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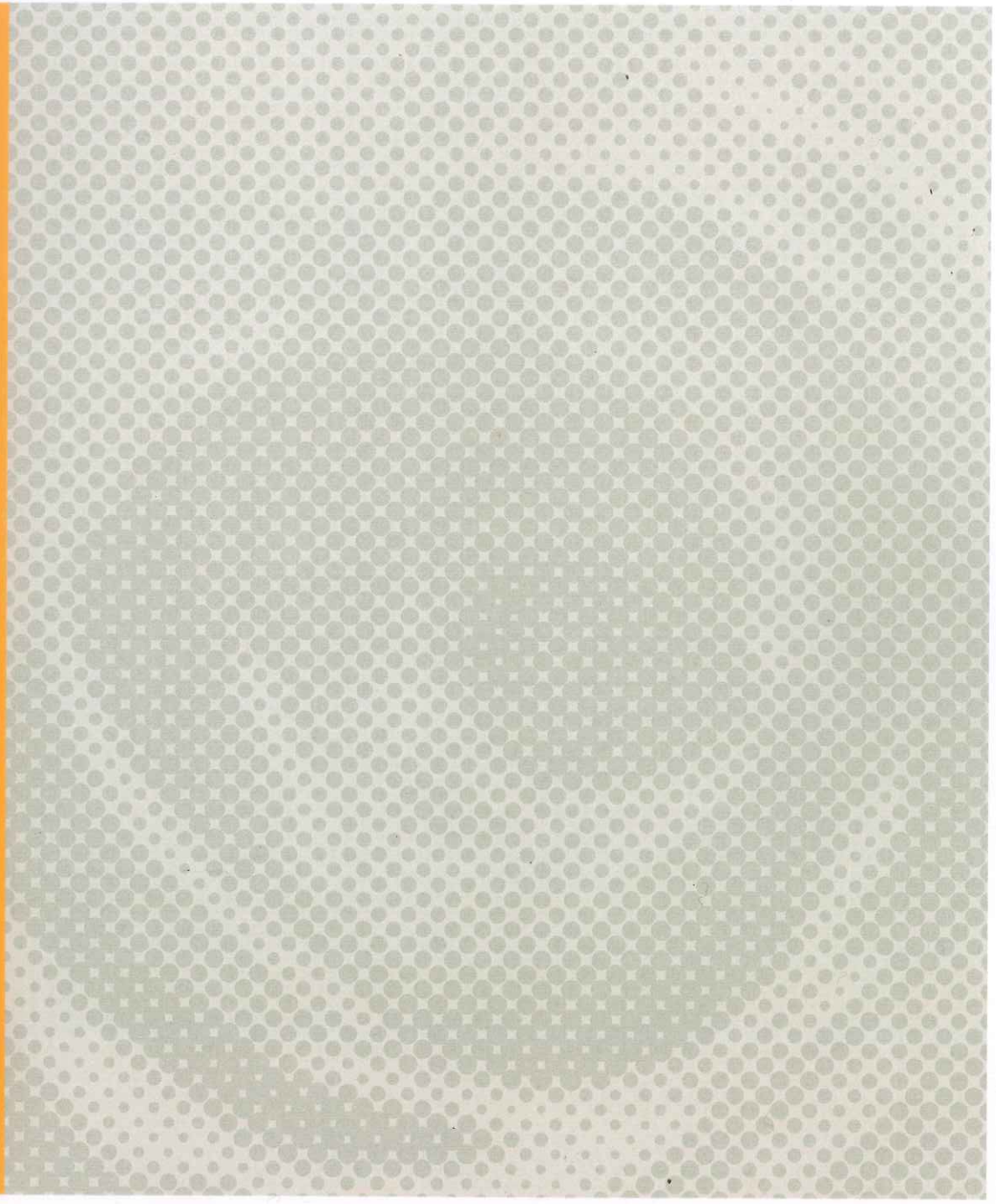
Master Thesis 60 credits 2013



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Universitetet for miljø og biovitenskap

Master Thesis

The involvement of light signaling components in thermoperiodic control of shoot elongation in pea (Pisum sativum)

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ABSTRACT

Production of short and compact plants is one of the major challenges in greenhouses. Due to high demand of such plants by the customers, this has been subjected to significant interest in the greenhouse industry, and temperature drops in the morning is commonly used in production of ornamental pot plants and transplants. Stem elongation is controlled by both temperature and light parameters. Earlier studies have demonstrated a differential elongation response to temperature drop in light and darkness, but the knowledge on the mechanism underlying the thermoperiodic control of shoot elongation is still limited. The aim of this study was to investigate the effect of day and night temperature drops as well as the interaction between temperature drop and irradiance on the wild type (WT) pea (*Pisum sativum*) and pea plants mutated in central photomorphogenesis-related genes, *PHYTOCHROME A* and *B* as well as the the *HY5* ortolog *LONG1* and the *COP1* ortolog *LIP1* (the *long1, lip1, phyA, phyAlong1, phyB* and *phyBlong1* mutants). It was observed that a temperature drop in the middle of the day resulted in a significant reduction of stem elongation at day 15 in the WT (about 15%) as well as in the *lip1* (23%) and *phyA* (15%) mutants. In *phyB* there were no differences between day and night drop but stem elongation at day 15 was significantly reduced in both treated groups in comparison to the control. The treatment did not any have significant effect on the other mutants. In the WT and *phyA* mutant there was no effect of a temperature drop in the night compared to the control. So a day and night drop affect elongation differently in these two genotypes. Our results indicate that *PHYA* is not important in thermoperiodic control of shoot elongation, while *PHYB* seems to be needed for the plant to be able to distinguish between a day drop and a night drop. *Long1* mutant did not show any effect of a temperature drop, neither in the day or the night, indicating that the presence of the *LONG1* gene is essential for a response to a temperature drop. *Lip1* mutant, on the other hand, reacted with inhibition of elongation growth both in response to a drop in the day and night, indicating that *LIP1* gene, which at least in Arabidopsis is known to be involved in down-regulation of HY5/LONG1 in the dark, must be present for a normal thermoperiodic response in pea.

