-B radiation compared to in pre-experiment 1. Ten plant of each of WT and *long1* and five plants of *le* per treatment were exposed to 6 different treatments (Table 3). A constant temperature of 20 ˚C was used, The **first treatment** was the control treatment. In the **second-sixth treatment** 15 min, 30 min, 1 h, 1 h 30 min or 6 h of UV-B radiation was given at 0,35 W m^{-2}.

Table 3. Experimental conditions during the second pre-experiment with pea. UV-B radiation was provided at a fluence rate of 0.35 W m^{-2}.

Genotype	Treatment	Light period	UV-B radiation period	Temperature
WT ***long1*** ***le***	Control	9.00-21.00	-	20 ˚C
WT ***long1*** ***le***	15 min UV-B	9.00-21.00	12.00-12.15	20 ˚C
WT ***long1*** ***le***	30 min UV-B	9.00-21.00	12.00-12.30	20 ˚C
WT ***long1*** ***le***	1 h UV-B	9.00-21.00	12.00-13.00	20 ˚C
WT ***long1*** ***le***	1 h 30 min UV-B	9.00-21.00	12.00-13.30	20 ˚C
WT ***le***	6 h UV-B	9.00-21.00	12.00-18.00	20 ˚C

After these experiments it was clear that the *long1* mutant did only survive well after the 15 or 30 min treatments with the lowest fluence rate of UV-B radiation. Neither WT, *le* or *lip1* mutants showed any major damage after the 6 h treatment.

Experiment with WT and *lip1*

The aim of this thesis was not only to investigate the effects of UV-B radiation, but mainly to investigate the effects of an interaction between UV-B radiation and a temperature drop treatment.

The aim of this experiment was therefore to illustrate the effect of the interaction between UV-B radiation and temperature drop on WT and the *lip1* mutant under 4 different treatments. 15 plants per treatment of each of WT and the *lip1* mutant were exposed to 4 different treatments (Table 4). This experiment was done twice. The **first treatment** was the control treatment at constant temperature (20 ˚C). In the **second treatment** 6 h UV-B radiation was given at a fluence rate of 0.50 W m^{-2} at constant temperature (20 ˚C). In the **third treatment** 6 h UV-B radiation was given at a fluence rate of 0.50 W m^{-2} together with a temperature drop for 6 h from 21 ˚C to 13 ˚C in the middle of the light period. In the **fourth treatment** a temperature drop was given for 6 h from 21 ˚C to 13 ˚C in the middle of the light period.

Table 4. Experimental conditions during an experiment with pea exposed to 6 h of UV-B radiation at a fluence rate of 0.50 W m^{-2} either alone or in combination with a temperature drop treatment.

Genotype	Treatment	Light period	UV-B radiation period	Temperature
WT ***lip***	Control	9.00-21.00	-	20 ˚C
WT ***lip***	6 h UV-B	9.00-21.00	12.00-18.00	20 ˚C
WT ***lip***	6 h UV-B and 6 h T-drop	9.00-21.00	12.00-18.00	12.00-18.00: 13 ˚C 18.00-12.00: 21 ˚C
WT ***lip***	6 h T-drop	9.00-21.00	-	12.00-18.00: 13 ˚C 18.00-12.00: 21 ˚C

Experiment with WT, long*1* and *le*

The aim of this last experiment was to illustrate the effect of the interaction between UV-B and temperature drop on WT, *long1* and *le* mutants under 6 different treatments. 10 or 15 plants of each of WT, *long1* and/or *le* mutants per treatment were exposed to 6 different treatments. This experiment was done twice. The **first treatment** was the control treatment at constant temperature (20 ˚C). In the **second treatment** 30 min UV-B radiation was given at a fluence rate of 0.35 W m^{-2}. In the **third treatment** 30 min UV-B radiation was given at a fluence rate of 0.35 W m^{-2} together with a temperature drop for 6 h from 21 ˚C to 13 ˚C in the middle of the light period. In the **fourth treatment** 6 h UV-B radiation was given at a fluence rate of 0.35 W m^{-2}. In the **fifth treatment** 6 h UV-B radiation was given in a fluence rate of 0.35 W m^{-2} together with a temperature drop for 6 h from 21 ˚C to 13 ˚C in the middle of

the light period. In the **sixth treatment** a temperature drop was given for 6 h from 21 ˚C to 13 ˚C in the middle of the light period (Table 5).

Table 5. Experimental conditions during an experiment with pea exposed to different durations of UV-B at a fluence rate of 0.35 W m^{-2} either alone or in combination with a temperature drop treatment.

Genotype	**Treatment**	**Light period**	**UV-B radiation period**	**Temperature**
WT ***long1*** ***le***	Control	9.00-21.00	-	20 ˚C
WT ***long1***	30 min UV-B	9.00-21.00	15.00-15.30	20 ˚C
WT ***long1***	30 min UV-B and 6 h T-drop	9.00-21.00	15.00-15.30	12.00-18.00: 13 ˚C 18.00-12.00: 21 ˚C
WT ***le***	6 h UV-B	9.00-21.00	12.00-18.00	20 ˚C
WT ***le***	6 h UV-B and 6 h T-drop	9.00-21.00	12.00-18.00	12.00-18.00: 13 ˚C 18.00-12.00: 21 ˚C
WT ***long1*** ***le***	6 h T-drop	9.00-21.00	-	12.00-18.00: 13 ˚C 18.00-12.00: 21 ˚C

Registrations

Figure 16 shows a growth chamber with pea plants of the WT and the *lip1* mutant. To ensure the dwarf mutant, *lip1*, was receiving the same amount of UV-B radiation as the WT a construction was built under the pots. Thus, the tops of both the WT and the *lip1* mutant plants were constantly at a similar height.

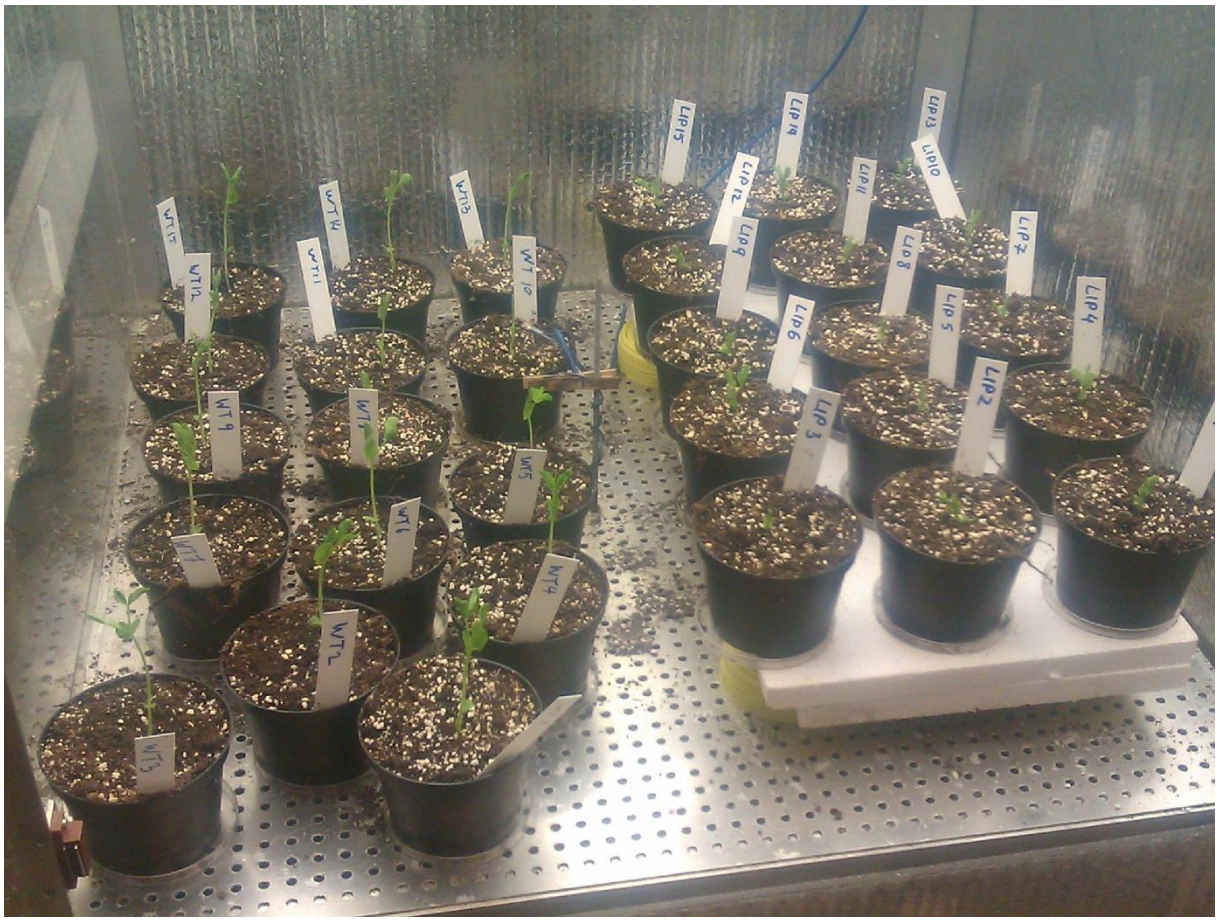


Figure 16. Picture of the pea plants grown in a growth chamber.

Visible damage

The visible damage caused by the UV-B radiation on the pea plants was classified at day 10 on a scale from 0 to 3: (0) No visible damage (1) Little damage with curled leaf edges (2) More severe damage with curled leaf edges and yellow, chlorotic spots (3) Severe damage with very curled leaf edges, brown necrotic spots, yellow stem/ dead plants.

On day 10 photographs of the pea plants were taken with a SRL camera EOS 400D (Canon, Tokyo, Japan).

Plant height

During day 0, day 3 and day 7 and at the end of the experiments (day 10) the plant height was measured from the pot edge to the apex, and number of leaves was registered.

Total leaf area

On day 10 the leaf area was measured by an area meter (Li Cor Inc., Lincoln, Nebrasca, USA).

Total dry weight

The stems and the leaves were separately dried in a drying cabinet (Termaks, Bergen, Norway) at 70 ˚C for 2 days. Thereafter they were weighed on a scale (Sartorius, Goettingen, Germany).

Content of phenolic compounds in the epidermis measured by Multiplex

In the experiment with WT and the *lip1* mutant, we used a Multiplex Instrument to assess the amount of phenolic compounds in the epidermis. The Multiplex sensor (Force-A, Orsay, France) consists of a fluorimeter with four light-emitting diodes in the UV-A (370 nm), the blue (460 nm), the green (515 nm) and the red (637 nm) spectral regions.

One leaf of the third pair of leaves was measured by the Multiplex sensor. The leaf was held in plane with the mask of the Multiplex sensor during measurement. The formulae used to calculate the percentage of UV-A absorbance was:

FLAVONOID CONTENT = log (FRFred/FRFuv)

Content of phenolic compounds in the leaf measured by HPLC

In order to determine the concentration of phenolic compounds in leaves, at the end of the experiment (day 10) the 3. mature leaf pair from the soil was harvested with WT and *lip1* mutants. The 2. leaf pair was harvested at at the end of the experiments with WT, *long1* and *le* mutants. The change of leaf pair was because of the fact that there was more visible damage on the *long1* mutant on the 3. mature leaf than on the 2. mature leaf pair form the soil. Thereafter the leaves were dried in a drying cabinet at 30 ˚C for 2 days. The petiole and main vein of the leaves were cut away and the leaf was weighed on a scale (Mettler Toledo, Oslo, Norway). The leaf material was

put in a Precellys-vial, 600 µl of methanol (MeOH) for liquid chromatography was added and homogenised 30 sec. with Precellys 24 (Bertin technologies, Montigny le Bretonneux, France) and the vials were placed on ice for 15 min. The vials were centrifuged for 3 min. at the highest setting (18000 rpm min^{-1}) in a centrifuge (Hettich, Tuttlingen, Germany).The supernatant was transferred into a labeled test tube (6-10 ml). Addition of 600 µl methanol to the precipitate and transfer of the supernatant to the test tube was repeated three more times.

In the next step the methanol was evaporated from the test tubes with the Concentrator (Eppendorf AG, Hamburg, Germany) at 30 ˚C for 1 hour. For use in the HPLC analysis, the following solutions were made:

A-solution: 5 ml Orthophosphoric acid (Merck KGaA, Darmstadt, Germany), 30 ml Tetrahydrofyran (Merck KGaA, Darmstadt, Germany) and 1965 ml HPLC-water from the Purelab Maxima HPLC (Elga Labwater, Bucks, HP, USA).

B solution: methanol for liquid chromatography (Merck KGaA, Darmstadt, Germany).

The sample was dissolved in 200 µl MeOH + 200 µl HPLC-water by using an ultrasonic cleaner (VWR, Leuven, Belgium). A Pasteur pipette was used to transfer the extract to an Eppendorf-vial and the extract was centrifuged 3 min. at maximum speed in the centrifuge. Thereafter the extract was transferred to an HPLC-vial. The vials and the solutions were placed at the tray in the HPLC (Agilent 1200, Agilent Technologies, CA, USA) and the machine was turned on.

The phenols were analyzed following the method of Julkunen-Tiitto (2001). The column used was Hypersil ODS Kappa Capillary HPLC Column, serial number: 12154391Q3, dimension 50 mm x 4,6 mm, particle size: 3 um (Thermo scientific, Waltham, Massachusetts, USA). The samples were injected by an autoinjector and detection of the compounds of interest was done by using a detector (Agilent 1200, RID G1362A, Agilent Technologies, Santa Clara, CA, USA). The chromatographic peaks were used to measure the quantities of the different compounds by using the following formula:

$$\text{Quantity} = \frac{\text{A x RF x total solution}}{Amount\ of\ sample\ x\ weight\ dry\ leaf\ material}$$

A = the area under the peak (calculated by the computer)

RF = the response factor for each specific phenolic. The response factor for each phenolic compound was found by comparing with standards

The total solution = here 400 µl (200 µl MeOH + 200 µl HPLC water)

The amount of the sample = here 20 µl (for the used method RJT)

Weight = the weight of the dry leaf material used

Statistical analysis

All data were analyzed statistically using the general linear model procedure with two-ways analysis of variance (ANOVA) to test significance ($p \leq 0.05$) of effects of UV-B radiation and temperature drop treatments (Table 11 to 46 in appendix). The effects of the treatments on phenolic compounds measured by HPLC were tested statistically for each of the 18 compounds detected. Since the same tendency was observed for each component within a group, these components were pooled and the statistical analysis of the pooled values are presented. Prior to analysis data were tested with respect to homogeneity of variances and normal distribution.

Results

Results pre-experiment 1: Effect of different UV-B radiation durations on WT and *long1*

Visible effects of UV-B radiation

To investigate the role of *LONG1* in response to UV-B radiation in pea, effects of different duration of UV-B radiation were studied on plants mutated in this gene compared to the WT. In a preliminary experiment these genotypes were exposed to 2 and 4 h of 0.50 W m^{-2} UV-B radiation daily in the middle of the photoperiod (12 h of 100 µmol m^{-2} s^{-1}).

The WT plants treated with 4 h UV-B radiation at 0.50 W m^{-2} showed severe damage (Table 6). The stem was shortened and yellow-brownish. The leaves were curled and had yellow-brown spots (necrosis). The plants of the *long1* mutant showed severe damage already when treated with 2 h UV-B radiation at 0.50 W m^{-2}. The stem was shortened and yellow brownish. The leaves were curled together and had light brown, necrotic spots.

Table 6. Classification of visible damage on the wild type (WT) and the *long1* mutant grown for 10 days with 2 different durations of 0.50 W m^{-2}. Damage is classified as (0) No visible damage (1) Little damage with curled leaf edges (2) More severe damage with curled leaf edges and yellow, chlorotic spots (3) Severe damage with very curled leaf edges, brown necrotic spots, yellow stem/ dead plants.

	WT	***long1***
Control	0	0
2 h UV-B radiation (0.50 W m^{-2})	2	3
4 h UV-B radiation (0.50 W m^{-2})	3	3

Effect of UV-B radiation on leaf area

In figure 17 the leaf area of both the WT and the *long1* mutant decreased significantly after daily treatment with UV-B radiation for 2 h. In the WT the decrease was 61% compared to WT-control and in *long1* the decrease was 91% compared to the *long1*-control. Because of the curling of the leaves it was not possible to measure the leaf area for the treatments with 4 h UV-B radiation

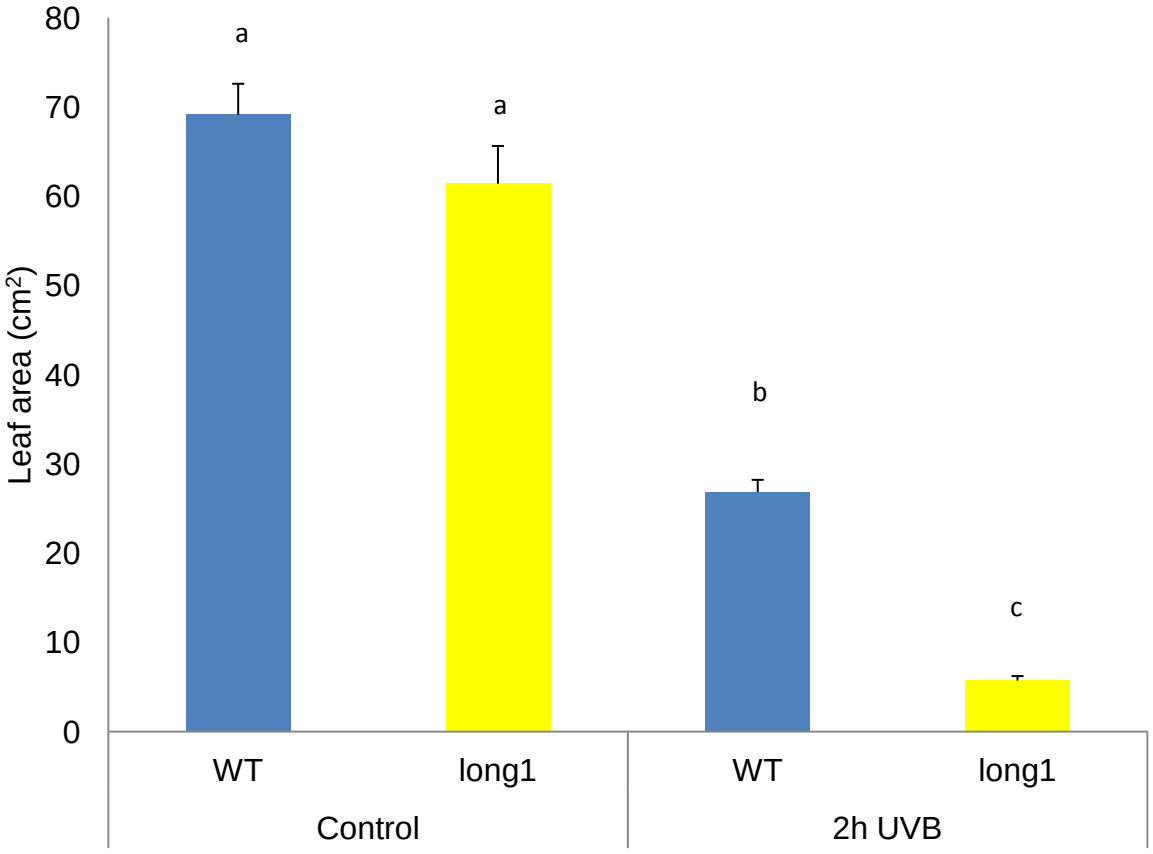


Figure 17. Effect of UV-B radiation (0.50 W m^{-2}) for 2 h on leaf area in the wild type (WT) and the *long1* mutant of pea. Results are mean of 15 plants in 1 experiment and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$)

Effect of UV-B radiation on dry weight

The total dry weight (DW) decreased significantly in the *long1* mutant treated with UV-B radiation for 2 h. The total DW was 65% reduced compared to the *long1*-control. In contrast, the WT showed no reduction in the total DW after the treatment with 2 h UV-B radiation. However, the ratio of DW leaves/ DW stem changed in both genotypes after the treatment with 2 h UV-B radiation. More dry matter had then been allocated to the stem compared to the control treatment (Figure 18). The DW was not measured after the treatment with 4 h UV-B radiation because of the many necrotic spots.

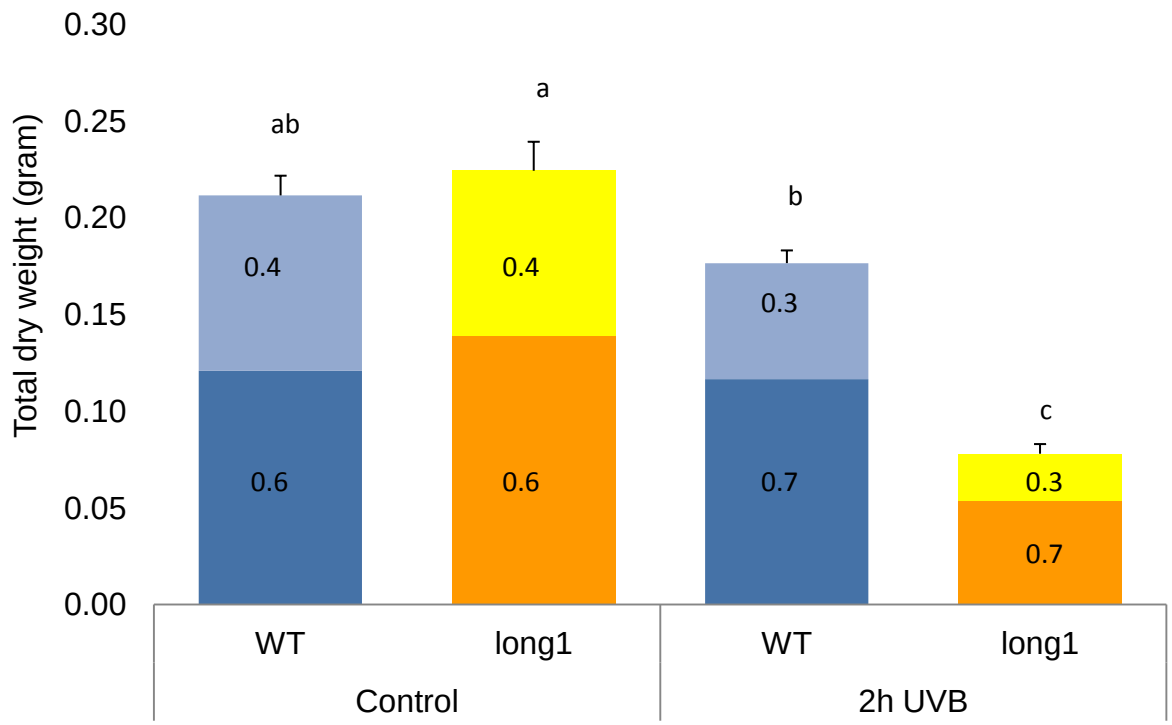


Figure 18: Effect of UV-B radiation (0.50 W m^{-2}) for 2 h daily in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on dry weight (DW) in the wild type (WT) and the *long1* mutant in pea. The upper and lower part of the bar shows the dry weight of the leaves and stem, respectively. The numbers in the table are the ratio of the dry weight of the leaves or the stem compared to the total dry weight (=1). Results are mean of 15 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation on shoot elongation

When exposed daily to different durations of UV-B treatment the WT showed a significant decrease in shoot elongation for all treatments compared to the control. 28% and 81% reduction in shoot elongation were observed for the treatments with 2 h and 4 h UV-B radiation, respectively. The *long1* mutant showed a significant decrease in shoot elongation after UV-B treatment for 2 and 4 h. Shoot elongation was then reduced with 67% and 89%, respectively, compared to the control (Figure 19).

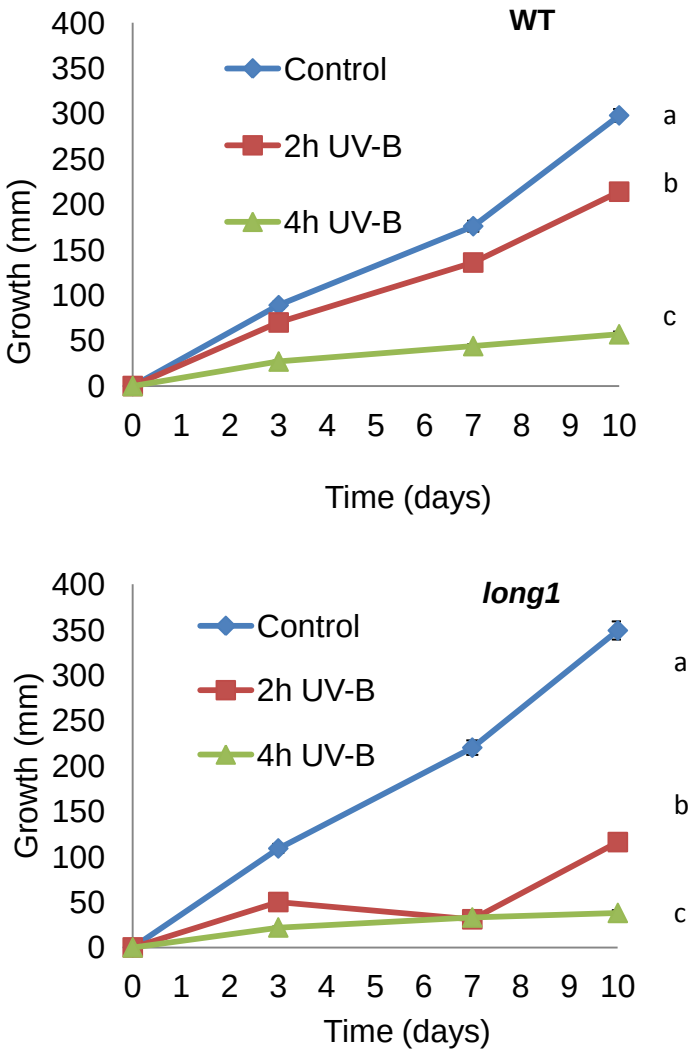


Figure 19. Effect of UV-B radiation (0.50 W m^{-2}) for 2 h or 4 h on shoot elongation in wild type (WT) and *long1* mutant in pea. Results are mean of 15 plants and ±SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Considerable damage was registered in the WT as well as in the *long1* mutants after this experiment. One aim with the thesis work was to investigate the role of LONG1 in production of UV-protecting compounds in response to exposure to UV-B and

temperature drop. However, since the plants in this pre-experiment were heavily damaged, it was not possible to do a reliable test of the levels of phenolic compounds in the leaves by HPLC.

The next pre-experiment was therefore changed in several ways; the fluence rate of UV-B radiation was lowered from 0.50 W m^{-2} to 0.35 W m^{-2} . This was done by wrapping aluminum foil around the UV-B radiation fluorescent tubes.

Another adjustment was made by making the gaps between the walls of the chamber and the UV-C-cutting cellulose acetate film smaller. This was to avoid the possibility that UV-C radiation from the fluorescent lamps could reflect on the aluminium foil-covered walls and reach the plants. UV-C radiation can cause severe damage and although only very little UV-C radiation is emitted by the fluorescent UV- lamps it is of great importance to exclude this.

Results Pre-Experiment 2: Effect of different UV-B radiation durations on WT, *long1* and *le*

Visible effects of UV-B radiation

To investigate the sensitivity to different UV-fluence rates in the WT and the *long1* mutant in more detail as well as to shed light on the role of GA in response to UV-B radiation, the WT, *long1* and the GA biosynthesis (*GA20ox*) mutant *le* were exposed to different durations daily of 0.35 W m^{-2} UV-B radiation. Table 7 shows no visible damage in any genotype grown for 10 days with 15 min UV-B radiation treatment. In the WT and *long1* a slight curling of leaves started to appear after the treatment of 30 min or longer with UV-B radiation. The damage became more severe in the *long1* mutant after the treatment with 1 h or 1 h 30 min UV-B radiation. The *le* mutant showed a little leaf curling after the treatment with 6 h UV-B radiation only, but not in the shorter UV-B durations.

Table 7: Classification of visible damage on WT, *long1* and *le* mutant grown for 10 days with 6 different treatments. Damage is classified as (0) No visible damage (1) Little damage with curled leaf edges (2) More severe damage with curled leaf edges and yellow, chlorotic spots (3) Severe damage with very curled leaf edges, brown necrotic spots, yellow stem/ dead plants.

	WT	***long1***	***le***
Control	0	0	0
15 min UV-B radiation (0.35 W m^{-2})	0	0	0
30 min UV-B radiation (0.35 W m^{-2})	1	1	0
1 h UV-B radiation (0.35 W m^{-2})	1	2	0
1 h 30 min UV-B radiation (0.35 W m^{-2})	1	2	0
6 h UV-B radiation (0.35 W m^{-2})	1	-	1

Effect of UV-B radiation on leaf area

After daily exposure to different relatively short durations of UV-B the leaf area in WT was significantly smaller after the treatments with UV-B radiation (Figure 20). Compared to the WT-control, a decrease in leaf area of 55% and 36% after the daily treatments with 15 and 30 min UV-B radiation, respectively, was recorded. Furthermore, after treatment with 1 h and 1 h 30 min of UV-B exposure 42% and 33%, respectively were measured. The differences between the different UV-B treatments were not significant.

Similarly, the leaf area of *long1* decreased significantly after the treatments with UV-B radiation, but there was no difference in leaf area between the different UV-B treatments. Compared to the *long1*-control, we measured a decrease in leaf area of 62% and 50% after the treatment with 15 and 30 min UV-B radiation, respectively, as well as a decrease of 62% and 69% after the treatment with 1 h and 1 h 30 min UV-B radiation, respectively.

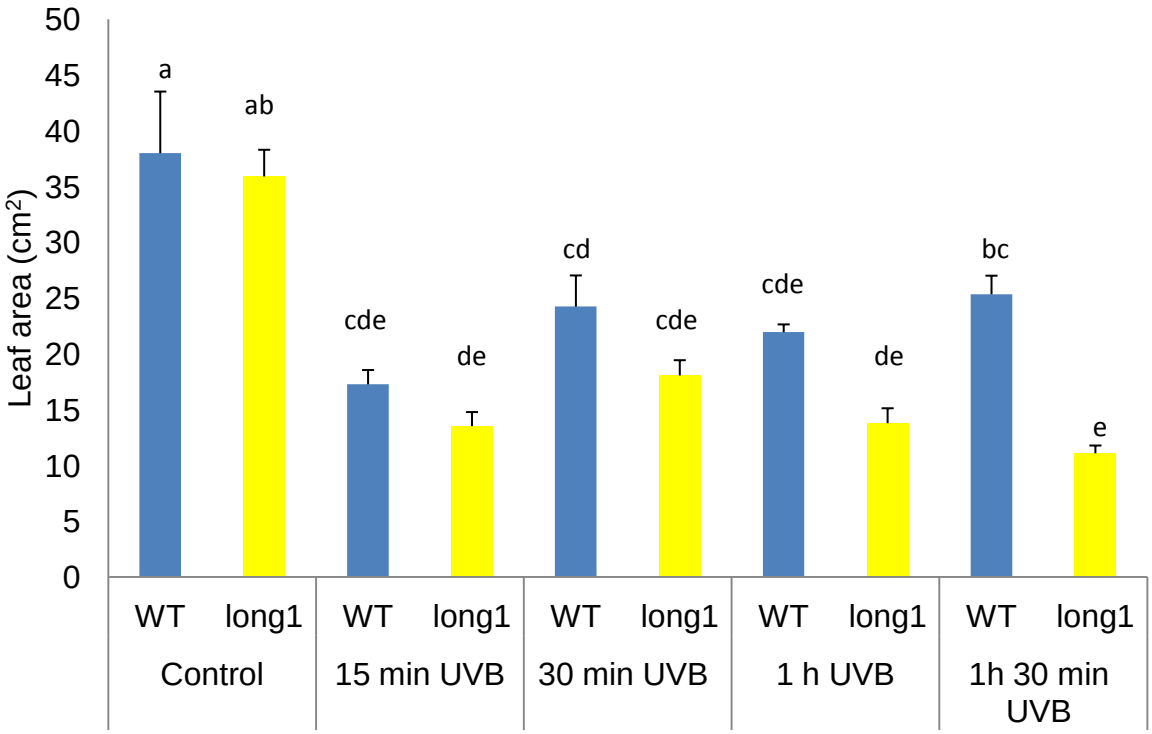


Figure 20. Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on leaf area in the WT and the *long1* mutant in pea. Results are mean of 5 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

As stated above, the leaf area in WT was significant smaller after the different treatments with UV-B radiation compared to WT-control. In the *le* mutant the leaf area was not significantly different between the control and any of the different daily treatments with UV-B radiation (0.35 W m^{-2}) lasting from 30 min up to 6 h (Figure 21).

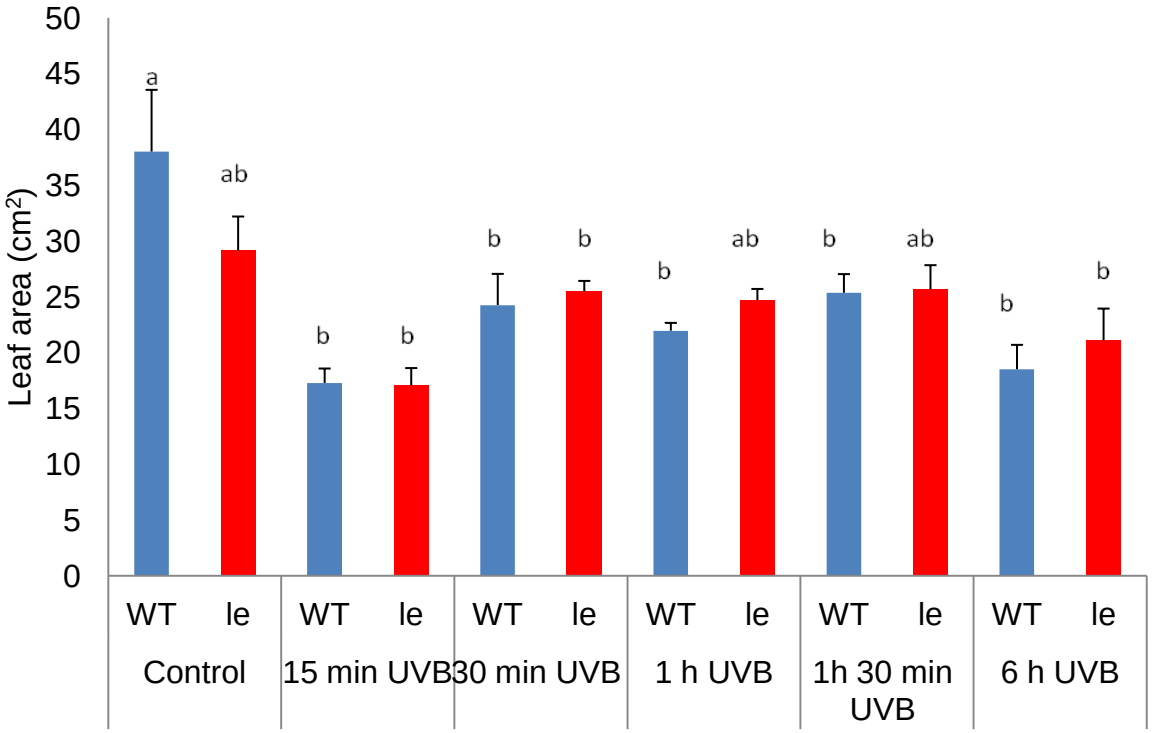


Figure 21. Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on leaf area in the WT and the *le* mutant in pea. Results are mean of 5 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation on dry weight

In the WT, there were no significant differences in total DW after the different treatments with UV-B radiation. In the *long1* mutant significant differences were measured in total DW between the control treatments and the treatments with 30 min or longer UV-B radiation. After the treatment with 30 min UV-B radiation the decrease in total DW was 33% compared with the *long1*-control. The decrease was 27% after the treatment with 1 h UV-B radiation and 37% after the treatment with 1 h 30 min UV-B radiation compared with *long1*-control (Figure 22).

The ratio between DW of the stem and DW of the leaves was quite stable regardless of the treatment. For the WT the ratio fluctuated between 0.6-0.7 for the DW of the stem/ total DW and 0.3- 0.4 for the DW of the leaves/ total DW. In *long1* a larger amount of the DW was in the stem. Here the ratio DW of the stem/ total DW fluctuated between 0.7- 0.8 and the ratio DW of the leaves/ total DW varied between 0.2- 0.3.

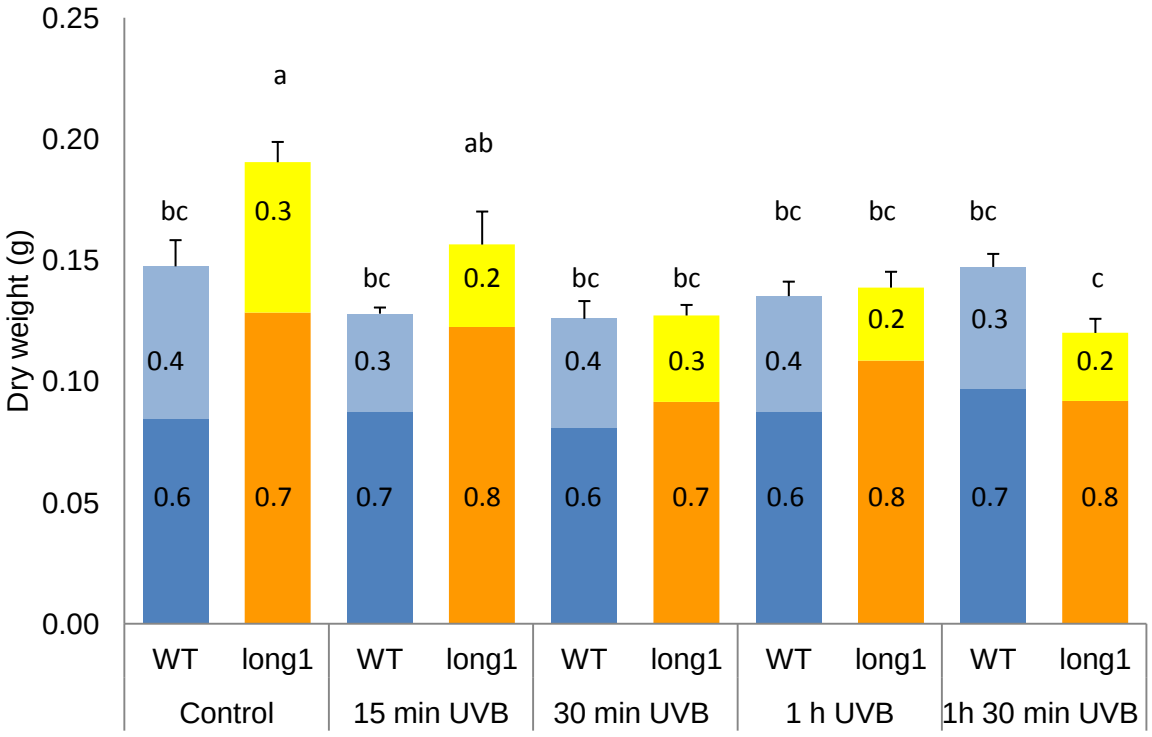


Figure 22. Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on total dry weight in the wild type (WT) and the *long1* mutant in pea. The upper and lower part of the bar shows the dry weight of the leaves and stem, respectively. The numbers in the bars are the ratio of the dry weight of the leaves or the stem compared to the total dry weight (=1). Results are mean of 5 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

There were no significant differences in total DW after the different treatments with UV-B radiation in neither the WT nor the *le* mutant (Figure 23).

In both genotypes the ratio between DW of the stem and DW of the leaves compared to total DW was stable regardless of the treatment. For the WT the ratio was 0.6-0.7 for the DW of the stem/ total DW and 0.3-0.4 for the DW of the leaves/ total DW. In *le* the ratio between DW of the stem and DW of the leaves compared to total DW was on average about 0.5 for the DW of the stem/ total as well as for the DW of the leaves/ total DW.