Our results show that the other measured growth parameters were not significantly affected by temperature drop treatments. Furthermore, the combination of day temperature drop and increased irradiance resulted in a stronger inhibitory effect on shoot elongation in comparison to a temperature drop treatment only. In the WT increased irradiance in combination with a

day drop decreased stem elongation significantly by about 45% at day 15, while the reduction in stem elongation was even more pronounced in *lip1*, *phyA* and *phyB* mutants (about 60% at day 15). In plants mutated in *LONG1* gene, no effect of increased irradiance alone or in combination with a temperature drop was observed, indicating a central role of this gene in response to increased irradiance as well as a day drop. The other growth parameters measured both in WT and other mutants were also more significantly affected when day drop was applied with increased irradiance suggesting that this combination of treatments might be more stressful to the plants, thus affecting the growth parameters.

ACKNOWLEDGEMENT

First and foremost, I would like to give many thanks to my supervisor Prof. Jorunn Elisabeth Olsen, for the patient guidance, encouragement and advices she has provided throughout my time as her student. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly.

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Most of all, I would like to thank my family: my sister Maja, my mother Milka and my grandmother Višnja to whom I dedicate this thesis. I would have never achieved anything without their love and support.

ABBREVIATIONS

COP1	Constitutive Photomorphogenesis 1
GA	gibberellic acid
GA2ox2	GA2-oxidase 2
DIF	difference between DT and NT
DT	day temperature
FR	far-red light
HIRs	high irradiance responses
HFR1	LONG HYPOCOTYL IN FAR-RED
HY5	Long HYpocotyl 5
LAF1	LONG AFTER FARRED LIGHT
LIP1	COP1 orthologous protein in pea
LFRs	low fluence responses
LONG1	HY5 orthologous protein in pea
NT	night temperature
PAS	Per/Arnt/Sim
PhyA-E	phytochromes A to E
PIF	Phytochrome Interacting Factors
Pfr	phytochrome in its far-red light absorbing form
Pr	phytochrome in its red light absorbing form
R	red light
R: FR ratio	ratio of red light to far-red light
RH	relative humidity
VLFRs	very low fluence responses
WT	wild type

TABLE OF CONTEST

1. INTRODUCTION

1.1. Control of stem elongation and it practical implications

Stem elongation is an important physiological process in plant development. Control of stem elongation is commonly required in greenhouse grown transplants and pot plants. The horticulture industry favors short and compact plants since, they are easier to handle, pack and transport in comparison to more elongated plants. In addition, smaller plants need less space and the initial costs are accordingly reduced. Thus, compact plants are ideal throughout the whole production process.

Plant height depends of several factors. Light, temperature, hormones (e.g. gibberellins (GAs)) and nutrients all contribute to the regulation of growth and development of the stem. In the last 40-50 years, plant growth has been very much controlled in greenhouses through application of chemical growth retardants. In the recent few years, a lot of attention has been given to non-chemical regulators of plants growth. The use of some growth retardants such as daminozide and paclobutrazol are restricted in many European countries, because of potential negative effects to human health and the environment (Erwin et al., 1995). Due to this, many experiments have been conducted to find other practical means of regulating plant height. For example, (Mortensen and Stromme, 1987) have suggested that manipulation of environmental factors would affect control of plant height. Much of the today's applied research on greenhouse crops is dealing with effects of environmental conditions on plant growth and quality.

Plants are sessile organisms which are constantly bombarded by numerous environmental signals (reviewed by Koornneef et al., 2002). As mentioned above there are several factors known to contribute to the regulation of growth and development of the stem. As a consequence, plants have developed a complex system of different receptors and signal transduction pathways that help them respond properly to each of the signals (Briggs and Olney, 2001). In the following text I will summarise the latest knowledge about two important environmental factors affecting stem elongation in horticulture, namely light and temperature.

1.2. Pisum sativum as model plant to study stem elongation

Pisum sativum, the common pea, has been a model organism in plant research for more than a century and it has been shown to be a good model for studying stem elongation due to several reasons: 1) it responds well to different temperature and light regimes (different day and night temperatures (DIF), temperature drop treatments etc.), 2) several mutants are available, 3) many important genes involved in control of stem elongation have been characterized (light receptors, hormone metabolism genes, photomorphogenesis-genes), 4) it is easy to grow and it grows quickly.