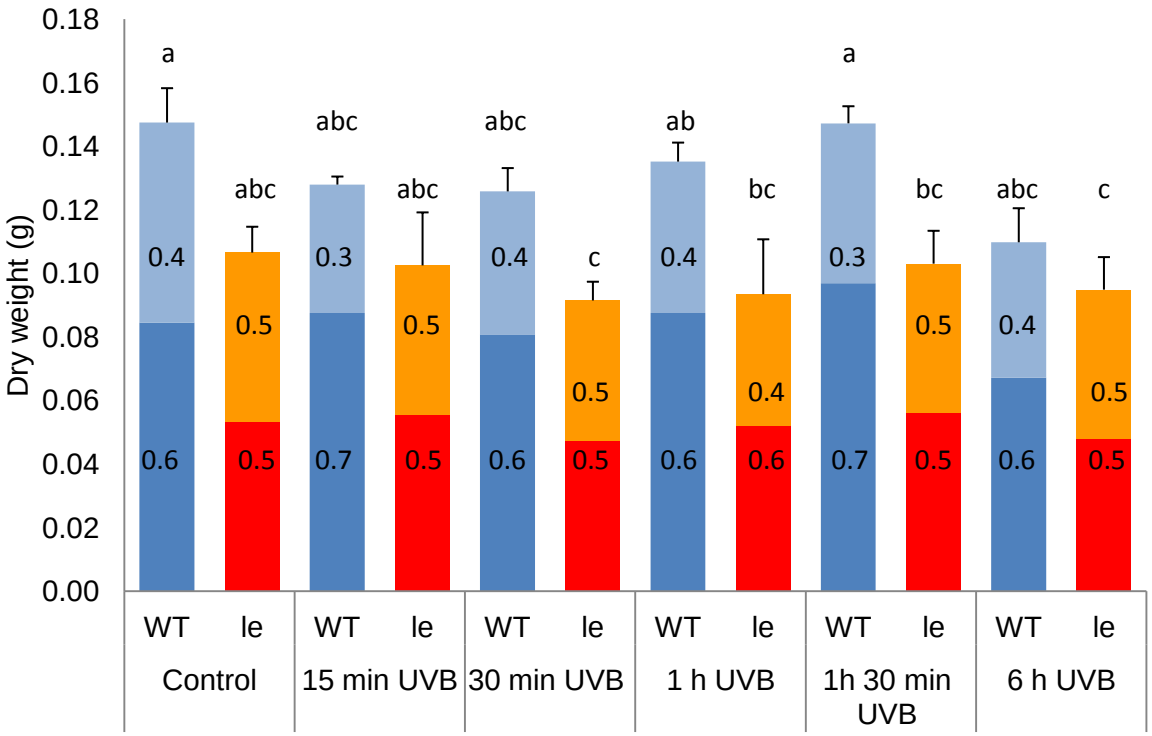


Figure 23: Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on dry weight in the wild type (WT) and the *le* mutant in pea. The upper part of the bar is the dry weight of the leaves, the lower part is dry weight of the stem. The numbers in the bars are the ratio of the dry weight of the leaves or the stem compared to the total dry weight (=1). Results are mean of 5 plants in 1 experiment and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation on shoot elongation

The growth curve for the treatment with 15 min UV-B was adjusted after the experiment. The reason for this was because we discovered a temperature difference of +1˚ C in this chamber compared to the other chambers and thus more elongation growth. The data from day 0 were used to calculate the estimated growth curve for this treatment. On day 0 the plants in the control chamber were only 86% of the height of the plants in the chamber with a temperature difference of +1˚ C. Therefore the measured data from this chamber were multiplied by 0.86, such that the values for day 0 were the same as those from the control chamber. This was done for all days.

In WT, there was only a significant decrease in height (25%) of the plants after the treatment with UV-B radiation for 6 h compared to the control (Figure 24). Significant changes in shoot elongation were neither measured in the *long1* mutant (Figure 25), nor in the *le* mutant (Figure 26).

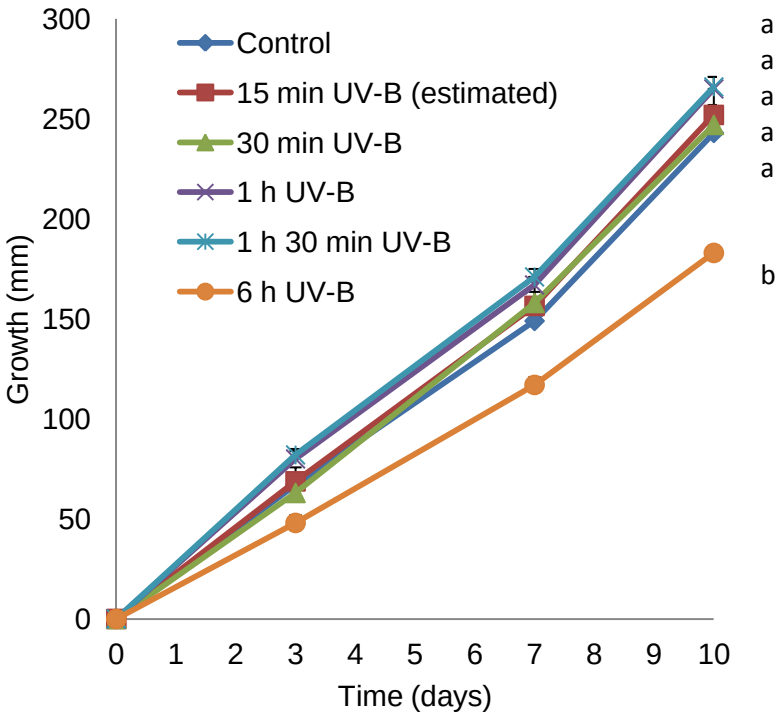


Figure 24. Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on shoot elongation in wild type in pea. Results are mean of 10 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

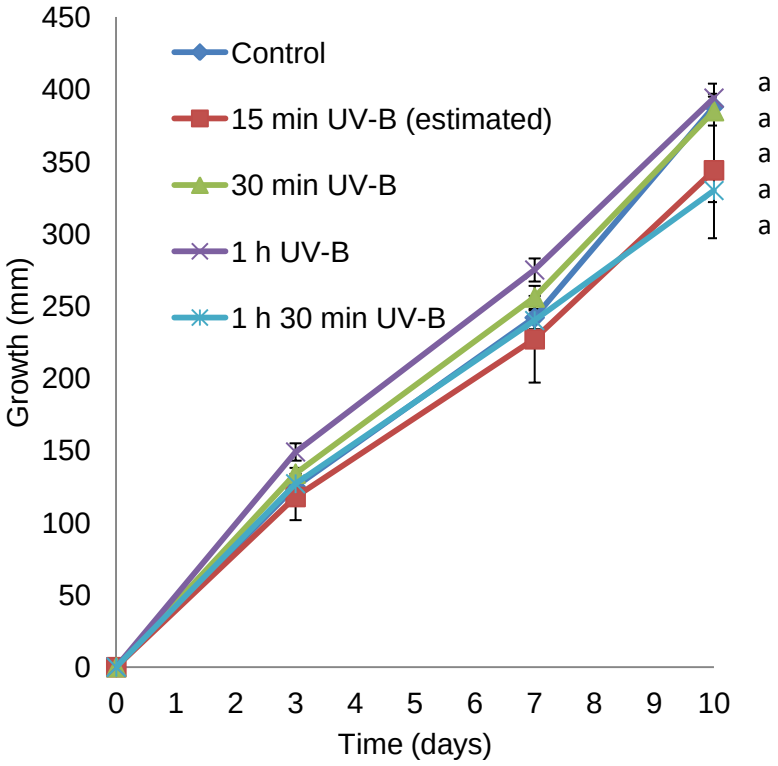


Figure 25: Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on shoot elongation in the *long1* mutant in pea. Results are mean of 10 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p≤ 0.05).

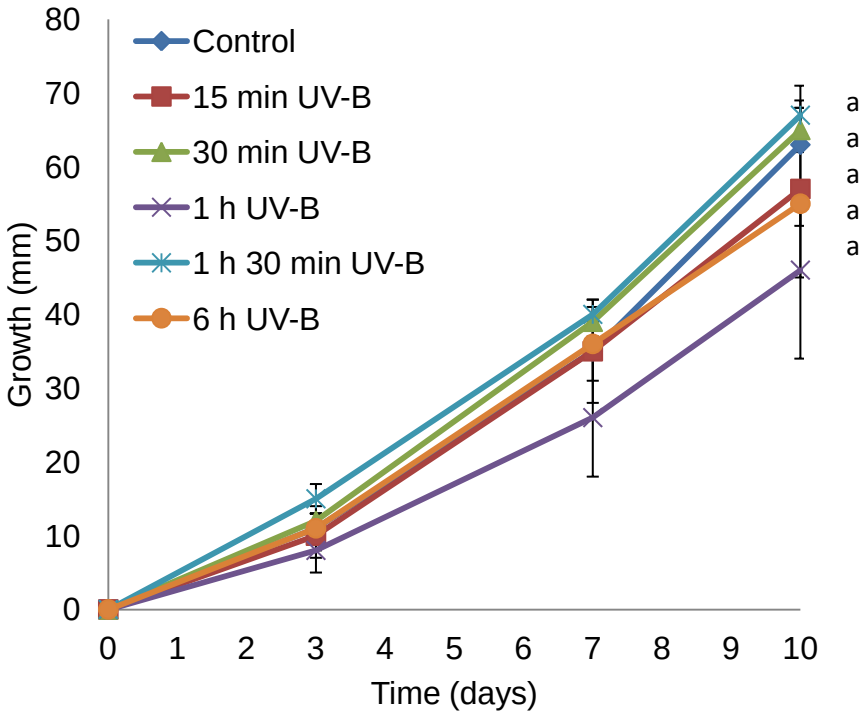


Figure 26: Effect of UV-B radiation (0.35 W m^{-2}) for different durations in the middle of a 12 h photoperiod of 100 µmol m^{-2} s^{-1} on shoot elongation in the *le* mutant in pea. Results are mean of 5 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p≤ 0.05).

Results Experiment WT and *lip1*

Visible effects of UV-B radiation and temperature drop

To shed light on the role of *LIP1* in response to UV-B exposure, the *lip1* mutant and the WT were exposed to daily UV-B radiation. WT treated with 6 h UV-B radiation of 0.50 W m^{-2} showed some damage with curled leaf edges. This damage was more severe in the treatment without 6 h temperature drop (Figure 27, table 8). Thus, temperature drop treatment reduced the leaf curling induced by UV-B in the WT. No visible damage was registered in the *lip1* mutant after the different treatments (Figure 28, table 8).

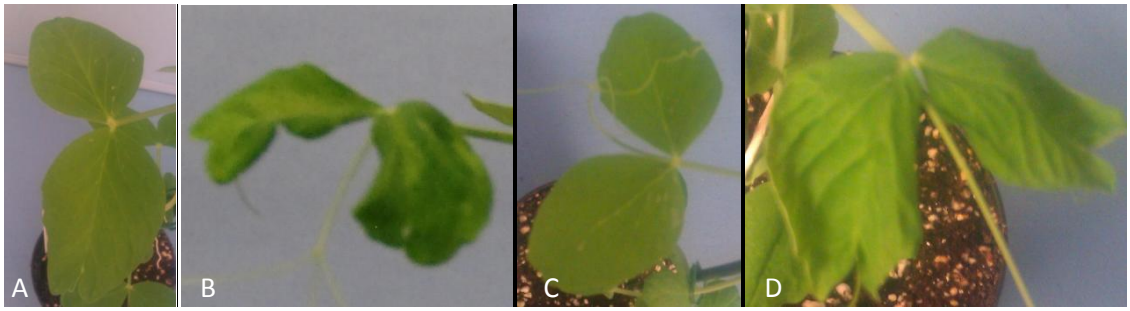


Figure 27. Leaves of WT in pea plants grown for 10 days with 4 different treatments, (A) control, (B) UV-B radiation (0.50 W m^{-2}) for 6 h, (C) 6 h temperature drop (from 21 to 13 °C), and (D) UV-B radiation (0.50 W m^{-2}) for 6 h combined with a 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

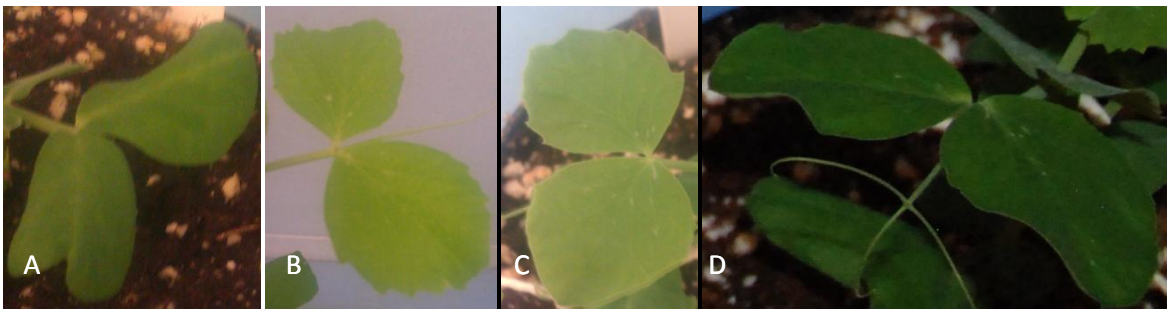


Figure 28. Leaves of the *lip1* mutant in pea grown for 10 days with 4 different treatments, (A) control, (B) UV-B radiation (0.50 W m^{-2}) for 6 h, (C) temperature drop (from 21 to 13 °C) and (D) UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

Table 8. Classification of visible damage on WT and in the *lip1* mutant grown for 10 days with 4 different treatments. Damage is classified as (0) No visible damage (1) Little damage with curled leaf edges (2) More severe damage with curled leaf edges and yellow, chlorotic spots (3) Severe damage with very curled leaf edges, brown necrotic spots, yellow stem/ dead plants.

	WT	***lip1***
Control	0	0
6 h UV-B radiation ($0.50\ W\ m^{-2}$)	2	0
6 h UV-B radiation ($0.50\ W\ m^{-2}$) combined with 6 h temperature drop	1	0
6 h temperature drop	0	0

Effect of UV-B radiation and temperature drop on leaf area

The leaf area of the WT plants was in all cases larger compared with that of the *lip1* mutant. After the treatment with 6 h UV-B radiation at 0.50 W m^{-2} the WT showed a significant reduction of 49% in leaf area compared to the WT-control. A significant reduction of 52% in leaf area was also measured when 6 h UV-B radiation was given together with a 6 h temperature drop. There was no significant difference in leaf area after the treatment with only 6 h temperature drop compared to the control treatment. In the *lip1* mutant, there were no significant differences in leaf area after the exposure to UV-B radiation only or with 6 h temperature drop only compared to the control treatment. However, when 6 h UV-B radiation was given together with a 6 h temperature drop a significant reduction in leaf area by 38% was measured compared to the control (Figure 29).

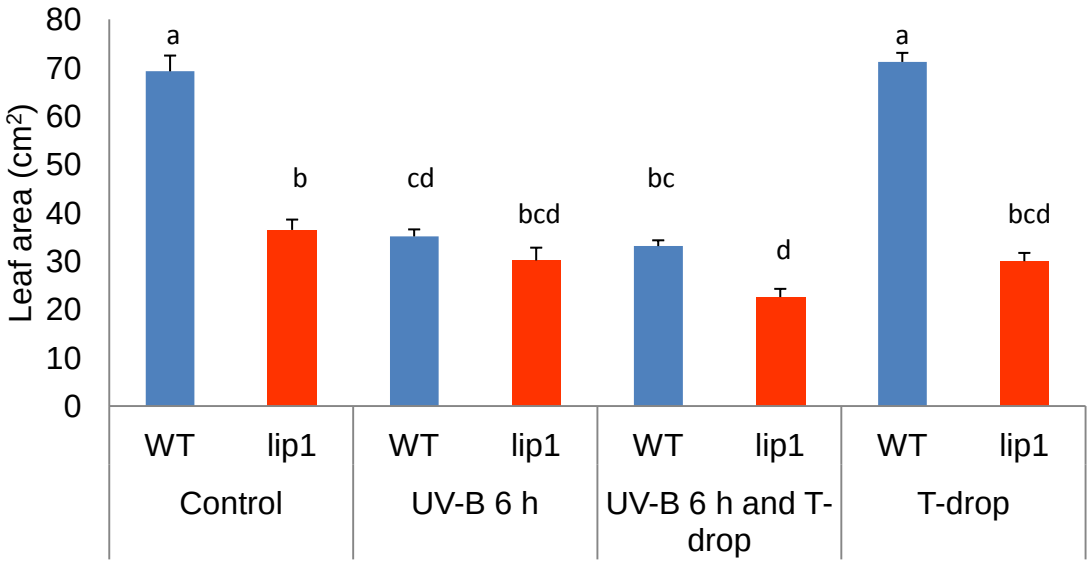


Figure 29. Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on leaf area in the wild type (WT) and the *lip1* mutant in pea. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 15 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation and temperature drop on dry weight

Comparing the total DW of WT plants and *lip1* mutants within the same treatment gave in all cases a larger total DW for the WT plants. The total DW of both genotypes did not differ between the control treatment and after a 6 h temperature drop. In the WT there was a significant reduction in DW after the treatment with 6 h UV-B radiation (34% reduction compared to WT-control) and less reduction in DW after the treatment with both 6 h UV-B radiation and 6 h temperature drop (23% reduction compared to WT-control). There were no significant differences in DW after the different treatments in *lip1* mutant compared to the control (Figure 30).

The ratio between DW of the stem and DW of the leaves compared to total DW was quite stable in both genotypes, regardless of the treatment. For the WT the ratio was 0.6 for the DW of the stem/ total DW and 0.4 for the DW of the leaves/ total DW. In *lip1* the ratio between DW of the stem and DW of the leaves compared to total DW was on average about 0.4 for the DW of the stem/ total DW and 0.6 for the DW of the leaves/ total DW.

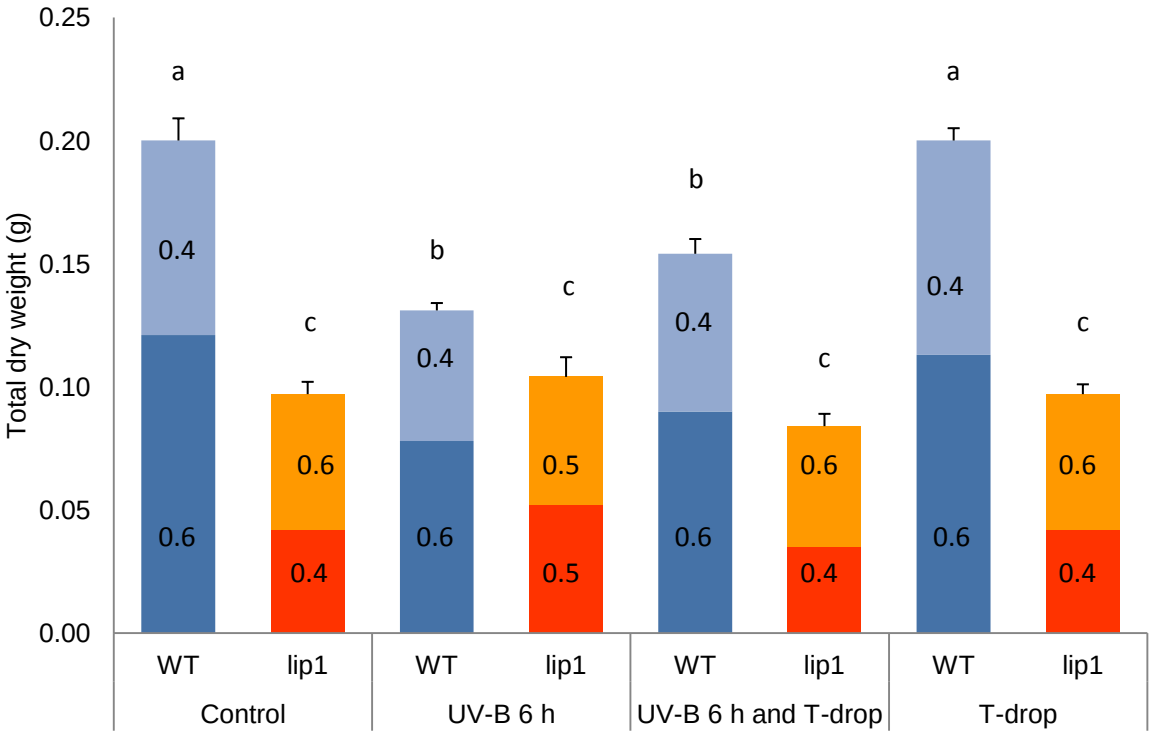


Figure 30. Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on total dry weight (in gram) in WT and *lip1* mutant. The upper and lower part of the bar shows the dry weight of the leaves and stem, respectively. The numbers in the bars are the ratios of the dry weight of the leaves or the stem compared to the total dry weight (gives 1 in total). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 15 plants and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation and temperature drop on shoot elongation

To investigate the effect of UV-B radiation and temperature on shoot elongation the height of the plants was measured at four different times.

After 10 days of different treatments the differences in in height in WT plants of pea were obvious. The plants treated with UV-B radiation were shorter than the other plants (Figures 31 and 32). The highest plants were measured in the control treatment, and then in order: 6 h temperature drop, UV-B radiation for 6 h, and 6 h UV-B radiation combined with 6 h temperature drop, with significant reduction of 15%, 36% and 54%, respectively, compared to the control.