Isolation and characterization of mutants played important part in our understanding of the light signal transduction. Several different phytochrome and photomorphogenesis mutants have been identified in pea. Such mutants were compared with the wild type (WT) in this thesis in order to study the signaling associated with the effects of light, temperature and irradiance on stem elongation. The mutants included the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1*. Each mutant will be explained in more details further in the text.

1.3. The effects of light on stem elongation

Light is a very important abiotic factor in most ecosystems. It is used as a source of energy but also provides information about the environment. Plants display different growth behaviors in dark and light. In the dark they have elongated stems, undifferentiated chloroplasts and unexpanded leaves. The process is called skotomorphogenesis (dark development). On the other hand, light-regulated plant development involves the inhibition of stem elongation, the differentiation of chloroplasts and accumulation of chlorophyll, and the expansion of leaves, a phenomenon known as photomorphogenesis.

Plants have developed many different light-absorbing molecules to sense light intensity, light duration, light direction and spectral composition. These processes are coordinated by several classes of photo-receptors: the red- (R) and far-red (FR) light absorbing phytochromes, blue (B) light-U-A receptors (cryptochromes, phototropins and others) as well as at least one UV-B receptor (UV-resistant locus (UVR8)) (Whitelam et al., 1998; Kendrick and Kronenberg, 1994; Briggs and Olney, 2001; Briggs et al., 2001; Rizzini et al., 2011). Among the most extensively studied family of photoreceptors that plant use to distinguish the presence of light and light quality are the phytochromes.

1.3.1. Phytochrome biochemistry and functions

Phytochromes were biochemically characterised already in the late 1950s and considered to be very important in mediating various physiological and developmental processes in plants (Borthwick et al., 1952). During 40 years of extensive research it was generally assumed that a single phytochrome mediates the many R and FR reversible photoresponses. By 1989, scientists discovered the existence of more phytochromes, reporting two different phytochromes in pea and five different phytochromes in *A. thaliana* (Sharrock and Quail, 1989; Clack et al., 1994). Even though all phytochromes share some common characteristics, they also show varying amino acid sequence. Phytochromes are soluble chromoproteins consisting of a light-absorbing pigment named chromophore and a polypeptide chain named the apoprotein. Phytochromes exist in two inter-convertible forms, Pr and Pfr (Quail, 1997). In the first form, phytochrome absorbs R light while in the second, it absorbs FR light. When a molecule of Pr absorbs a photon of R light (660 nm), it is instantly converted into a molecule of Pfr, and when a molecule of Pfr absorbs a photon of FR light (730 nm), it is instantly converted to Pr. This reversible process is referred to as photoconversion (Smith, 1995) (Figure 1). Pfr is biologically active and Pr is regarded biologically inactive.

In its native state, phytochrome is a dimer which consists of four main regions; a bilin lyase domain, a phytochrome (PHY) domain, a Per/Arnt/Sim (PAS) domain and a kinase domain. In the Pr form, phytochrome is localized in the cytosol, but when Pr is converted to Pfr, a cis/trans isomerization occurs that exposes two nuclear localization signals in the PAS domain. This allows the molecule to be transported into the nucleus where it functions as a transcription factor (Rudiger et al., 1983; Nakasako et al., 2005).

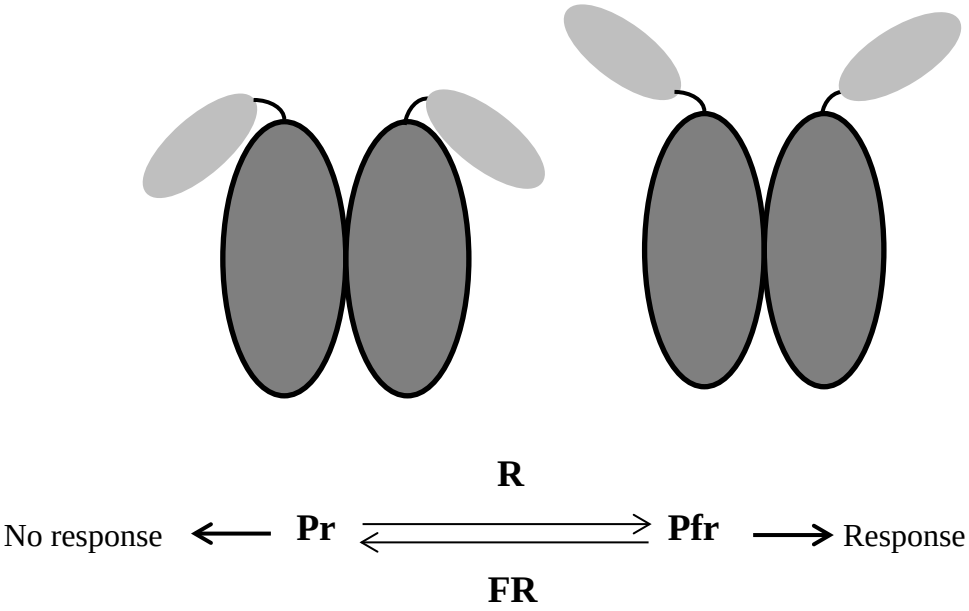
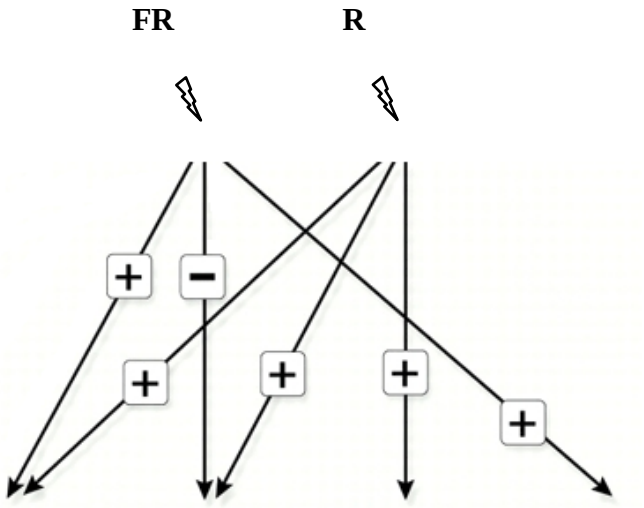