Figure 31. Plants of the wild type (WT) in pea grown for 10 days with 4 different treatments, from left to right: control, UV-B radiation (0.50 W m^{-2}) for 6 h, 6 h temperature drop (from 21 to 13 °C), and UV-B radiation (0.50 W m^{-2}) for 6 h combined with a 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

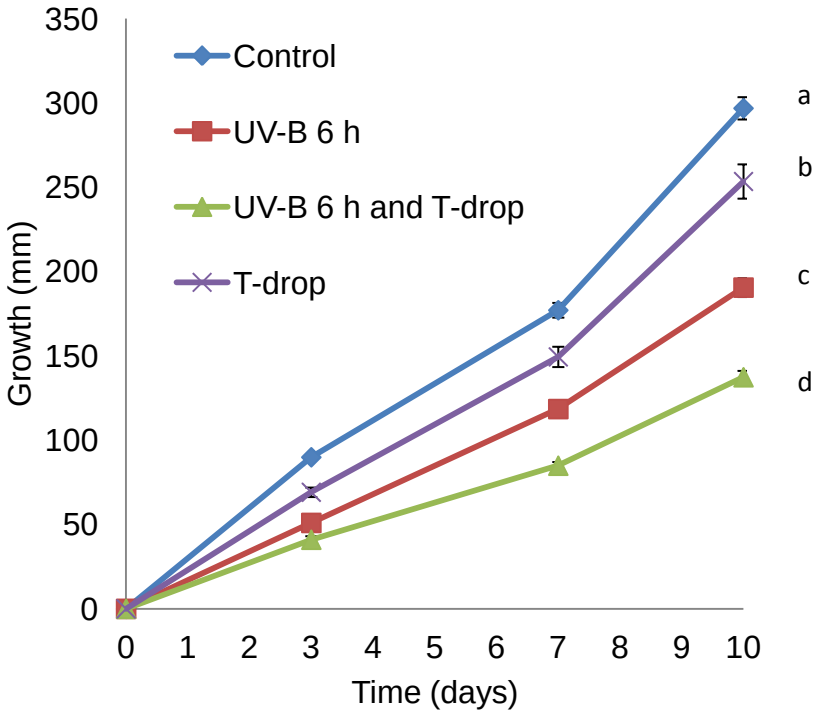


Figure 32. Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (T-drop; from 21 to 13 °C) on shoot elongation in the wild type of pea. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 15 plants in each of 2 replicate experiments and ±SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

The *lip1* dwarf mutant plants grown exposed to 6 h UV-B radiation or only to a 6 h temperature drop showed no significant differences in height compared to the *lip1*-control treatment. However, the treatment with UV-B radiation for 6 h as well as a 6 h temperature drop showed a significant height reduction of 49% compared to the control (Figures 33 and 34).

Figure 33. Plants of the *lip1* mutant in pea grown for 10 days with 4 different treatments, from left to right: control, UV-B radiation (0.50 W m^{-2}) for 6 h, temperature drop (from 21 to 13 °C), and UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

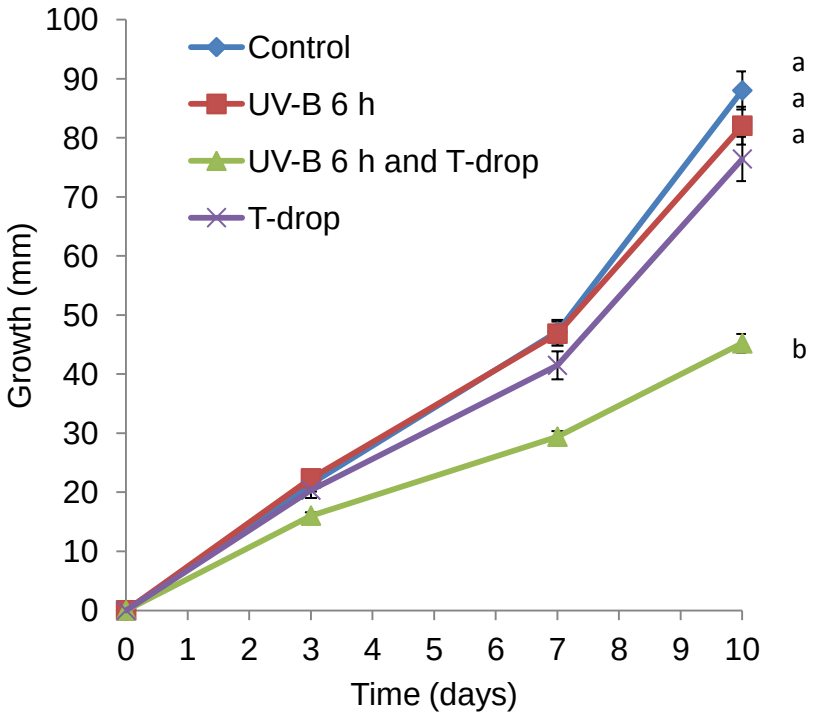


Figure 34: Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (T-drop; from 21 to 13 °C) on shoot elongation in *lip1*. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 15 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation and temperature drop on the content of phenolic compounds

To investigate the effect of the UV-B and temperature drop treatments on the content of known UV-B-protecting phenolic compounds, these were analysed by HPLC. Eighteen different such phenolic compounds were detected (Figure 35, table 9). Following the research of Hertog (1994), our focus was on 5 groups; luteolins, apigenins, quercetins, kaempferols and myrcetins, which all occurred in their glycosylated forms. To simplify the terminology we describe the phenolic compounds as e.g. apigenin instead of their more correct name apigenin-glycoside. The different glycosides of a specific flavonoid were grouped.

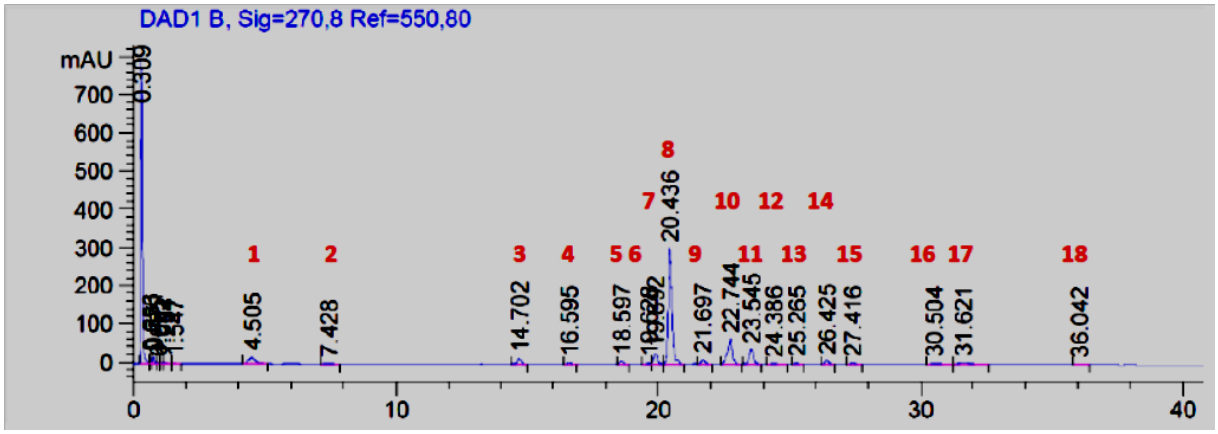


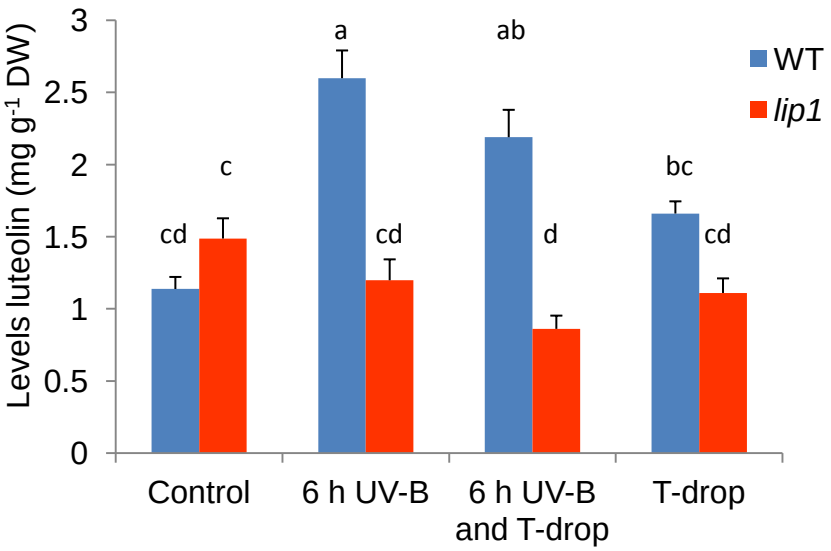
Figure 35: Chromatogram showing different chromatographic peaks of different phenolic compounds in pea from the treatment with 6 h UV-B radiation and 6 h temperature drop in the *lip1* mutant.

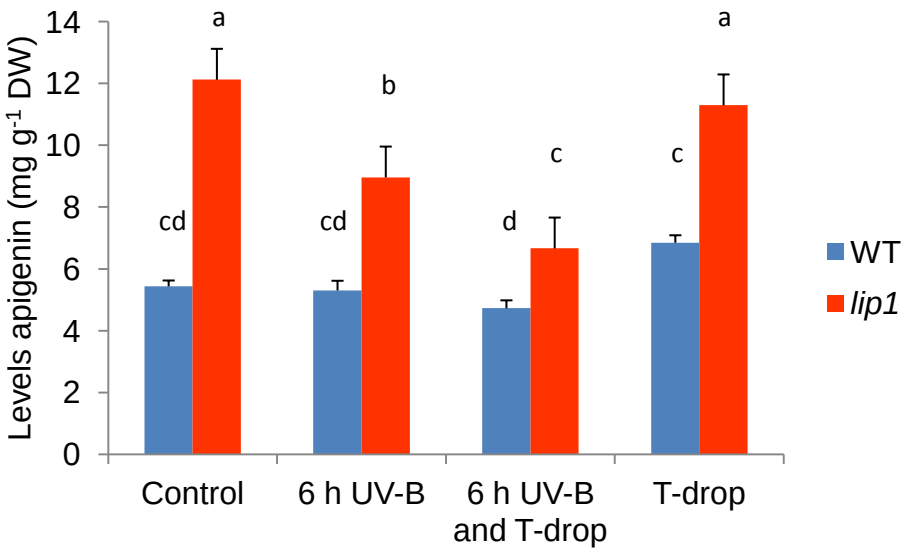
Table 9: The detected phenolic compounds in pea from the chromatogram in figure 35.

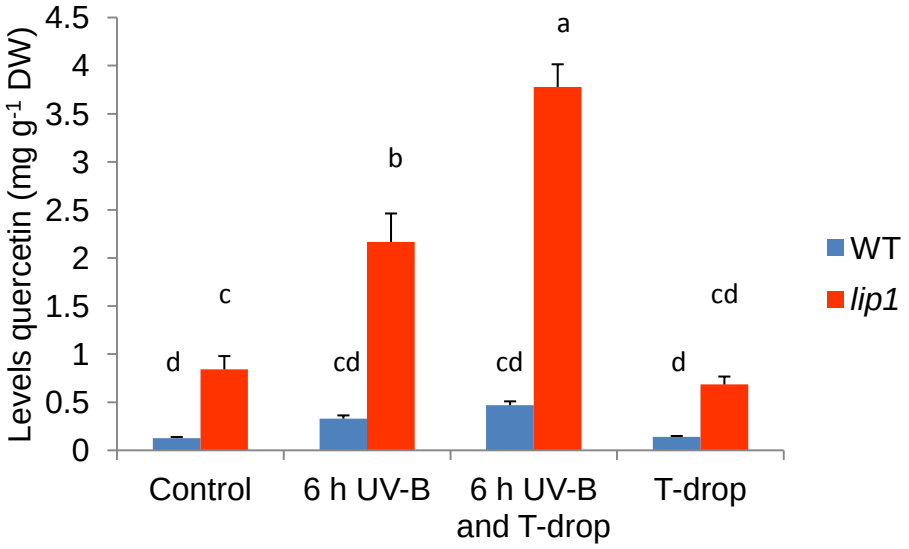
Peak number	Detected compound
1	Tryptophan
2	Unknown
3	Quercetin-glycoside
4	Kaempferol-glycoside
5	Luteolin-glycoside
6	Luteolin-glycoside
7	Luteolin-7- glycoside
8	Apigenin-7-glycoside
9	Luteolin-7-glycoside
10	Apigenin-7-glycoside
11	Kaempferol-glycoside
12	Myricetin-glycoside
13	Myricetin-glycoside
14	Apegenin-glycoside
15	Unknown
16	Phenolic acid
17	Phenolic acid
18	Phenolic acid

Compared to the control treatment the levels of luteolins in the WT were significantly higher after the UV-B treatments, with or without a temperature drop. By contrast, in *lip1* the levels of luteolins were significantly reduced by 6 h UV-B radiation combined with a 6 h temperature drop, as compared to the control. The levels of apigenins in the WT were not affected by the treatments, while the levels in *lip1* were significantly lowered under UV-B irrespective of temperature regime. In the WT none of the treatments resulted in significantly different levels of quercetin compared to the control. On the other hand, in *lip1* the levels of quercetins were significantly higher after the UV-B treatments, with or without a temperature drop, compared to the control. The levels of kaempferols in the WT as well as the *lip1 mutant* were significantly higher after the UV-B treatments under both temperature regimes, compared to their respective controls. In the WT and *lip1* the levels of myricetins were significantly higher after the UV-B treatments irrespective of temperature regime as compared to their controls (Figure 36). Comparing the content of phenolic compounds, WT had significantly higher levels of luteolins and myricetins (although not in the control treatments) then *lip1*, while *lip1* had significantly higher levels of apigenins, quercetins and kaempferols than the WT

According to figure 37 there were no significant differences in the total contents of phenolic compounds in WT and *lip1* after the different treatments.




Levels apigenin (mg g-1 DW)
14
12
10
8
6
4
2
0
a
b
c
c
cd
cd
d
a
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
lip1


Levels quercetin (mg g-1 DW)
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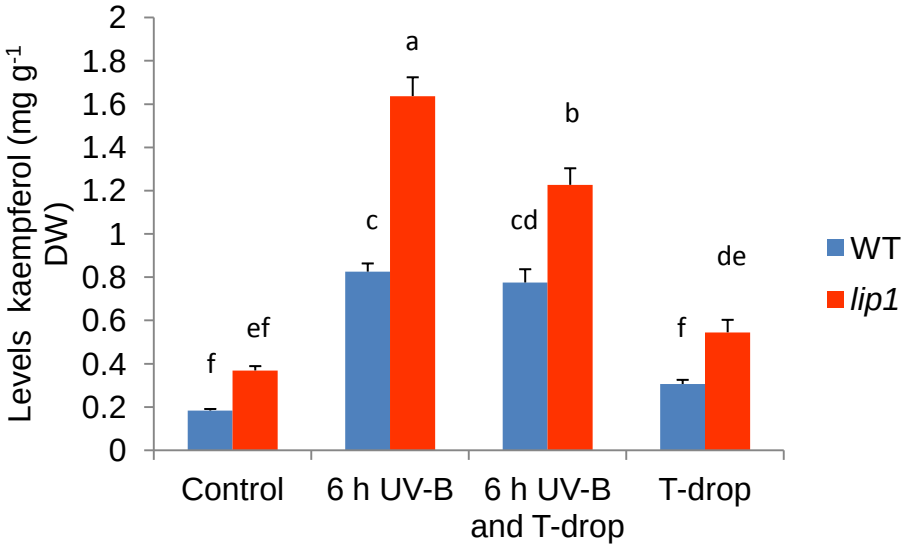
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
cd
d
cd
cd
d
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
lip1


Levels kaempferol (mg g-1 DW)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
b
c
cd
de
ef
f
f
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
lip1

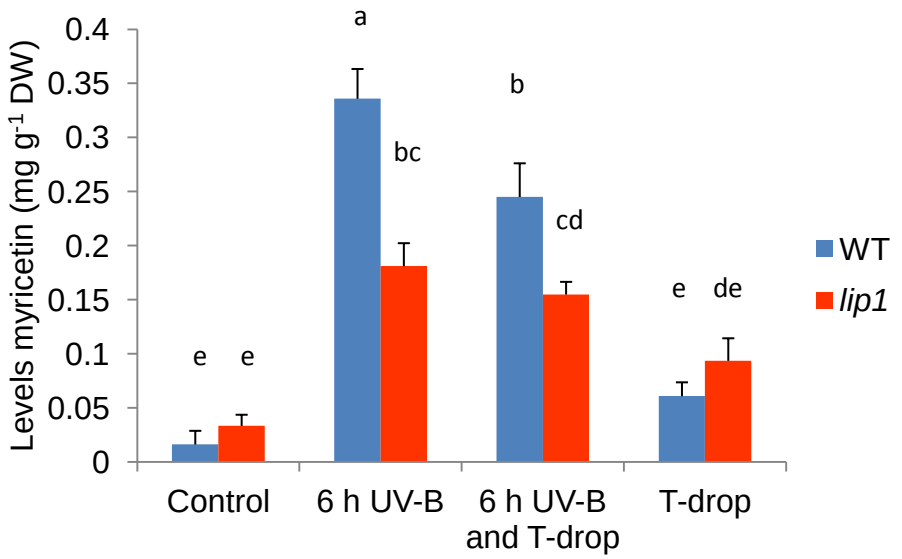


Figure 36: Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on the levels of phenolic compounds (in mg g^{-1} DW) in the third leaf pair from the soil in WT and *lip1* in peas as measured by HPLC. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

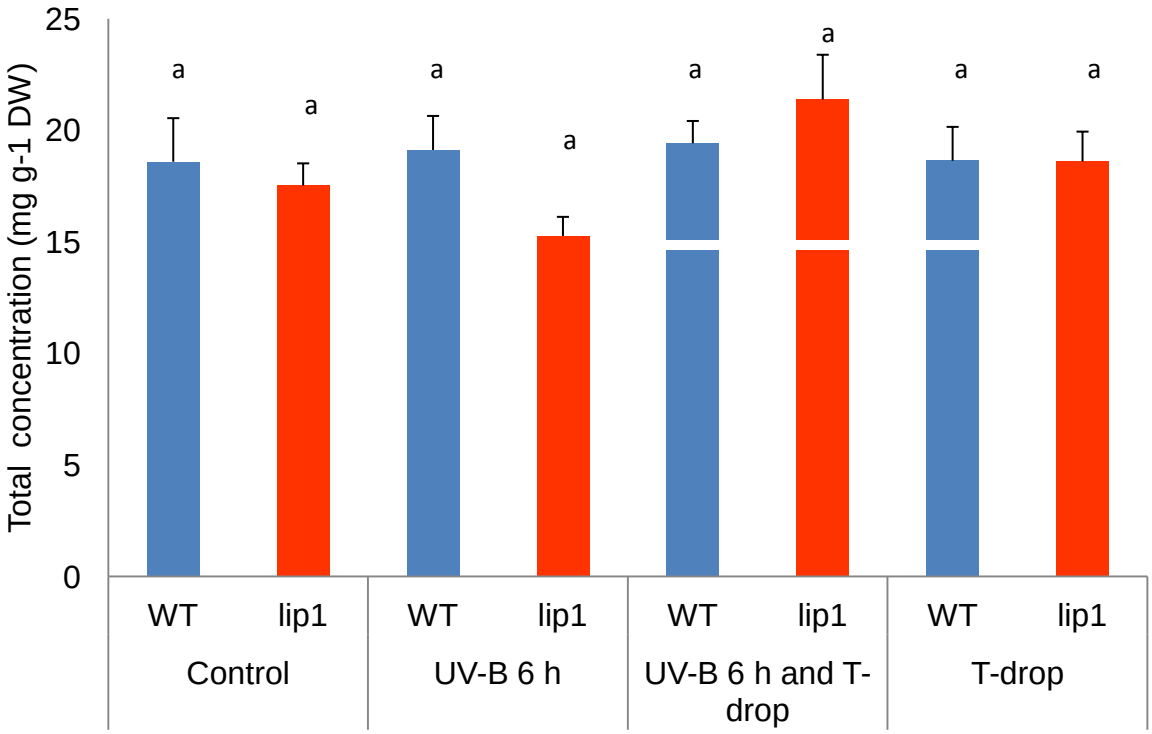


Figure 37. Effect of UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on the the total levels of all flavonoids, tryptophan and phenolic acids (in mg g^{-1} dry weight) in the third leaf pair from the soil in WT and *lip1* in peas as measured by HPLC. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effect of UV-B radiation and temperature drop on the absorbance of UV-A

To investigate the presence of UV screening components in epidermis in the WT and *lip1*, a Multiplex instrument was used to measure UV-A absorbance in leaf surfaces. In both genotypes no difference was measured between the control-treatments and the treatments with temperature drop (Figure 38). WT showed a significant increase in UV-A absorbance after the treatment with UV-B radiation for 6 h (48 %) or when combined with a temperature drop (28 %) as compared to the WT-control. Similarly, *lip1* also showed a significant increase in UV-A absorbance under UV-B radiation for 6 h (16 %) and the combined treatment with UV-B radiation and temperature drop (20%). Furthermore, in *lip1* no significant differences were measured between the two UV-B radiation treatments indicating that the amount of phenols in epidermis is only dependent on the UV-B radiation and that a temperature drop has no influence.

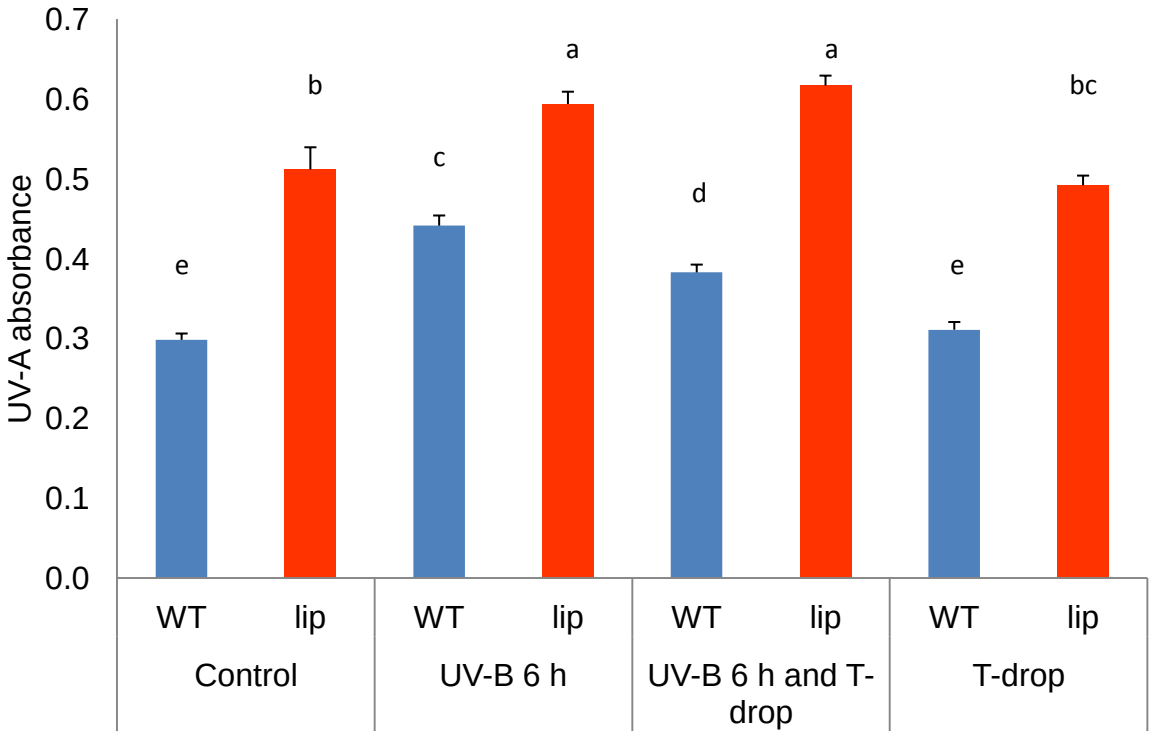


Figure 38. Effect of daily UV-B radiation (0.50 W m^{-2}) for 6 h and temperature drop (from 21 to 13° C) on UV-A absorbance in leaves of WT and the *lip1* mutant of pea as measured by a Multiplex instrument. The control and plants exposed to UV-B only were grown at 20° C (diurnal average of the temperature drop treatments). Results are mean of 3 plants in 1 experiment and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Results Experiment WT, *long1* and *le*

Visible effects of UV-B radiation and temperature drop

To investigate the effects of UV-B radiation combined with a temperature drop in the WT, the *long1* and the GA biosynthesis (*GA3ox*) mutant *le*, these genotypes mutants were exposed daily to 0.35 W m^{-2} UV-B radiation combined with a temperature drop treatment. The only visible damage in the WT, like curled leaf edges, was observed after the treatment with 6 h UV-B radiation at 0.35 W m^{-2}. However, when 6 h UV-B treatment was combined with a 6 h simultaneous temperature drop from 21 to 13 °C, no damage was observed, indicating a temperature modulation of the response to UV-B radiation (Figure 39, table 10).

There was some visible damage in *long1* after treatment with 30 min UV-B radiation. Here the leaf edges were curled and some yellow, chlorotic spots were apparent. However, when 30 min UV-B treatment was combined within a 6 h T-drop from 21 to 13° C, less damage was observed (Figure 40, table 10). The *le* mutant appeared very resistant to UV-B radiation, no visible damage on the leaves of *le* was observed after the different UV-B radiation treatments (Figure 41, table 10).

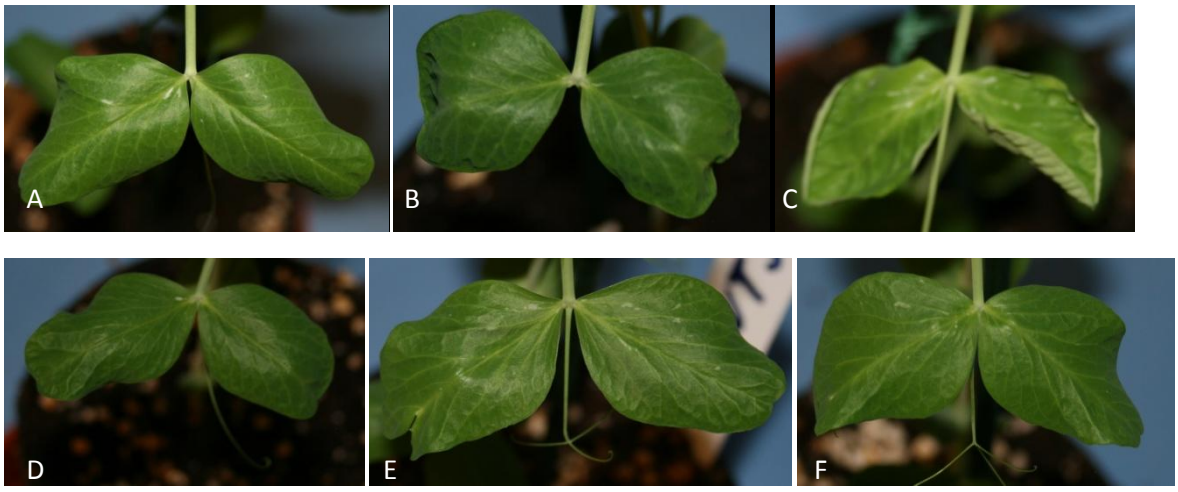


Figure 39. Leaves of wild type in pea plants grown for 10 days with 3 different treatments, (A) control, (B) UV-B radiation (0.35 W m^{-2}) for 30 min, (C) UV-B radiation (0.35 W m^{-2}) for 6 h, (D) UV-B radiation (0.35 W m^{-2}) for 30 min and a 6 h temperature drop period (from 21 to 13 °C) (E) UV-B radiation (0.35 W m^{-2}) for 6 h temperature drop (from 21 to 13 °C) and (F) only temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

Figure 40. Leaves of *long1* mutant in pea plants grown for 10 days with 4 different treatments, (A) control, (B) UV-B radiation (0.35 W m^{-2}) for 30 min, (C) UV-B radiation (0.35 W m^{-2}) for 30 min combined with a 6 h temperature drop (from 21 to 13 °C) and (D) only 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the temperature drop treatments).

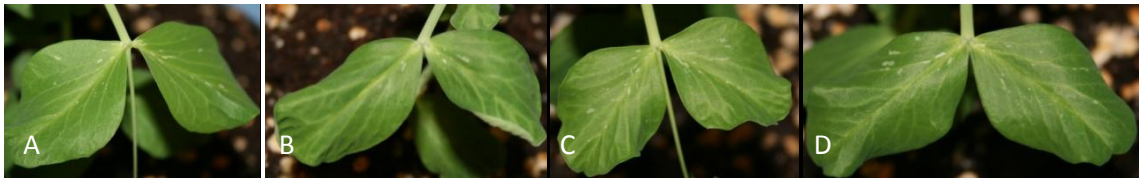


Figure 41. Leaves of the *le* mutant in pea grown for 10 days with 4 different treatments, (A) control, (B) UV-B radiation (0.35 W m^{-2}) for 6 h, (C) UV-B radiation (0.35 W m^{-2}) for 6 h combined with a a 6 h temperature drop (from 21 to 13 °C) and (D) only 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments).

Table 10. Classification of visible damage on WT, the *long1* and *le* mutant grown for 10 days with 4 different treatments. Damage is classified as (0) No visible damage (1) Little damage with curled leaf edges (2) More severe damage with curled leaf edges and yellow, chlorotic spots (3) Severe damage with very curled leaf edges, brown necrotic spots, yellow stem/ dead plants.

	WT	***long1***	***le***
Control	0	0	0
30 min UV-B radiation (0.35 W m^{-2})	0	1	-
6 h UV-B radiation (0.35 W m^{-2})	1	-	0
30 min UV-B radiation (0.35 W m^{-2}) combined with 6 h temperature drop	0	1	-
6 h UV-B radiation (0.35 W m^{-2}) combined with 6 h temperature drop	0	-	0
6 h temperature drop	0	0	0

Effects of UV-B radiation and temperature drop on leaf area

Figure 42 shows that both WT and *long1* mutant gave a similar trend for the effects of 30 min UV-B radiation and temperature drop on leaf area. The treatment with a 6 h temperature drop did not give a significant difference compared to the control treatment, both in WT and in *long1* mutant. There was a significant decrease in leaf area after the treatment with 30 min UV-B radiation (55% for the WT and 60% for the *long1* mutant compared to their controls). When a 6 h temperature drop was given together with 30 min UV-B radiation, there was also a significant decrease in leaf area, but this was less than after exposure to UV-B radiation only (29 % for WT and 44% for *long1* compared to their controls).

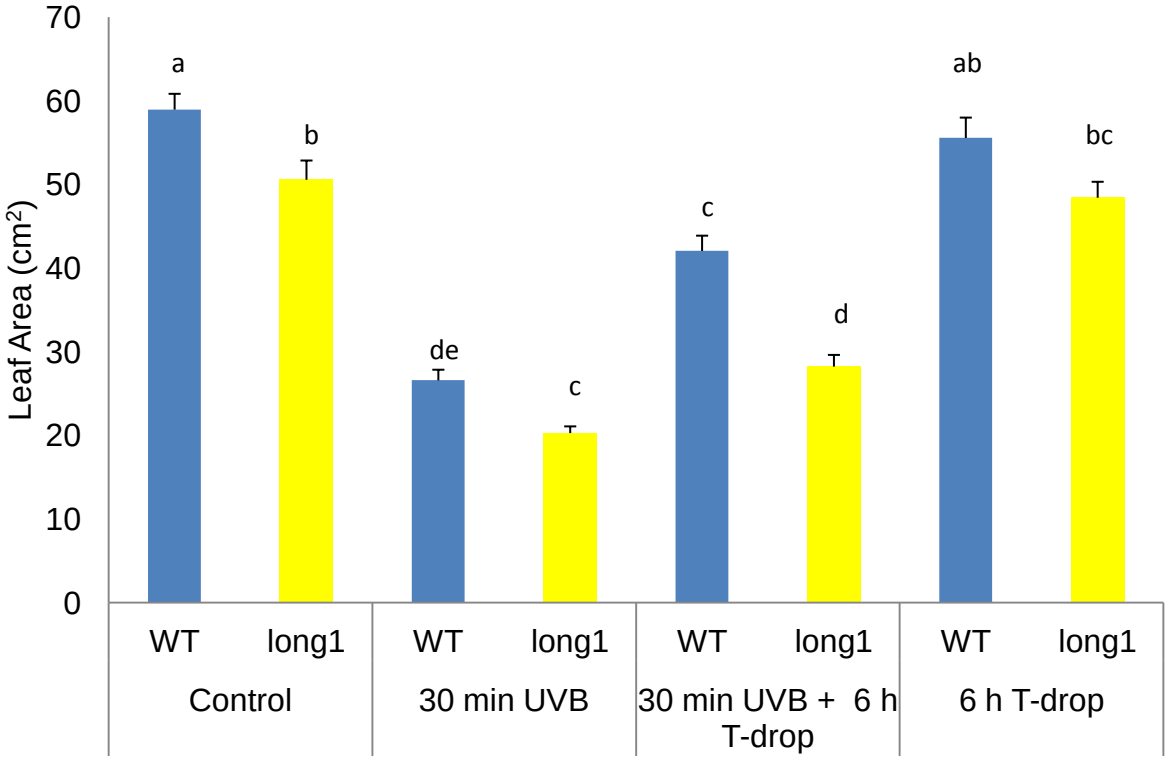


Figure 42. Effect of UV-B radiation (0.35 W m^{-2}) for 30 min and a 6 h temperature drop (from 21 to 13 °C) on leaf area in the wild type (WT) and the *long1* mutant in pea. The control and plants exposed to UV-B radiation only were grown at 20° C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

In the WT significant decreases in leaf area were measured. After the treatment with 6 h UV-B radiation this was 56%, but when 6 h UV-B radiation was given together with a temperature drop this decrease was less, 32 % (both compared to WT-control). There was no significant difference between the temperature drop treatment and the control. Although the trend was similar in the *le* mutant, there were no significant differences between the different treatments (Figure 43).

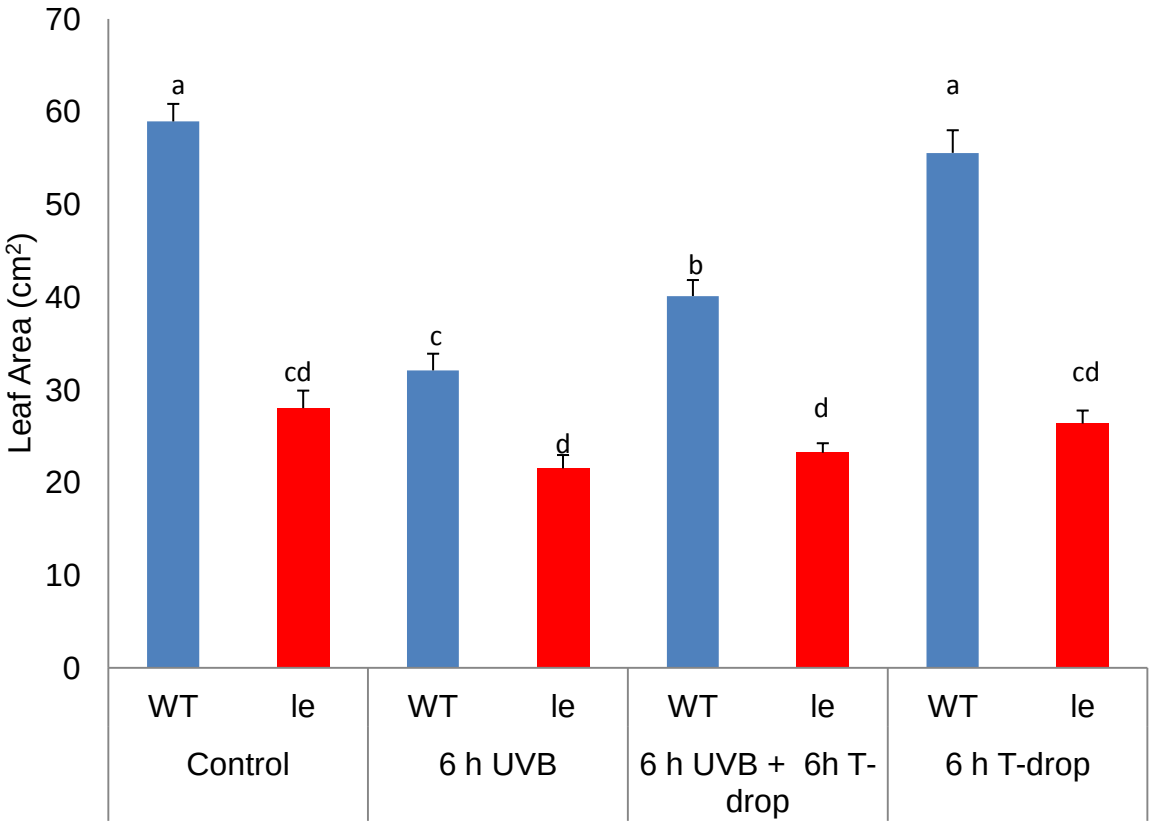


Figure 43. Effect of UV-B radiation (0.35 W m^{-2}) for 6 hand a 6 h temperature drop (from 21 to 13 °C) on leaf area in the wild type (WT) and the *le* mutant in pea. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effects of UV-B radiation and temperature drop on dry weight

The total DW of both genotypes did not differ between the control treatment and the 6 h temperature drop treatment. This indicated that the temperature drop had no effect on the DW of the plants in this experiment. The WT did not show a significant reduction in DW after the treatment with 30 min UV-B radiation, but there was a significant reduction of 22% after the treatment with both 30 min UV-B radiation and temperature drop (compared to control). The *long1* mutant showed a similar trend as the WT; but here the reduction in DW was significant both after the treatment with 30 min UV-B radiation and after the treatment with 30 min UV-B radiation and temperature drop (18% and 21% compared to the *long1*-control, respectively) (Figure 44). For WT, the ratio between DW of the stem and DW of the leaves compared to total DW was quite stable. The average was 0.6 for the DW of the stem/ total DW and 0.4 for the DW of the leaves/ total DW. In *long1* the ratio between DW of the stem and DW of the leaves compared to total DW is in average about 0.7 for the DW of the stem/ total DW and 0.3 for the DW of the leaves/ total DW. The treatment with only UV-B radiation gave a ratio of 0.8 for the DW of the stem/total DW and 0.2 for the DW of the leaves/ total DW.

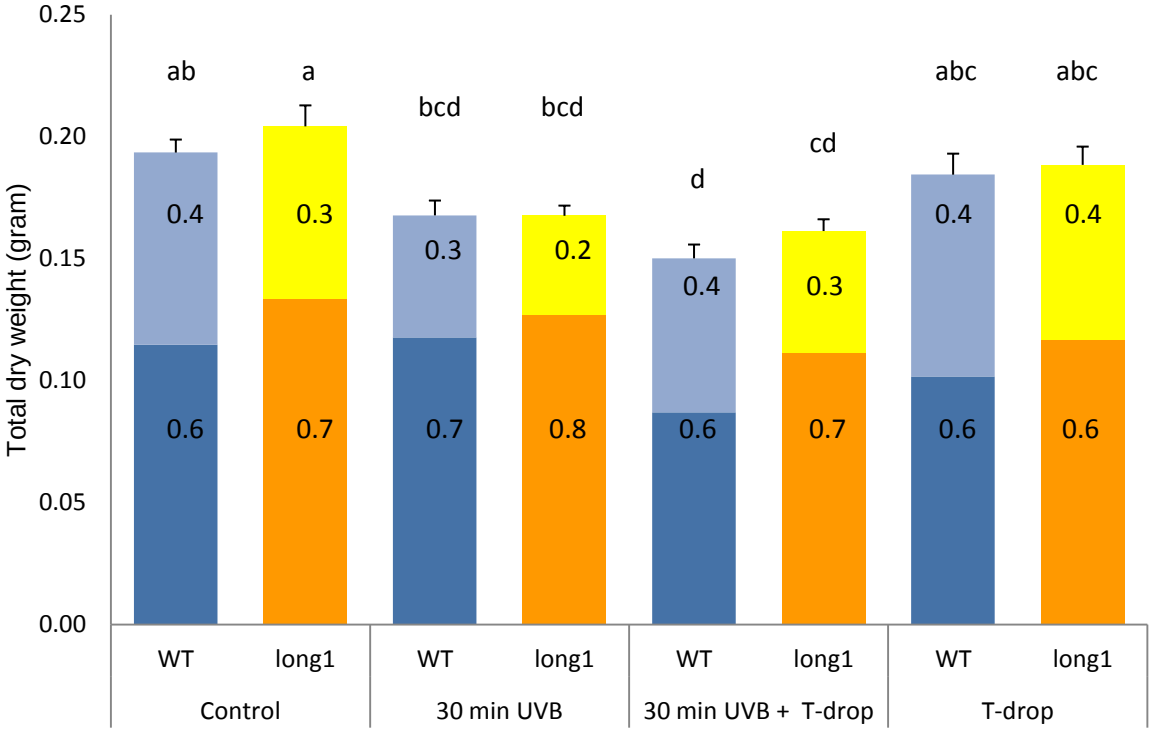


Figure 44. Effect of UV-B radiation (0.35 W m^{-2}) for 30 min and a 6 h temperature drop (from 21 to 13 °C) on total dry weight (in gram) in the wild type (WT) and the *long1 mutant* of pea. The upper and lower part of the bar shows the dry weight of the leaves and stem, respectively. The numbers in the table are the ratio of the dry weight of the leaves or the stem compared to the total dry weight (=1). The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

In the WT a significant reduction of 22% in DW was measured after the treatment with only 6 h UV-B radiation and after the treatment with 6 h UV-B radiation combined with a temperature drop (compared with WT-control). Thus, there was no interactive effect of UV-B and temperature drop. *le* reacted somewhat differently from the WT; there was no significant reduction in DW after the different treatments compared to control (Figure 45).