Figure 1. The isomerization of phytochrome. Phytochrome acts as a molecular switch in response to red (R) and far-red (FR) lights. Absorption of R light by Pr (R light absorbing form) converts the protein to the Pfr. Absorption of FR light by Pfr (FR absorbing form) converts the conformation back to Pr. The figure is adapted from Bae et al. (2008).

By measuring phytochrome conversion between the active and inactive state, plants are able monitor the light conditions they are exposed to. It has also been shown that the spectra significantly overlap to some extent, which means that an absorbed photon can affect the photoconversion in both directions. For example, saturating R converts about 80% of the total phytochrome to the Pfr form, whereas saturating FR results in about 97% Pr and 3% Pfr (Hartmann and Cohnen Unser, 1973).

The physiological and developmental responses that are known to be mediated by phytochromes include chloroplast development, initiation of germination, inhibition of cell elongation, regulation of gene expression and photoperiodic control of flowering (Mullet, 1988; Chory, 1991; Thompson and White, 1991). Most of the mentioned responses can be divided into 3 different groups: 1) very low fluence responses (VLFRs), 2) low fluence responses (LFRs) and 3) high irradiance responses (HIRs) (Casal et al., 1998). Germination of *A. thaliana* is an example of VLFRs, the control of lettuce seed germination is a good example of LFRs, while inhibition of hypocotyl elongation growth is an example of HIR (Neff et al., 2000). In addition, phytochromes are also classified according to their stability upon light exposure. The light stable phytochrome is termed Type II phytochrome and the light-labile phytochrome is termed Type I phytochrome (Furuya et al., 1989; Clough et al., 1997; Sullivan et al., 2003). The difference in stability of the two groups of phytochromes

upon light exposure allows them to carry out different functions in the plant during its development.



Response mode:	VLFR	LFR	R-HIR	FR-HIR
Phytochrome:	phyA	phyB	phyB	phyA

Figure 2. The effects of red (R) and far-red (FR) light on very low fluence responses (VLFR), low fluence responses (LFR), R-high irradiance responses (HIR) and FR-HIR modes and the involvement of phytochrome A (phyA) and phytochrome B (phyB). The picture is adapted from (doi:10.1093/embo-reports/kvf222).

1.3.2. Different phytochrome types

Phytochromes are encoded by a small multigene family. In *A. thaliana*, five members have been described (*PHYA* to *PHYE*; (Mathews et al., 1997)). Among them, phyA and phyB are the best characterized (Smith, 2000; Quail, 2002) and they will be discussed in more details in the following text. The light-labile phyA molecule is the most abundant phytochrome in dark-grown plants (Clough and Viestra, 1997). Furuya and Schäfer (1996) reported phyA as the sensor for very low fluoresce responses and for absorption of continuous FR light. On the other hand, the phyB molecule is in charge for the photoperception of R light and it has been shown to be classical R/FR light reversible molecular switch (Furuya and Schäfer, 1996). In dark-grown plants the abundance of the light-stable phyB protein is about 50 times lower than that of the phyA protein. In *A. thaliana* both phyA and phyB control seed germination, hypocotyl growth, cotyledon unfolding, greening, hook opening, flowering and the gene expressions of light-harvesting proteins and β-tubulin (Casal et al., 1998). phyB has the most

important role in regulating seedling de-etiolation under high-irradiance R light. Under low-irradiance or continuous R light, phyA and phyB act together in regulating the process. In pea, however, phyA exerts a greater influence in R light responses compared to *A. thaliana* (Weller et al., 2001), showing both similar but also different functions in both plants.