The ratio between DW of the stem and DW of the leaves compared to total DW was similar in both genotypes, regardless of the treatment. For both types the ratio is 0.6 for the DW of the stem/ total DW and 0.4 for the DW of the leaves/ total DW.

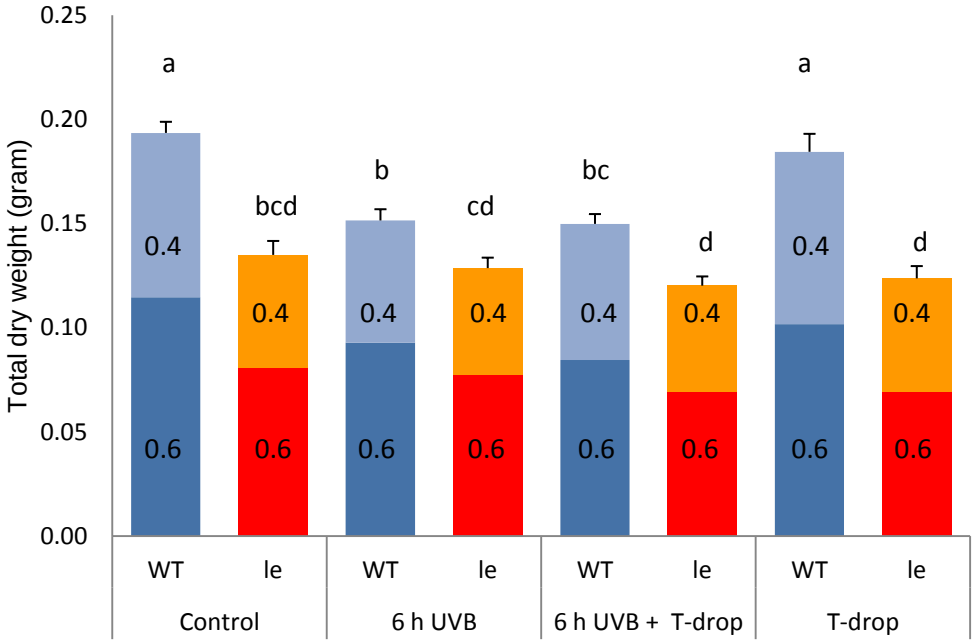


Figure 45. Effect of UV-B radiation (0.35 W m^{-2}) for 6 h and a 6 h temperature drop (from 21 to 13 °C) on total dry weight (in gram) in the wild type (WT) and *le* mutant in pea. The upper and lower part of the bar shows the dry weight of the leaves and stem, respectively. The numbers in the bars are the ratio of the dry weight of the leaves or the stem compared to the total dry weight (=1). The control and plants exposed to UV-B radiation only were grown at 20° C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

Effects of UV-B radiation and temperature drop on shoot elongation

After 10 days daily exposure to UV-B radiation of 0.35 W m^{-2} for different periods alone or in combination with a 6 h temperature drop, the differences in height in WT plants of pea were obvious. The tallest plants were the control plants, the shortest plants were those treated with UV-B radiation for 6 h together with temperature drop (Figures 46 and 47).Only the treatment with 30 min UV-B radiation gave no significant decrease in shoot elongation, but when 30 min UV-B radiation was combined with a temperature drop the decrease was 16%. The treatment with only 6 h UV-B radiation gave a significant decrease of 25 % and this decrease was almost doubled when 6 h UV-B radiation was given combined with a temperature drop (46%). The treatment with only a temperature drop gave a significant decrease of 11 % (all compared with WT-control). This indicated that a temperature drop inhibits shoot elongation and given with a treatment of UV-B radiation (30 min or 6 h) this effect is more than doubled.

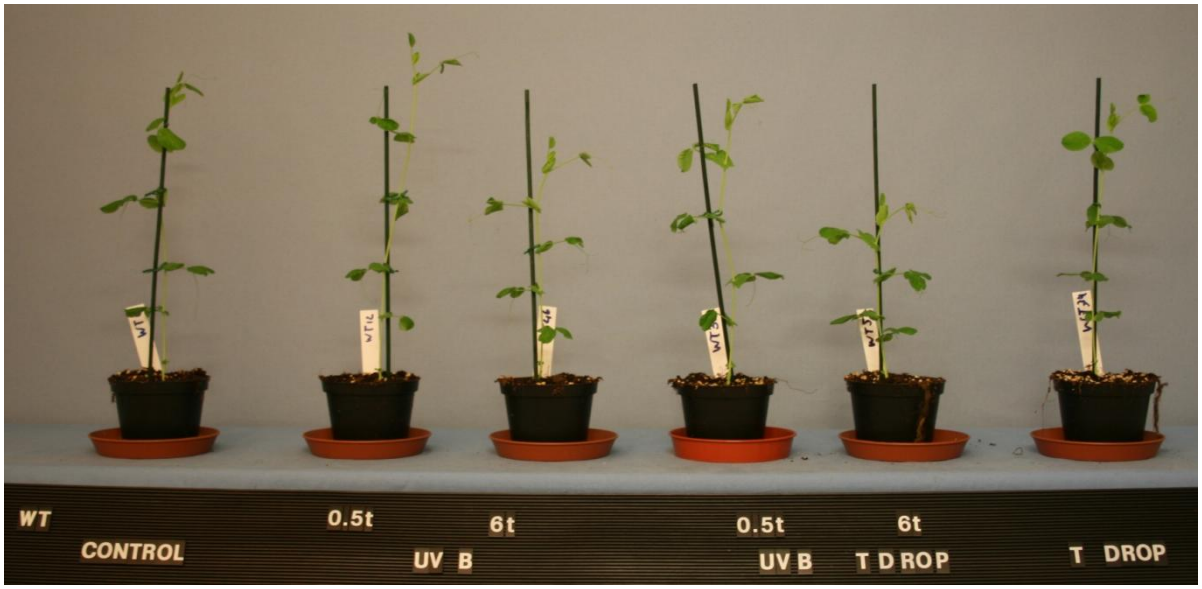


Figure 46. Plants of WT in pea grown for 10 days with 6 different treatments, from left to right: control, UV-B radiation (0.35 W m^{-2}) for 30 min, UV-B radiation (0.35 W m^{-2}) for 6 h, UV-B radiation (0.35 W m^{-2}) for 30 min combined with a 6 h temperature drop (from 21 to 13 °C), UV-B radiation (0.35 W m^{-2}) for 6 h combined with a 6 h temperature drop (from 21 to 13 °C) and only temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the drop treatments).

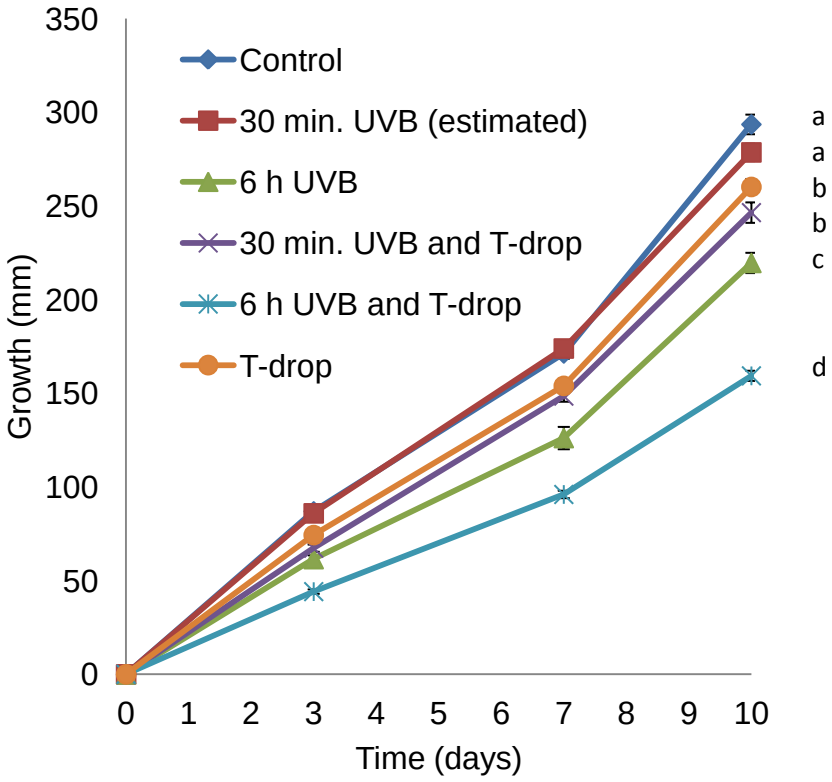


Figure 47. Effect of UV-B radiation (0.35 W m^{-2}) for 30 min or 6 h and a 6 h temperature drop (from 21 to 13 °C) on shoot elongation in the WT of pea. The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

In the *long1* mutant, there were no significant differences between the control treatment, the treatment with 30 min UV-B radiation or the treatment with 30 min UV-B radiation combined with 6 h temperature drop. However, the treatment with only a temperature drop showed a significant decrease (8%) in shoot elongation compared to the control (Figures 48 and 49).

Figure 48. Plants of the *long1* mutant in pea grown for 10 days with 4 different treatments, from left to right: control, UV-B radiation (0.35 W m^{-2}) for 30 min, UV-B radiation (0.35 W m^{-2}) for 30 min combined with a 6 h temperature drop treatment (from 21 to 13 °C) and only 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the drop treatments).

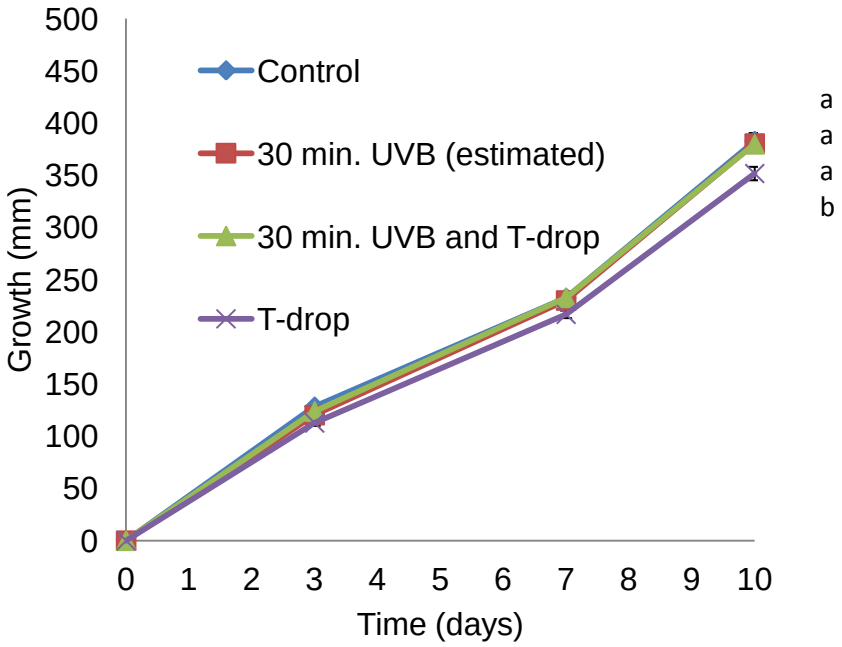


Figure 49: Effect of UV-B radiation (0.35 W m^{-2}) for 30 min and temperature drop (from 21 to 13° C) on shoot elongation (in mm) in the *long1* mutant of pea. The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p≤ 0.05).

In the *le* mutant only a combination of 6 h UV-B radiation and 6 h temperature drop resulted in a significant reduction in shoot elongation (36% compared to the *le*-control) (Figures 50 and 51).

Figure 50. Plants of *le* mutant in pea grown for 10 days with 4 different treatments, from left to right: control, UV-B radiation (0.35 W m^{-2}) for 6 h, UV-B radiation (0.35 W m^{-2}) for 6 h combined with a 6 h temperature drop (from 21 to 13 °C) and only 6 h temperature drop (from 21 to 13 °C). The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the drop treatments).

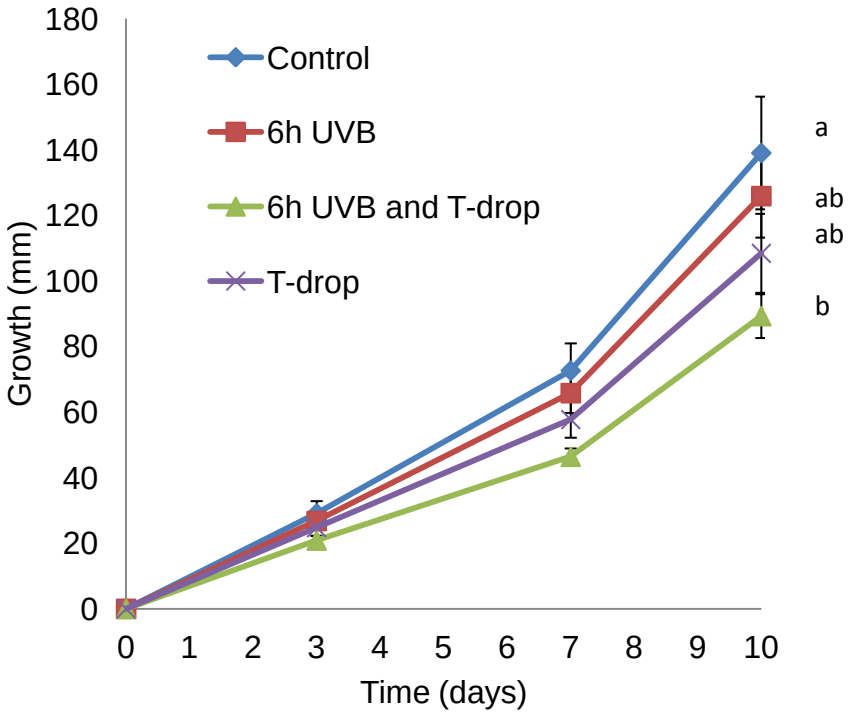


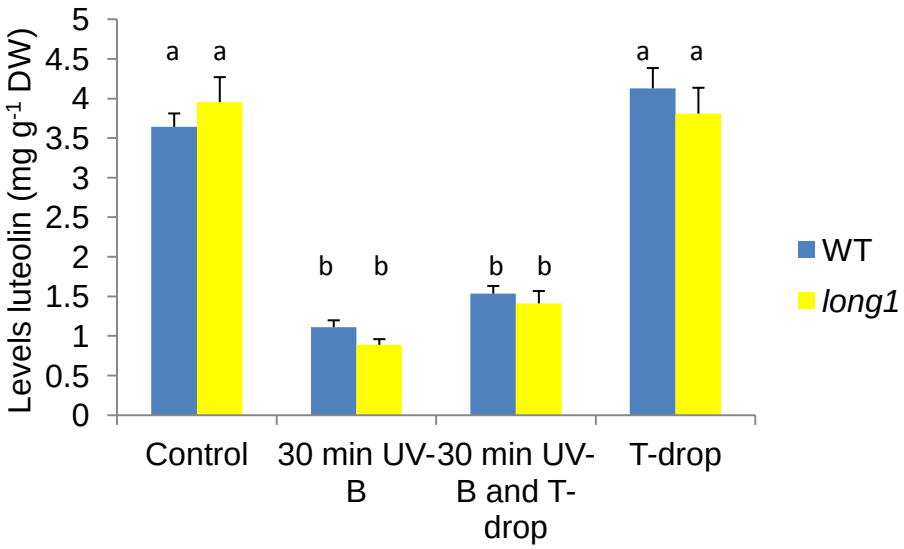
Figure 51. Effect of UV-B radiation (0.35 W m^{-2}) for 6 h and a 6 h temperature drop (from 21 to 13 °C) on shoot elongation (in mm) in the *le* mutant in pea. The control and plants exposed to UV-B radiation only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

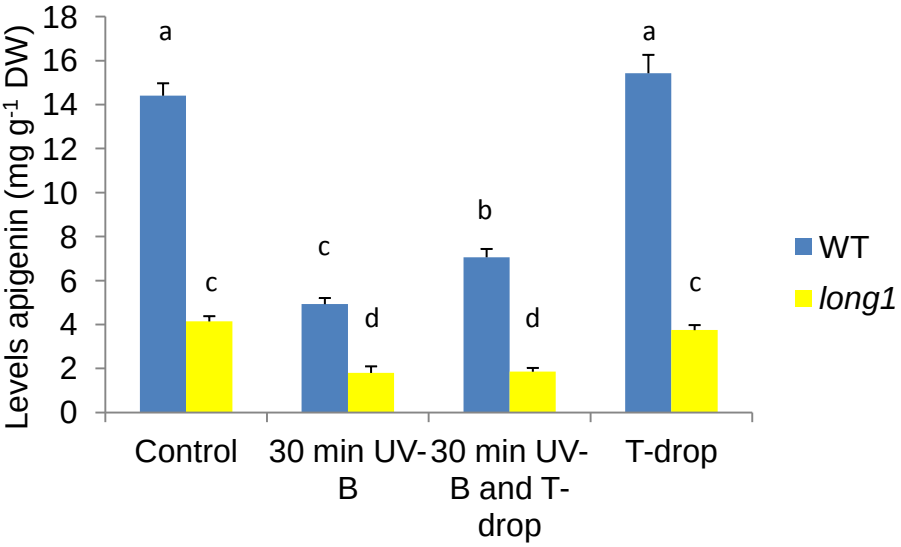
Effects of UV-B radiation and temperature on the total content of phenolic compounds

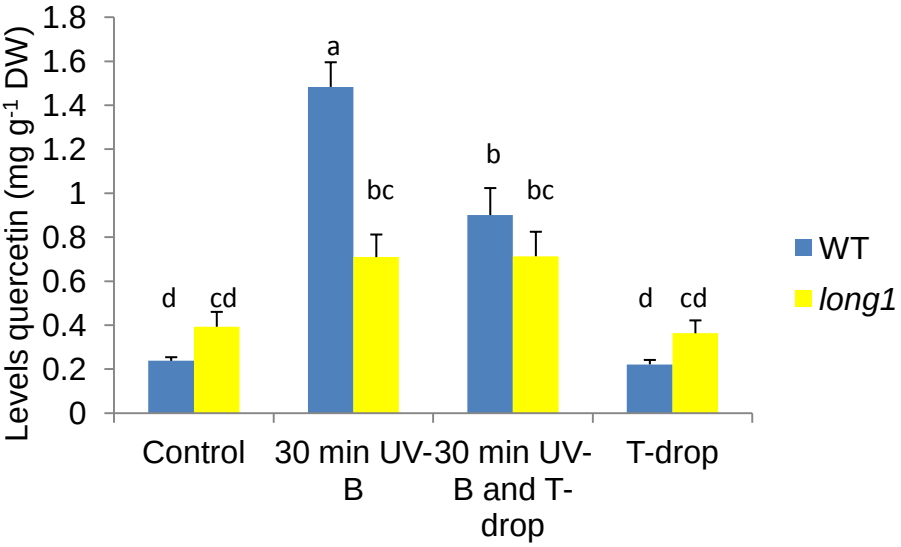
The levels of phenolic compounds (flavonoids) were compared in the *long1* mutant and WT exposed to 30 min UV-B at 0.35 W m^{-2} either alone or in combination with a 6 h temperature drop. There were some significant differences in the levels of phenols between the WT and *long1* and after most treatments WT has a higher concentration of phenols than WT.

In WT and in *long1* the levels of luteolins and apigenins were significantly lower after the UV-B treatment, with or without a temperature drop, compared to the control treatment. In WT the levels of quercetins and kaempferols were significantly higher after the UV-B treatments, with or without a temperature drop, compared to the control treatment, but in *long1* there were no significant differences in the levels of quercetins and kaempferols compared to the control. In WT and in *long1* the levels of myricetins showed no significant differences compared to the control (Figure 52).

In WT and in *long1* the total content of all flavonoids, tryptophan and phenolic acids were significantly lower after the UV-B treatment, with or without a temperature drop, compared to the control treatment. The treatment with the temperature drop did not result in any significant differences from the control (Figure 53).




Levels apigenin (mg g-1 DW)
18
16
14
12
10
8
6
4
2
0
a
c
c
d
b
d
a
c
Control
30 min UV-B
30 min UV-B and T-drop
T-drop
WT
long1


Levels quercetin (mg g-1 DW)
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1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
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bc
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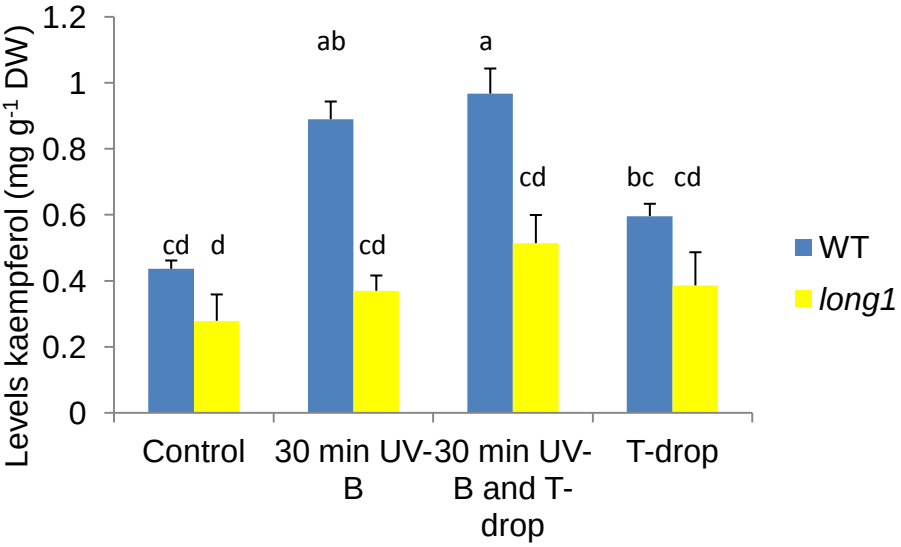
Control
30 min UV-B
30 min UV-B and T-drop
T-drop
WT
long1


Levels kaempferol (mg g-1 DW)
1.2
1
0.8
0.6
0.4
0.2
0
cd
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ab
cd
a
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bc
cd
Control
30 min UV-B
30 min UV-B and T-drop
T-drop
WT
long1

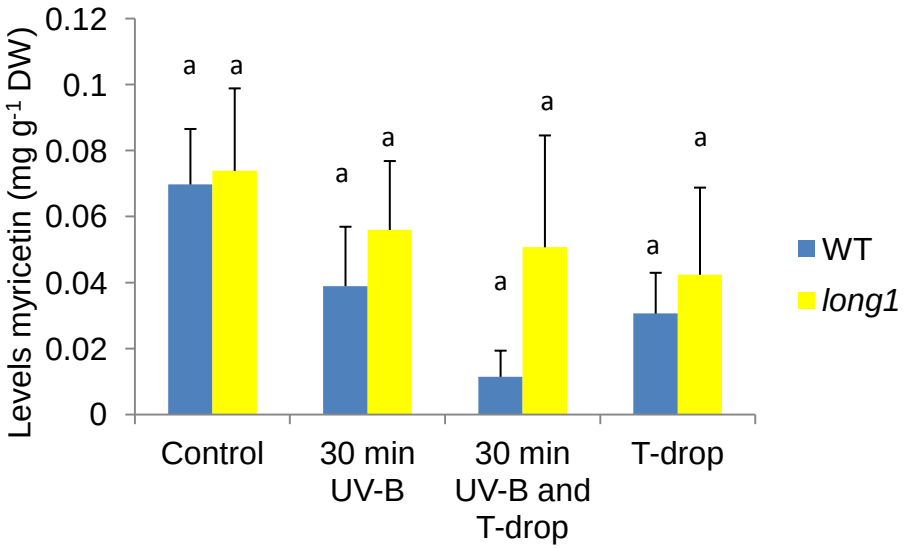


Figure 52. Effect of daily UV-B radiation (0.35 W m^{-2}) for 30 min and a 6 h temperature drop (from 21 to 13 °C) on the levels of phenolic compounds (in mg g^{-1} DW) in the second leaf pair from the soil in the wild type (WT) and *long1* mutant in pea as measured by HPLC. Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p≤0.05).

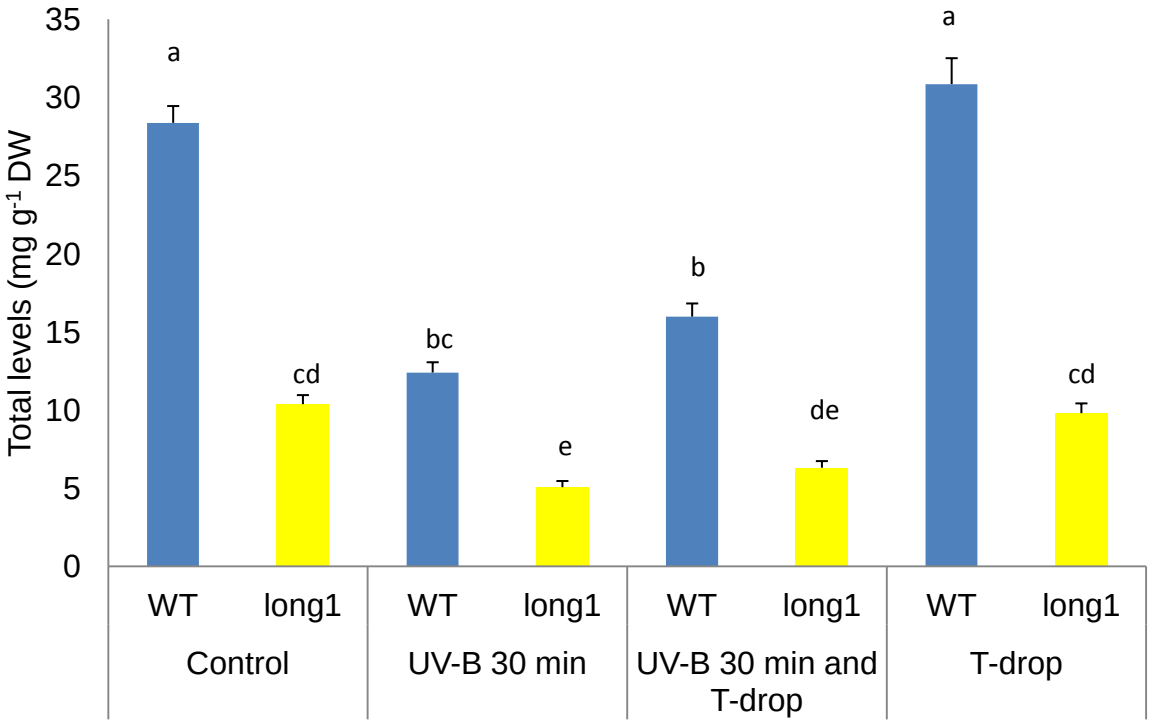
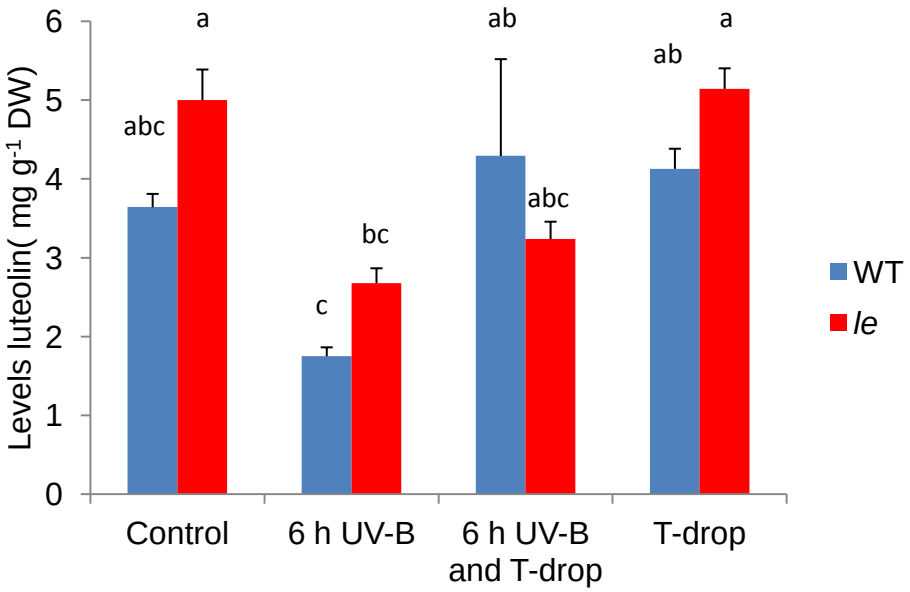


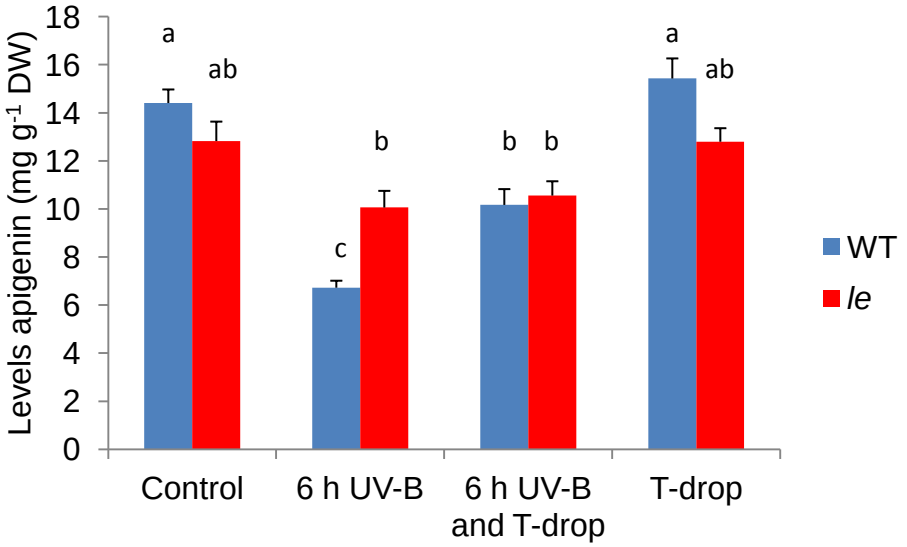
Figure 53. Effect of UV-B radiation (0.35 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on the total levels of all flavonoids, tryptophan and phenolic acids (in mg g^{-1} DW) in the second leaf pair from the soil in WT and *long1* in peas as measured by HPLC. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p ≤0.05.)

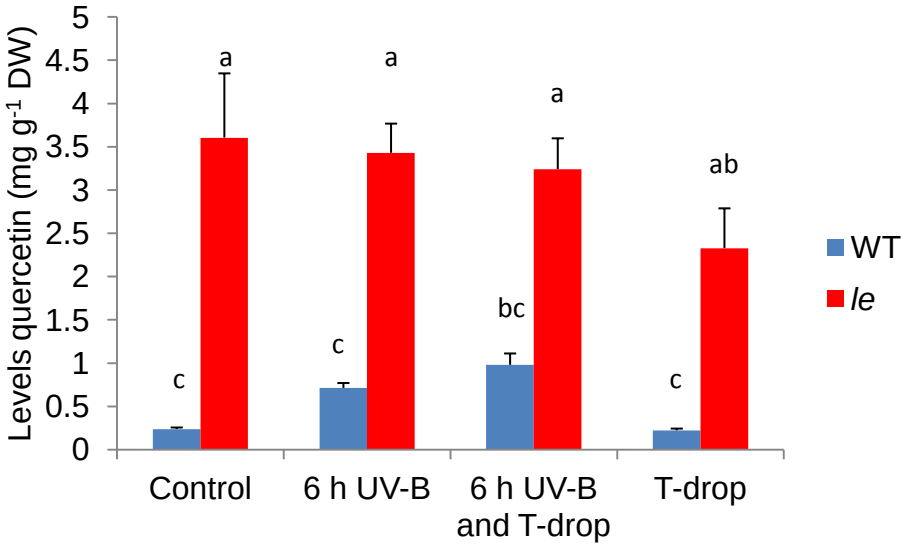
The levels of phenolic compounds (flavonoids) were compared in the *le* mutant and WT exposed to 6 h UV-B radiation at 0.35 W m^{-2} either alone or in combination with a 6 h temperature drop. There were some significant differences in the levels of phenols between the WT and *le* and after most treatments *le* had a higher level of phenols than WT.

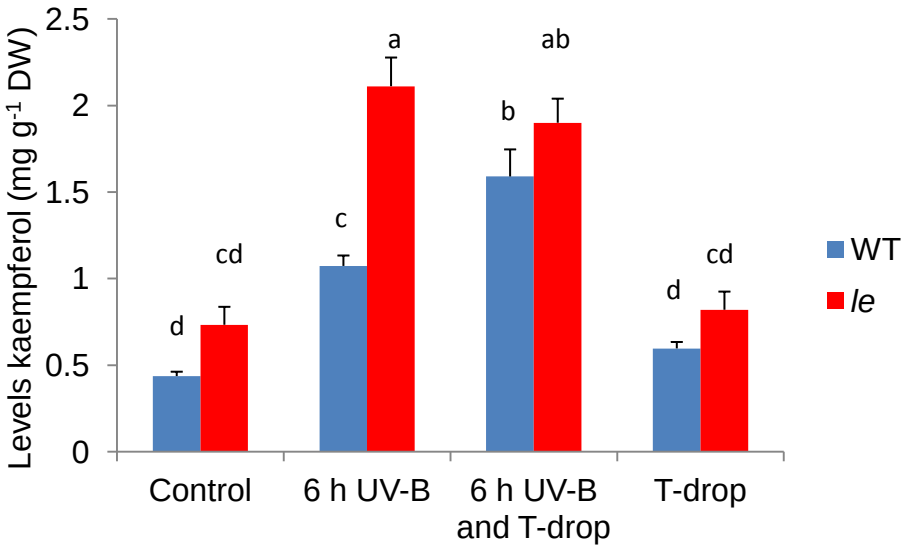
In WT there were no significant differences in the levels of luteolins, compared to the control treatment. However, in *le* the level of luteolins were significant lower after the UV-B treatment without a temperature drop, compared to the control treatment. In WT the levels of apigenins was significant lower after the UV-B treatments, with or without a temperature drop, compared to the control treatment, but here there were no significant differences in *le* compared to the control. In WT and *le* the level of quercetins had no significant differences compared to the control. In WT and in *le* the levels of kaempferols and myricetins were significantly higher after the UV-B treatments, irrespective of temperature treatment, compared to the control treatment (Figure 54).

In WT the total content of all flavonoids, tryptophan and phenolic acids were significantly lower after the 6 h UV-B treatment without a temperature drop, compared to the control treatment. In *le* no significant differences were measured in total contents of all flavonoids, tryptophan and phenolic acids after the different treatments (Figure 55).




Levels apigenin (mg g-1 DW)
18
16
14
12
10
8
6
4
2
0
a
ab
c
b
b
b
a
ab
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
le


Levels quercetin (mg g-1 DW)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
a
a
a
ab
c
c
bc
c
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
le


Levels kaempferol (mg g-1 DW)
2.5
2
1.5
1
0.5
0
a
ab
b
c
cd
cd
d
d
Control
6 h UV-B
6 h UV-B and T-drop
T-drop
WT
le

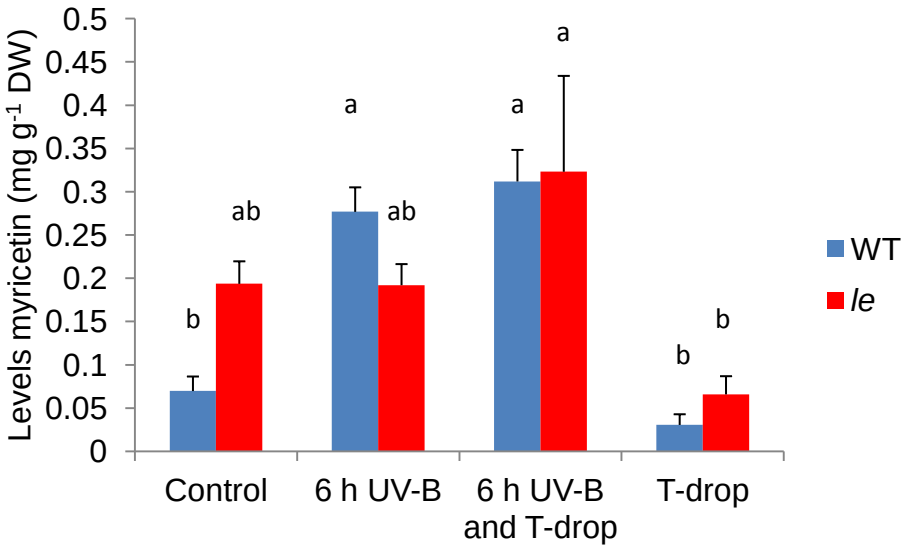


Figure 54. Effect of daily UV-B radiation (0.35 W m^{-2}) for 6 h and a 6 h temperature drop (from 21 to 13 °C) on the levels of phenolic compounds (in mg g^{-1} DW) in the second leaf pair from the soil in the wild type (WT) and *le* mutant in pea as measured by HPLC. Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p≤ 0.05).

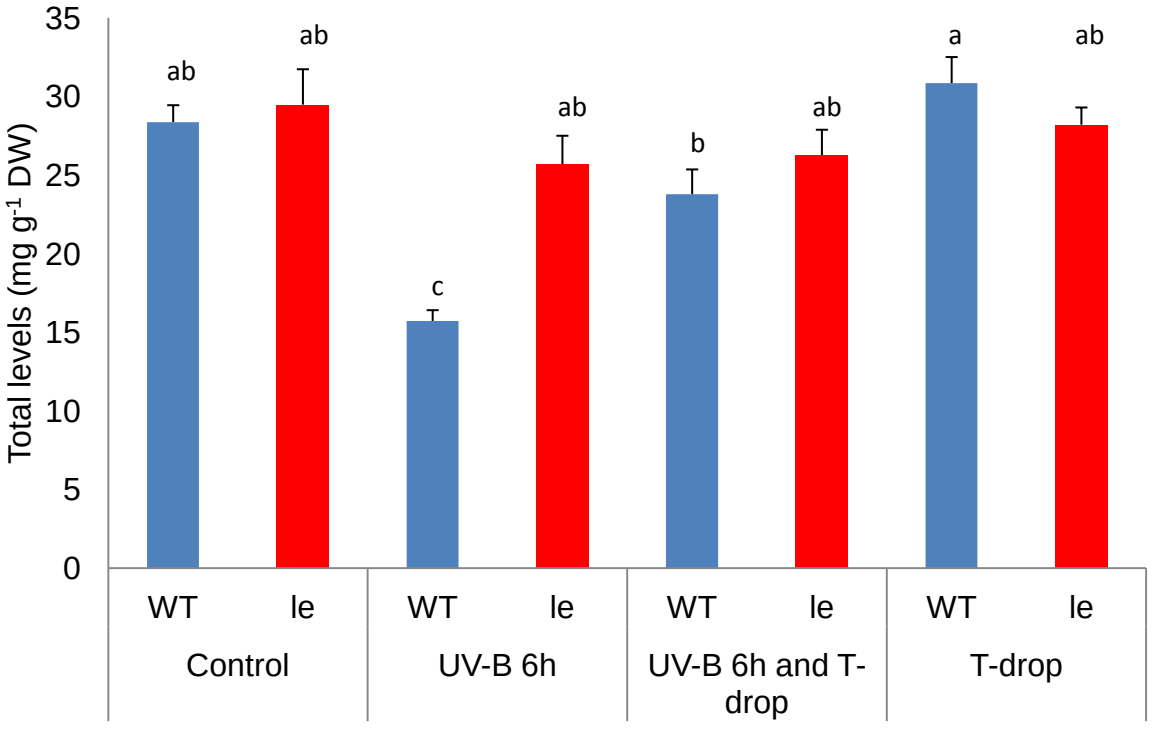


Figure 55: Effect of UV-B radiation (0.35 W m^{-2}) for 6 h and temperature drop (from 21 to 13 °C) on the total levels of all flavonoids, tryptophan and phenolic acids (in mg g^{-1} DW) in the second leaf pair from the soil in WT and *le* in peas as measured by HPLC. The control and plants exposed to UV-B only were grown at 20 °C (diurnal average of the temperature drop treatments). Results are mean of 10 plants in each of 2 replicate experiments and SE is shown. Different letters indicate significant differences and the same letters indicate no statistically significant difference (p ≤0.05).

Discussion

Effects of UV-B radiation and temperature drop

In order to investigate the effects of UV-B radiation combined with a temperature drop on pea morphology and the level of phenolic compounds, WT pea as well as three pea mutants were exposed to different lengths of periods with UV-B radiation, given in a high dose (0,50 W m^{-2}) or a low dose (0,35 W m^{-2}) in combination with a temperature drop for 6 h from 21 ˚C to 13 ˚C in the middle of the light period. Different morphological parameters were tested; leaf area, dry weight and stem elongation. The levels of phenolic compounds in the leaves were measured by HPLC.

Visible effects of UV-B radiation and temperature drop

The effects of daily treatment with only UV-B radiation showed some damage, like curled leaf edges on both WT and the *long1* mutant. This is in agreement with previous experiments (Jansen et al., 1998). When UV-B radiation was combined with a temperature drop from 21 to 13° C less or even no damage was observed. This is indicating a temperature modulation of the response to UV-B radiation.