1.3.3. Homologous genes: HY5 in Arabidopsis and LONG1 in pea

Even though phytochromes are considered as being well characterized, the phytochrome signal transduction pathways are still unclear. As mentioned above, phyA is regulating numerous responses to FR light, whereas phyB is the predominant phytochrome regulating reactions to R light. In light conditions, both phyA and phyB act as suppressors of COP1 (CONSTITUTIVELY PHOTOMORPHOGENIC 1) and PIFs (PHYTOCHROME INTERACTING FACTORS). The COP1 protein acts as an E3 Ub ligase, targeting several photomorphogenesis-promoting transcription factors; HY5 (LONG HYPOCOTYL 5), LAF1 (LONG AFTER FARRED LIGHT) and HFR1 (LONG HYPOCOTYL IN FAR-RED 1). Once activated, phytochromes react with PIFs leading to PIFs' phosphorylation and degradation. On the other hand, COP1 has a positive effect on regulation of PIFs' protein levels. COP1 regulates HY5, a transcription factor which has a very important role in light signaling and photomorphogenesis, by being present in different levels in the light and dark periods. In darkness, COP1 labels HY5 in the nucleus for degradation (Bae et al., 2008), while in the light, COP1 is excluded from the nucleus, letting HY5 to activate light-responsive genes (von Armin et al., 1994). High throughput methods, like microarray studies, demonstrated a big overall overlap between light-regulated and COP1-regulated genes, proposing COP1 as a master gene repressing photomorphogenesis (Ma et al., 2002).

Recently, two orthologous transcription factors to *A. thaliana* HY5 and COP1 were found in pea and named LONG1 and LIP1, respectively (Weller et al., 2009). Even though LONG1 and HY5 were shown to have many similar functions in regulating photomorphogenesis, they can still be distinguished from each other. For example, LONG1 has an additional N-terminal domain in comparison to HY5. Generally, LONG1 acts downstream of the photoreceptors phyA and phyB and interacts with LIP1. LONG1 is essential for de-etiolation under R, B, and FR light (Weller et al., 2009). There are two different phases described during de-etiolation in pea 1) a very fast initial drop in active GA_1 content, 2) followed by a gradual recovery to dark levels. During the light-regulation phase of GA-levels, it was found that LONG1 targets *GA2-oxidase 2* (*GA2ox2*), although other *GA2ox* genes are also regulated by light in *A. thaliana*.

LIP1 is necessary to maintain high GA level during etiolated growth in the dark, and it functions by somehow repressing LONG1. This repression is most likely to occur at a post-transcriptional stage (Weller et al., 2009).

The *long1* mutants described in Weller et al. (2009) are not completely unresponsive to light, but exhibit a weaker transient down-regulation of GA, and the elongation of the stem is slightly inhibited, which suggests that other genes act together with LONG1 in regulating the process of de-etiolation.

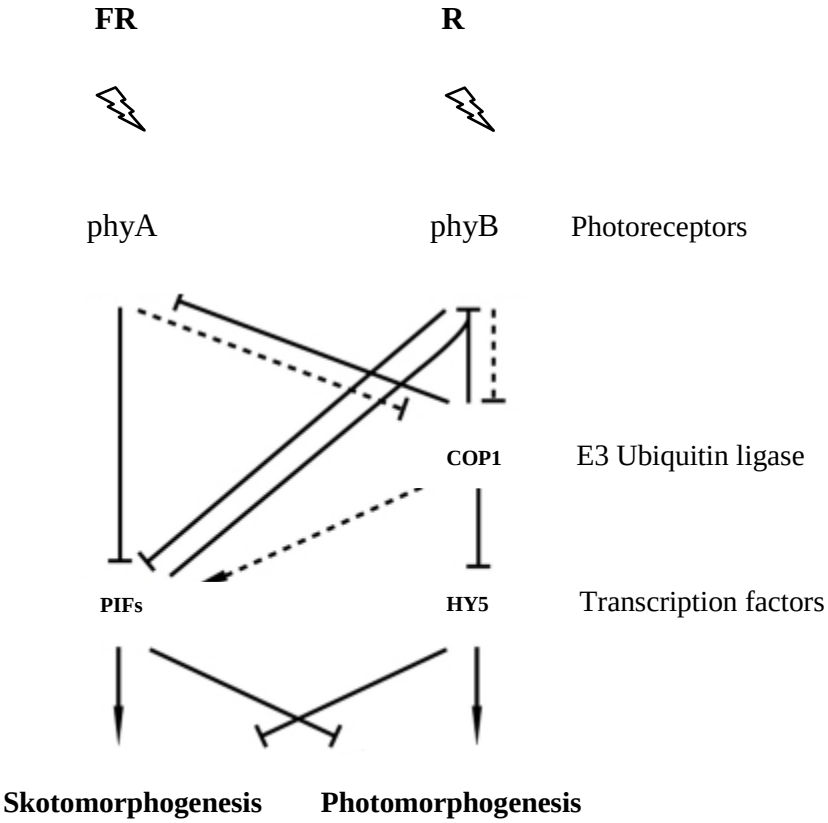


Figure 3. Phytochrome signaling pathway, simplified version. Under FR and R light, phyA and phyB respectively, suppress two main light signaling pathways: COP1 and PIFs. COP1 controls the level of the transcription factor HY5, and the degree of photomorphogenic development. PIFs are involved in skotomorphogenesis. Arrow, positive regulation; bar, negative regulation; solid line, direct regulation; dotted line, indirect regulation. Image adapted from Lau and Deng (2010).

1.4. Thermoperiodic control of stem elongation

Temperature is another important factor affecting growth and development in plants. Optimal growing temperature is different for different plant species. In addition, these optimum temperatures are different for the different developmental stages during the life cycle of the plant. It is well accepted that temperature plays important part in morphology of greenhouse

crops. In addition, it has been found that morphology of a plant can be affected by the differences in day temperature (DT) and night temperature (NT) (Erwin et al., 1989).

It has been reported that stem elongation in many different plants, can be manipulated by the relation between DT and NT (Myster and Moe, 1995). In general, plants grown under a negative temperature difference [negative DIF; day temperature (DT) < night temperature (NT)] elongate less than those grown under positive DIF (DT > NT). Also, shorter periods with reduced temperature (temperature drops) are efficient to reduce shoot elongation in many species (Myster and Moe, 1995). The same authors showed that DIF do not only influence stem elongation and plant height, but also leaf orientation, shoot orientation, chlorophyll content, lateral branching, as well as flower stalk elongation. All these studies suggest that a negative DIF treatment and temperature drops can be a tool to produce compact plants. In this way chemical growth retardants are being replaced. For example, control of stem elongation, by temperature drop and DIF treatments have become central tools in Begonia x hiemalis and poinsettia (Euphorbia pulcherrima), which is one of the economically most important flowering pot plants worldwide (Myster et al., 1995).

Although being a very effective tool, in warmer periods and regions negative DIF and temperature drops are considered expensive methods due to the need for cooling to be able to obtain such temperature regimes. On the other hand, Northern countries are very good examples where a temperature drop can be obtained by simple and inexpensive techniques such as opening vents in greenhouses during the early morning period when the outdoors temperature is lower than inside the greenhouse. Such techniques were shown to be effective in controlling shoot elongation in a number of species. The best example is poinsettia, an ornamental plant very popular in Norway, with more than 6 million plants sold every year around Christmas time. Ueber and Hendriks (1992) reported that even a very short, 2 h, temperature drop from 24 °C to 8 °C, reduced the stem elongation by more than 50% in poinsettia.

1.4.1. Thermoperiodic control of GA metabolism

Even though thermoperiodic responses in plants have been studied during a number of years, still there is lack in knowledge about the basic mechanisms behind the process. Many studies have suggested that the effects of daily temperature alterations on stem elongation are related

to the metabolism and sensitivity to GA (Erwin et al., 1989; Jensen et al., 1996; Grindal et al., 1998; Stavang et al., 2005). GAs are hormones that control plant growth and development throughout their life cycle. Particularly they are well known to act in regulation of stem elongation by controlling cell elongation and cell division. Several genetic experiments, using GA mutants, have shown involvement of GA in thermoperiodic control of stem elongation (Tangerås, 1979; Zieslin and Tsujita, 1988; Moe, 1990; Ihlebekk et al., 1995; Grindal et al., 1998). Based on all these results, two different hypotheses were suggested 1) under negative DIF plants contain less endogenous bioactive GA, or 2) DIF alters the tissue sensitivity to endogenous GA. In order to test these two hypotheses Grindal et al. (1998) studied the effects of applied GA_3 on stem elongation in pea grown under both negative DIF and positive DIF. The authors concluded that tissue sensitivity does not play a big role in regulating stem elongation in response to DIF, since the differences on stem elongation were not big. On the other hand, several experiments done in *Campanula* (Jensen et al., 1996), tomato (Langton et al., 1997) and pea (Grindal et al., 1998) have shown that plants grown under positive DIF contained more endogenously bioactive GA_1 than those grown under negative DIF.

Furthermore, Stavang et al. (2005) did a study in pea as the model organism, where they looked at the effects of negative DIF (DT13°C/NT21°C) compared to positive DIF (DT21°C/NT13°C). Stem elongation was reduced by 30% after 12 days under negative DIF. In addition the same plants have 55% less of GA_1 content in the apical stem tissue. Under negative DIF as compared to positive DIF e a high expression of the *PsGA 2-oxidase 2* (*PsGA2ox2*) gene was observed. Another study done by the same research grope showed that the expression of *PsGA2ox2* was not only stimulated in negative DIF but also by a temperature drop treatment during the day. A temperature drop from 21°C to 13°C in the middle of the light period increased expression of *PsGA2ox2* and in this way reduced the stem elongation rate, even after only 2 h of the treatment. On the other hand, the same temperature drop in the night period did not increase expression of *PsGA2ox2* and this probably lead to smaller effect on stem elongation reduction. On basis of these studies, Stavang et al. (2005) suggested *PsGA2ox2* to be the main mediator of thermoperiodic effects on stem elongation in pea.

2. OBJECTIVES OF THE STUDY

As discussed above, Stavang et al. (2007) have shown that a temperature drop in the middle of the day is more efficient to reduce shoot elongation in pea than a temperature drop in the middle of the night. Also, increased irradiance or transfer of dark-germinated plants to light is known to affect elongation growth by inhibiting internode elongation (e.g. Weller et al., 2009). Such treatments may be practical tools in control of shoot elongation in greenhouses.