UV-B radiation induces the formation of cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PPs), which are two photoproducts of DNA damage. Constant, low temperature was previously shown to significantly decrease the formation of those two most frequent types of photoproducts (Li et al., 2002). The formation of CPDs and 6-4PPs is partly a temperature-independent process (the photochemical reaction) and partly a temperature-dependent process (the enzymatic process). This last process might explain the observation of less damage when UV-B radiation was combined with a temperature drop in the present study. On the other hand, the photorepair of CPDs and 6-4PPs is more efficient at higher temperatures than in lower temperatures. Therefore, less damage in the our treatment with a temperature drop from 21 to 13° C during the period of UV-B radiation than in a treatment with constant low temperature might also be due to repair of the damage caused by the UV-B radiation during the warmer period during the day.

Another investigation studied the effect of the combination of two stress factors; drought and UV-B radiation on pea. The growth parameters plant height, dry weight and leaf area showed that the combination of drought and UV-B radiation gave less reduction in all parameters than the treatment with UV-B radiation alone (Alexieva et al., 2001). This could be explained by thicker leaves caused by drought and therefore a reduction in UV-B radiation penetration. Other studies showed that exposure to two or more stress factors can either result in aggravated distress or increased cross-tolerance (Hideg et al., 2013). Thus, to predict the result of exposure to two or more stress factors is not so easy, and must be investigated in experiments.

Both the *le* and the *lip1* mutants appeared very resistant to UV-B, no visible damage on the leaves were observed when exposed to 6 h UV-B radiation, with or without a temperature drop. It would be very interesting to determine the amount of GA in the *le* and *lip1* mutants after the treatments with UV-B to investigate if there is a connection. It might be expected that UV-B affects the biosynthesis of GA. Both the *le* and *lip1* mutants are dwarfs having low levels of GA and it might be that this is beneficial for stress tolerance.

Effects of UV-B radiation and temperature drop on leaf area

In our experiment we did not measure any effect of temperature drop on leaf area in any genotype, but the effect of the UV-B radiation treatment was obvious. UV-B radiation gave a reduction in leaf area in WT and the *long1* mutant. This is consistent with previous studies (e.g. Caldwell, 2003). The reduction in leaf area in this genotype and the WT exposed to 30 min UV-B was less when the treatments were combined. This might be explained by the theory of increased cross-tolerance (Hideg et al., 2013)

The *le* mutant did not show such a reduction in leaf area upon UV-B exposure neither when given separately or in combination with a temperature drop. On the other hand, the *lip1* mutant showed a reduction in leaf area when the treatments were combined. This might be explained by the theory of aggravated distress (Hideg et al., 2013).

Effects of UV-B radiation and temperature drop on dry weight

The DW of the plants corresponded well with their heights; the tallest plants, the *long1* mutants, had a higher DW than the WT and both the dwarf mutants, *le* and *lip1*, had lower DW than the WT. The *lip1* mutants stored a smaller amount of dry matter in their stems than in their leaves compared to WT, the *le* mutant was similar to the WT in this respect. On the other hand, the tall mutant *long1* stored a larger amount of dry matter in its stem. The ratio DW leaves/ DW stem did not change after the different treatments. These results might suggest that GA is not a major determinant of biomass allocation to the different parts of the shoot. On basis of analogy with the situation in *A. thalinana*, in photomorphogenesis-related processes, LIP1 is anticipated to affect the content of LONG1 during the night. Thus it appears that lack of LONG1 in the *long1* mutant somehow is reducing the allocation to the leaves in pea, and that the anticipated higher levels of LONG1 during the night in the *lip1*-mutant, is increasing the allocation to the leaves. Although LONG1 is known to stimulate GA inactivation and thus reduce GA levels, the similar biomass allocation pattern in the *le* mutant and the WT suggests that other hormones than GA or other factors might be involved in controlling dry matter allocation to different parts of the shoot.

No effect of temperature drop on DW was measured, but the treatments with UV-B radiation gave a reduction in DW in WT and in the *long1* mutant compared to the control. This was in accordance with reactions of a range of other plants to UV-B

radiation (Caldwell, 2003). There were no significant differences between the UV-B treatments with or without temperature drop.

The DW of the *le* and *lip1* mutant was not affected by UV-B radiation, by temperature drop or by combination of those.

Effects of UV-B radiation and temperature drop on shoot elongation

In WT, the tallest plants were measured in the control treatment, and then in order: temperature drop, only UV-B radiation and UV-B radiation combined with temperature drop. In *long1* mutant there was only measured a significant decrease in shoot elongation after the treatment with temperature drop and in the *le* and *lip1* mutants the only significant reduction was measured when the treatments were combined. In the WT it has been shown that a temperature drop in the light period will give an increased expression of the GA-deactivation gene *GA2ox2* and will reduce the levels of the active GA_1 (Stavang et al., 2007). This will reduce the height of the plants. Thus, our results are consistent with this.

In the *long1* mutant the level of *GA2ox2* is far lower than in the WT (Weller et al., 2009) and it has been shown that this mutant due to the lack of LONG1 is not able to respond to a temperature drop in the light phase (Todorcevic, 2013).This did not agree with our measurements, but the measured reduction was only 8%, and although it was significant, it was very small.

The *lip1* mutant in pea has been mutated in the LIP1 gene and therefore the level of LONG1 should thus always be high, both day and night, and thus the GA2ox2 level is high and the active GA_1 is inactivated (Weller et al. 2009). Preliminary studies showed that the *lip1* mutant showed inhibited shoot elongation after treatments with a temperature drop (Todorcevic, 2013). In our experiment the only significant reduction in shoot elongation was measured after the combined treatment. This reaction was also measured in the *le* mutant. This mutant lacks GA 3-oxidase (GA3ox), which means that the active GA_1 is generally not made. We would therefore expect this mutant not to react on a temperature drop. It is difficult to find a possible answer why we measured this reduction in the combined treatments. However, the mutant is known to be somewhat leaky and still contain small amounts of GA (Ross et al., 1989).

Effects of UV-B radiation and temperature drop on the level of phenolic compounds

The three main questions to answer in this section are: (1) Are there significant differences in the level of the different phenolic compounds after the different treatments, (2) between the WT and the mutants and (3) do the WT and mutants react differently on the different treatments?

There were no differences in the levels of phenols after the treatment with only temperature drop compared with the control treatment. On the other hand, there were

obvious differences measured after the UV-B treatments with higher content of phenols compared to the control. The results were similar for the UV-B treatment without or the UV-B treatment with a temperature drop, so this indicates that a temperature drop had no clear influence on the phenols content.

The figures of the total content of phenolic compounds (Figure 37, 53 and 55) are totally overshadowed by only one phenol, apigenin, which occurred in the largest amounts among the phenols, and therefore these figures are not taken into account. That is why the phenolic compounds will be discussed separately.

We identified three major flavonols; quercetin, kaempferol and myricetin as well as two major flavones; luteolin and apigenin. Higher levels of the flavonols quercetin and myricetin were measured after the treatments with UV-B radiation in the WT and *lip1* mutant. The mutants *le* and *long1* did not show any significant differences. There were also higher levels of the third flavanol kaempferol after the treatments with UV-B radiation in the WT, *lip1* and *le* mutant. In these measurements the *long1* mutant did not show any significant differences. In WT and all the mutants the level of the flavone luteolin was lower compared to the control treatment. Furthermore, in the WT and *long1* a similar pattern was seen for the flavones apigenin, but the *lip1* and *le* mutants did not show any significant difference in content of apigenin between the different treatments.

In conclusion, the flavonols quercetin, kaempferol and myricetin have significantly higher levels or do not show any significant difference after the UV-B treatments in all pea plants. The flavones luteolin and apigenin show significantly lower levels or do not show any significant difference after the UV-B treatments in all pea plants. This is in line with the research done on *Arabidopsis* mutants; the level of quercetin and kaempferol increased after treatment with UV-B and it was suggested that especially flavonols protect *Arabidopsis* plants from UV-B damage (Ryan et al., 2001).

The *lip1* and *le* mutants had significantly higher levels of both quercetin and kaempferol compared to WT, whereas the opposite was the case for the *long1* mutant. This mutant has significant lower levels of all phenols compared to WT. Flavonoids protect plants against UV- B damage since they absorb UV-B radiation and act as a sunscreen. We observed more UV-B damage in the *long1* mutant and this can therefore be explained by the lower level of phenols. Less damage was observed in the *lip1* an *le* mutants and this corresponds with the higher level of phenols.

Conclusions

For the production of high-quality plants in greenhouses control of morphology is essential. This thesis have shown that a combination of UV-B radiation and temperature drop can potentially give such a desired effect, although it is important to find the optimum combination for each genotype. In general, less UV-B-related damage was observed when UV-B was provided together with the temperature drop. This might be explained by the fact that low temperature decreases the formation of thymidine dimers and 6-4-photoproducts in DNA, which are induced by UV-B radiation. However, this remains to be verified for pea in the experiments of the present work.

We can conclude that *lip1* and *le* mutants are less sensitive to UV-B radiation compared to WT and that the mutant *long1* is far more sensitive to UV-B radiation. This might be due to the levels of phenols; both the *lip1* and *le* mutant had higher levels of some flavonols, while the *long1* mutant had lower levels of phenolic compounds compared to WT.
In *Arabidopsis thaliana* HY5, which is required for photomorphogenic development, acts in UV-B signalling (Jenkins 2009). The E3 ubiquitin ligase COP1 regulates the HY5 turnover, resulting in high HY5 contents in light and degradation in the dark. The present thesis work demonstrates that their ortologs in pea, LONG1 and LIP1, respectively, also play a role in UV-B signalling.

Suggestions for future research

To learn more about the mechanisms involved in responses to UV-B-radiation under different temperature regimes, it would be interesting to study the involvement GAs and auxins, with or without combined with a temperature drop. It is known that temperature drop in the light period and negative DIF reduce the levels of GA in pea by increasing GA-inactivation. This regulation occurs at the transcript level; GA2ox2 in pea increases only during a temperature change in the light, not in the dark (Stavang et al 2005, 2007). Temperature rise in light reduced GA-inactivation in *A. thaliana* at the transcript level by a GA2ox1 (Stavang et al., 2009). However, the knowledge on interactive effects of UV-B and temperature alterations on hormone physiology is scarce and warrant further investigation.

To study this further a range of different experiments can be suggested:
a) Experiment with different applications (GA alone, GA biosynthesis inhibitor, GA + GA biosynthesis inhibitor and control). Can the effect of UV-B radiation with or without combined with a temperature drop be inhibited by addition of GA? If that is the case, it suggests that GA is important in the response.

b) Application of auxin and inhibition of auxin transport to the apex (similar reasoning as with GA).

b) Measurement of GA metabolism on transcript levels (as in Stavang et al., 2005; 2007; 2009).

c) Measurement of auxin metabolism on transcript levels (as in Stavang et al., 2009).

d) Study if GA or auxin affect the flavonoid content? Measure flavonoids after hormone application.

2) Examine whether there is less damage on DNA or more DNA damage repair by UV-B radiation combined with a temperature drop than treatments with UV-B radiation alone.

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Appendix

Pre-experiment 1: WT and *long1* (0.5 W m^{-2} UV-B radiation)

Table 11. Anova table for leaf area for WT and *long1* of pea

	DF	F-value	P-value
Treatment	1	314,16	0,0001
Genotype	1	26,67	0,0001
Treatment*Genotype	1	5,97	0,018
Error	55		
Total	58		

Table 12. Anova table for dry weight for WT and *long1* of pea

	DF	F-value	P-value
Treatment	1	86,00	0,0001
Genotype	1	19,59	0,0001
Treatment*Genotype	1	31,81	0,0001
Error	55		
Total	58		

Table 13. Anova table for total growth for WT and *long1* of pea

	DF	F-value	P-value
Treatment	2	915,90	0,0001
Genotype	1	16,78	0,0001
Treatment*Genotype	2	68,07	0,0001
Error	82		
Total	87		

Pre-experiment 2: WT, *long1* and *le* (0.35 W m^{-2} UV-B radiation)

Table 14. Anova table for total leaf area for WT and *long1* of pea

	DF	F-value	P-value
Treatment	4	27,27	0,0001
Genotype	1	21,62	0,0001
Treatment*Genotype	4	2,04	0,107
Error	40		
Total	49		

Table 15. Anova table for total leaf area for WT and *le* of pea

	DF	F-value	P-value
Treatment	5	8,92	0,0001
Genotype	1	0,06	0,807
Treatment*Genotype	5	1,39	0,247
Error	43		
Total	54		

Table 16. Anova table for dry weight for WT and *long1* of pea

	DF	F-value	P-value
Treatment	4	8,94	0,0001
Genotype	1	4,07	0,050
Treatment*Genotype	4	6,17	0,001
Error	40		
Total	49		

Table 17. Anova table for total dry weight for WT and *le* of pea

	DF	F-value	P-value
Treatment	5	8,25	0,0001
Genotype	1	64,00	0,0001
Treatment*Genotype	5	0,94	0,462
Error	45		
Total	56		

Table 18. Anova table for total growth in WT of pea

	DF	F-value	P-value
Treatment	5	17,74	0,0001
Error	53		
Total	58		

Table 19. Anova table for total growth in *long1* of

	DF	F-value	P-value
Treatment	4	1,51	0,216
Error	41		
Total	45		

Table 20. Anova table for total growth in *le* of pea

	DF	F-value	P-value
Treatment	5	1,20	0,336
Error	29		
Total	34		

Experiment WT and *lip1* (0.50 W m^{-2} UV-B radiation)

Table 21: Anova tabel for leaf area for WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	84,23	0,0001
Genotype	1	165,90	0,0001
Treatment*Genotype	3	45,00	0,0001
Error	101		
Total	108		

Table 22. Anova table for dry weight for WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	16,57	0,0001
Genotype	1	323,87	0,0001
Treatment*Genotype	3	17,81	0,0001
Error	112		
Total	119		

Table 23. Anova table for total growth in WT of pea

	DF	F-value	P-value
Treatment	3	101,91	0,0001
Error	116		
Total	119		

Table 24. Anova table for total growth in *lip1* of pea

	DF	F-value	P-value
Treatment	3	39,09	0,0001
Error	116		
Total	119		

Table 25. Anova table for total concentration of Luteolin in WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	7,45	0,0001
Genotype	1	58,70	0,0001
Treatment*Genotype	3	18,13	0,0001
Error	151		
Total	158		

Table 26: Anova tabel for total concentration of Apigenin in WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	26,40	0,0001
Genotype	1	187,13	0,0001
Treatment*Genotype	3	10,32	0,0001
Error	151		
Total	158		

Table 27: Anova tabel for total concentration of Quercetin in WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	59,64	0,0001
Genotype	1	238,80	0,0001
Treatment*Genotype	3	37,94	0,0001
Error	151		
Total	158		

Table 28: Anova tabel for total concentration of Kaempferol in WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	144,50	0,0001
Genotype	1	123,98	0,0001
Treatment*Genotype	3	14,06	0,0001
Error	151		
Total	158		

Table 29: Anova tabel for total concentration of Myricetin in WT and *lip1* of pea

	DF	F-value	P-value
Treatment	3	57,38	0,0001
Genotype	1	11,79	0,001
Treatment*Genotype	3	9,91	0,0001
Error	151		
Total	158		

Experiment WT, *long1* and *le* (0.35 W m^{-2} UV-B radiation)

Table 30: Anova tabel for leaf area for WT and *long1*

	DF	F-value	P-value
Treatment	3	138,28	0,0001
Genotype	1	50,83	0,0001
Treatment*Genotype	3	1,85	0,141
Error	150		
Total	157		

Table 31: Anova tabel for leaf area for WT and *le*

	DF	F-value	P-value
Treatment	3	39,92	0,0001
Genotype	1	311,57	0,0001
Treatment*Genotype	3	15,63	0,0001
Error	149		
Total	156		

Table 32: Anova tabel for total dry weight for WT and *long1*

	DF	F-value	P-value
Treatment	3	18,36	0,0001
Genotype	1	2,06	0,152
Treatment*Genotype	3	0,43	0,733
Error	191		
Total			

Table 33: Anova tabel for total dry weight for WT and *le*

	DF	F-value	P-value
Treatment	3	10,50	0,0001
Genotype	1	109,75	0,0001
Treatment*Genotype	3	5,67	0,0001
Error	188		
Total	195		

Table 34: Anova tabel for growth for WT

	DF	F-value	P-value
Treatment	5	156,79	0,0001
Error	153		
Total	158		

Table 35: Anova tabel for growth for *long1*

	DF	F-value	P-value
Treatment	3	34,79	0,0001
Error	96		
Total	99		

Table 36: Anova tabel for growth for *le*

	DF	F-value	P-value
Treatment	3	3,33	0,0023
Error	93		
Total	96		

Table 37: Anova tabel for total concentration of Luteolin in WT and *long1* of pea

	DF	F-value	P-value
Treatment	3	109,22	0,0001
Genotype	1	0,36	0,548
Treatment*Genotype	3	0,88	0,451
Error	151		
Total	158		

Table 38: Anova tabel for total concentration of Apigenin in WT and *long1* of pea

	DF	F-value	P-value
Treatment	3	114,04	0,0001
Genotype	1	629,37	0,0001
Treatment*Genotype	3	45,46	0,0001
Error	151		
Total	158		

Table 39: Anova tabel for total concentration of Quercetin in WT and *long1* of pea

	DF	F-value	P-value
Treatment	3	41,05	0,0001
Genotype	1	7,35	0,007
Treatment*Genotype	3	12,62	0,0001
Error	151		
Total	158		

Table 40: Anova tabel for total concentration of Kaempferol in WT and *long1* of pea

	DF	F-value	P-value
Treatment	3	11,92	0,0001
Genotype	1	48,63	0,0001
Treatment*Genotype	3	3,43	0,019
Error	151		
Total	158		

Table 41: Anova tabel for total concentration of Myricetin in WT and *long1* of pea

	DF	F-value	P-value
Treatment	3	1,38	0,252
Genotype	1	1,39	0,241
Treatment*Genotype	3	0,24	0,866
Error	151		
Total	158		

Table 42: Anova tabel for total concentration of Luteolin in WT and *le* of pea

	DF	F-value	P-value
Treatment	3	9,94	0,0001
Genotype	1	2,63	0,107
Treatment*Genotype	3	2,46	0,065
Error	145		
Total	152		

Table 43: Anova tabel for total concentration of Apigenin in WT and *le* of pea

	DF	F-value	P-value
Treatment	3	35,13	0,0001
Genotype	1	0,07	0,792
Treatment*Genotype	3	8,20	0,0001
Error	145		
Total	152		

Tabel 44: Anova tabel for total concentration of Quercetin in WT and *le* of pea

	DF	F-value	P-value
Treatment	3	2,28	0,082
Genotype	1	105,99	0,0001
Treatment*Genotype	3	1,24	0,297
Error	145		
Total	152		

Table 45: Anova tabel for total concentration of Kaempferol in WT pea and *le* of pea

	DF	F-value	P-value
Treatment	3	57,79	0,0001
Genotype	1	35,58	0,0001
Treatment*Genotype	3	6,15	0,001
Error	145		
Total	152		

Table 46: Anova table for total concentration of Myricetin in WT pea and *le* of pea

	DF	F-value	P-value
Treatment	3	14,16	0,0001
Genotype	1	0,48	0,489
Treatment*Genotype	3	2,00	0,117
Error	145		
Total	152		

Table 47. Reactions after treatment with only UV-B radiation of pea (different lengths of periods) compared to control. + means significant increase, ± means no significant difference and – means significant decrease.

Genotype and treatment	luteolin	apigenin	quercetin	kaempferol	myricetin
WT (0.50 W m^{-2}) for 6 h	+	-	±	+	+
lip1 (0.50 W m^{-2}) for 6 h	±	±	+	+	+
WT (0.35 W m^{-2}) for 30 min	-	-	+	+	±
long1 (0.35 W m^{-2}) for 30 min	-	-	±	±	±
WT (0.35 W m^{-2}) for 6 h	±	-	±	+	+
le (0.35 W m^{-2}) for 6 h	-	±	±	+	±

Table 48. Reactions after treatment with UV-B radiation of pea (different lengths of periods) and temperature drop compared to control. + means significant increase, ± means no significant difference and – means significant decrease.

Genotype and treatment	luteolin	apigenin	quercetin	kaempferol	myricetin
WT (0.50 W m^{-2}) for 6 h	+	-	±	+	+
lip1 (0.50 W m^{-2}) for 6 h	±	±	+	+	+
WT (0.35 W m^{-2}) for 30 min	-	-	+	+	±
long1 (0.35 W m^{-2}) for 30 min	-	-	±	±	±
WT (0.35 W m^{-2}) for 6 h	±	-	±	+	+
le (0.35 W m^{-2}) for 6 h	±	±	±	+	±

Table 49. Reactions after treatment with only temperature drop of pea (different lengths of periods) compared to control. + means significant increase, ± means no significant difference and – means significant decrease.

Genotype and treatment	luteolin	apigenin	quercetin	kaempferol	myricetin
WT	±	±	±	±	±
lip1	±	±	±	±	±
WT	±	±	±	±	±
long1	±	±	±	±	±
WT	±	±	±	±	±
le	±	±	±	±	±