The idea of the study in this thesis was to improve our understanding of the signaling mechanism underlying thermoperiodic control of shoot elongation and the interaction between temperature drop and irradiance in this respect. The main objective was to test how temperature drops during day and night as well as increased irradiance affect WT of pea and pea plants mutated in central photomorphogenesis-related genes, *PHYTOCHROME A* and *B* as well as the the *HY5* ortolog *LONG1* and the *COP1* ortolog *LIP1* (the *long1, lip1, phyA, phyAlong1, phyB* and *phyBlong1* mutants). Thus, we aimed to inquire the degree of reduction in elongation growth as well as the effect on other growth parameters, when plants were exposed to temperature drop treatments, and to the combination of increased irradiance and temperature drop.

3. MATERIALS AND METHODS

3.1. Plant materials and growing conditions

Seed of pea (*Pisum Sativum* L), wild type (WT) 'Torsdag' and different mutants *(long1, lip1, phyA, phyAlong1, phyB* and *phyBlong1*) were sown in 11 cm pots. The pots were placed in growth chambers (Conviron, Winnipeg, Canada) which had space for 8 trolleys (one trolley has space for 20 pots (size 11 cm). The plants were grown under the following environmental conditions: the light period was 12 h (from 07.00-19.00 h), with an irradiance of 50 μmol m^{-2} s^{-1} at 400-700 nm and a red:far red (R:FR) ratio of 1.7. Both fluorescent tubes (F96T12, Sylvania, Danvers, MA, USA) and incandescent lamps (Osram, Munich, Germany) were used. The air humidity was adjusted to approximately 0.5 kPa water vapour pressure deficit at this stage and during the experimental treatments described below. The plants were exposed to a constant temperature of 21 ± 0.5ºC and watered daily with a complete nutrient solution of EC= 1.5 mS cm^{-1}.

3.2. Experimental procedures

To investigate the role of different photomorphogenesis-related genes in response to temperature drops and increased irradiance, after a growing period of 6 days 10 plants of each of WTand the *long1, lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants were exposed to 5 different treatments as follow:

Treatment 1 *(control)* - constant irradiance (50 µmol m^{-2} s^{-1}); constant temperature (21°C); and constant relative humidity (RH) of 79-80%.

Treatment 2 *(increased irradiance and constant temperature)* - increased irradiance from 50 µmol m^{-2} s^{-1} to 150 µmol m^{-2} s^{-1} for 4 h in the middle of the light period; constant temperature of 21°C; and constant RH of 79-80%.

Treatment 3 *(constant irradiance and day drop)* - constant irradiance of 50 µmol m^{-2} s^{-1}; with a temperature drop from 21°C to 13°C for 4 h in the middle of the light period and RH of 79-80% at 21°C and 67% at 13°C.

Treatment 4 *(increased irradiance and day drop)* - increased irradiance from 50 µmol m^{-2} s^{-1} to 150 µmol m^{-2} s^{-1} and a temperature drop for 4 h from 21°C to 13°C in the middle of the light period and RH of 79-80% at 21°C and 67% at 13°C.

Treatment 5 *(constant irradiance and night drop*) - constant irradiance of 50 µmol m^{-2} s^{-1}; with a temperature drop from 21°C to 13°C for 4 h in the middle of the night (dark) period and RH of 79-80% at 21°C and 67% at 13°C.

Since 3 chambers only were available at the time, treatment 1, 3 and 5 was performed in one experiment and treatment 1, 2, 4 in another. Both experiments were done twice. In all replicate experiments 10 plants were included per treatment and genotype and registrations were performed on all plants if otherwise not mentioned in the following section.

3.3. Registrations

3.3.1. Plant height

Height of the pea plants (Figure 4) was measured from the pot edge to the shoot apical meristem at day 0, 3, 6, 10 and 15. If plants were lower than the pot edge the negative value

was noticed. This was important in order to correct all values when cumulative elongation growth was calculated. Cumulative growth relative to day 0 was calculated and a cumulative growth curve was plotted.

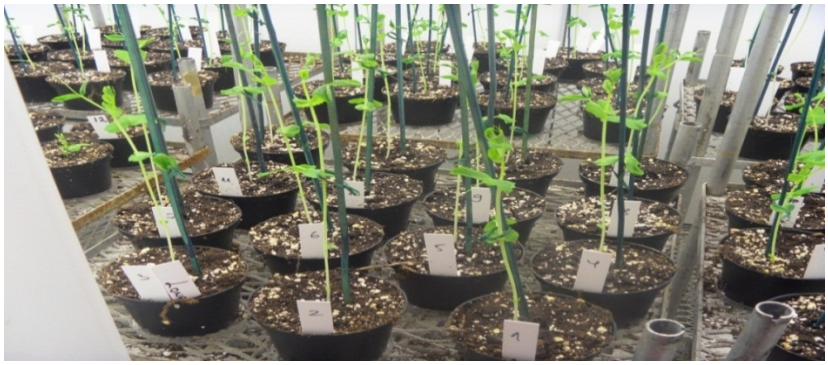


Figure 4. A wild type (WT) pea plant at day 6 of the experiment. The pot sizes are identical.

3.3.2. Number of leaves

Number of leaves in all plants was registered by making a spot with a permanent black pen on the uppermost unfolded leaf on each measurement day (Figure 5). Cumulative leaf formation was calculated and a cumulative leaf formation curve was plotted.

Figure 5. Pea plants marked with a black pan every time a new leaf was detected. The number of leaves was measured at days 3, 6, 10 and 15 after start of the experimental treatments. The pot sizes are identical.

3.4. Leaf area, dry weight, chlorophyll content

3.4.1. Total leaf area

On day 15 we measured total leaf area on 3 plants per treatment per genotype. Leaf area was determined with a Li-Cor LI-3100 area meter (Li-CorBiosciences, Lincoln, NE, USA; Figure 6).

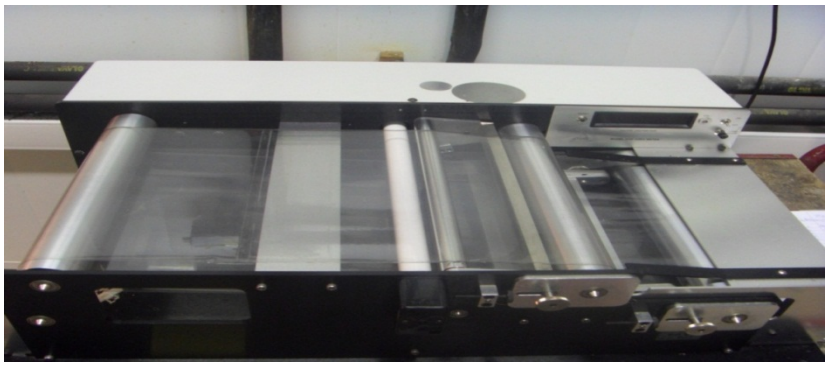


Figure 6. Li-Cor LI-3100 area meter (Li-CorBiosciences, Lincoln, NE, USA) used to measure total leaf area.

3.4.2. Total dry weight

The same plants that were used to calculate leaf area, were used also for total dry weight determination. The plants were then separated into leaves and stem tissue (stem tissue included stem tendrils and petioles) and were placed in paper bags left to dry in a tumble dryer for two days at a temperature of 65°C. After drying the total dry weight of stem and leaves were measured. Steam mass ratio and leaf mass ratio were calculated as follows:

Stem mass ratio=steam dry weight/(stem dry weight + leaf dry weight)

Leaf mass ratio=leaf dry weight/(stem dry weight + leaf dry weight).

3.4.3 Chlorophyll content

By using a Hansatech CL-01-chlorophyll content meter (Hansatech Instruments, King's Lynn,Norfolk; Figure 7) relative chlorophyll content was estimated for each leaf (from the bottom to the top) on 4 plants per treatment per genotype. Each measurement was done twice. In some cases chlorophyll levels could not be measured due to damaged leaves.

Figure 7. Hansatech CL-01-chlorophyll content meter (Hansatech Instruments, King's Lynn,Norfolk) used to measure chlorophyll content in leaves.

3.3. Statistical analyses

The effects of the two experimental factors, treatment and genotype, on measured growth parameters were analysed using a general linear model (GLM) approach ($p \leq 0.05$) in the Minitab statistical software (Minitab 15.1, Minitab Inc., PA, USA). For these analyses, values from two replicate experiments were pooled. Tukey's test was used for testing for differences between means.

4. RESULTS

To investigate the mechanism underlying thermoperiodic responses using pea as a model system the effects of temperature drop during day and night and increased irradiance were investigated in WT pea plants and different photomorphogenesis mutants of pea, namely *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1*.

4.1. Effects of temperature drop treatments

In the first experiment all plants were subjected the following conditions:

1) Constant temperature (21°C) and constant irradiance (50 μmol m^{-2} s^{-1})

2) Temperature drop (from 21°C to 13°C) for 4 h in the middle of light period and constant irradiance (50 μmol m^{-2} s^{-1})

3) Temperature drop (from 21°C to 13°C) for 4 h in the middle of night period and constant irradiance (50 μmol m^{-2} s^{-1})

In the results plants exposed to constant temperature and irradiance are referred to as control plants or simply control, while plants exposed to the temperature drop during the day and the night are called, day drop and night drop, respectively.

4.1.1. The effects of day and night temperature drops on stem elongation

In the WT day drop reduced the stem elongation significantly, with 15% reduction at day 15 compared to the control (Figure 8). In contrast the night drop did not affect elongation significantly. In the *long1* mutant there was no difference in stem elongation between the control and any of the temperature drop treatments. On the other hand, the biggest effects of day drop and night drop were recorded in *lip1* mutant. At day 15 day drop and night drop significantly reduced the elongation compared to the control by about 23% and 60%, respectively.

In *phyA* the stem elongation was significantly reduced by day drop by approximately 15% at day 15, while there were no noticeable differences between night drop and control. In the *phyAlong1* mutant there were no clear, significant differences between the treatments, only a slight tendency of reduced elongation in the day drop treatment at day 15. In *phyB* there were no differences between day and night drop but stem elongation at day 15 was significantly reduced in both treated groups in comparison to the control. Temperature drop did not have any significant effect on *phyBlong1* mutant.

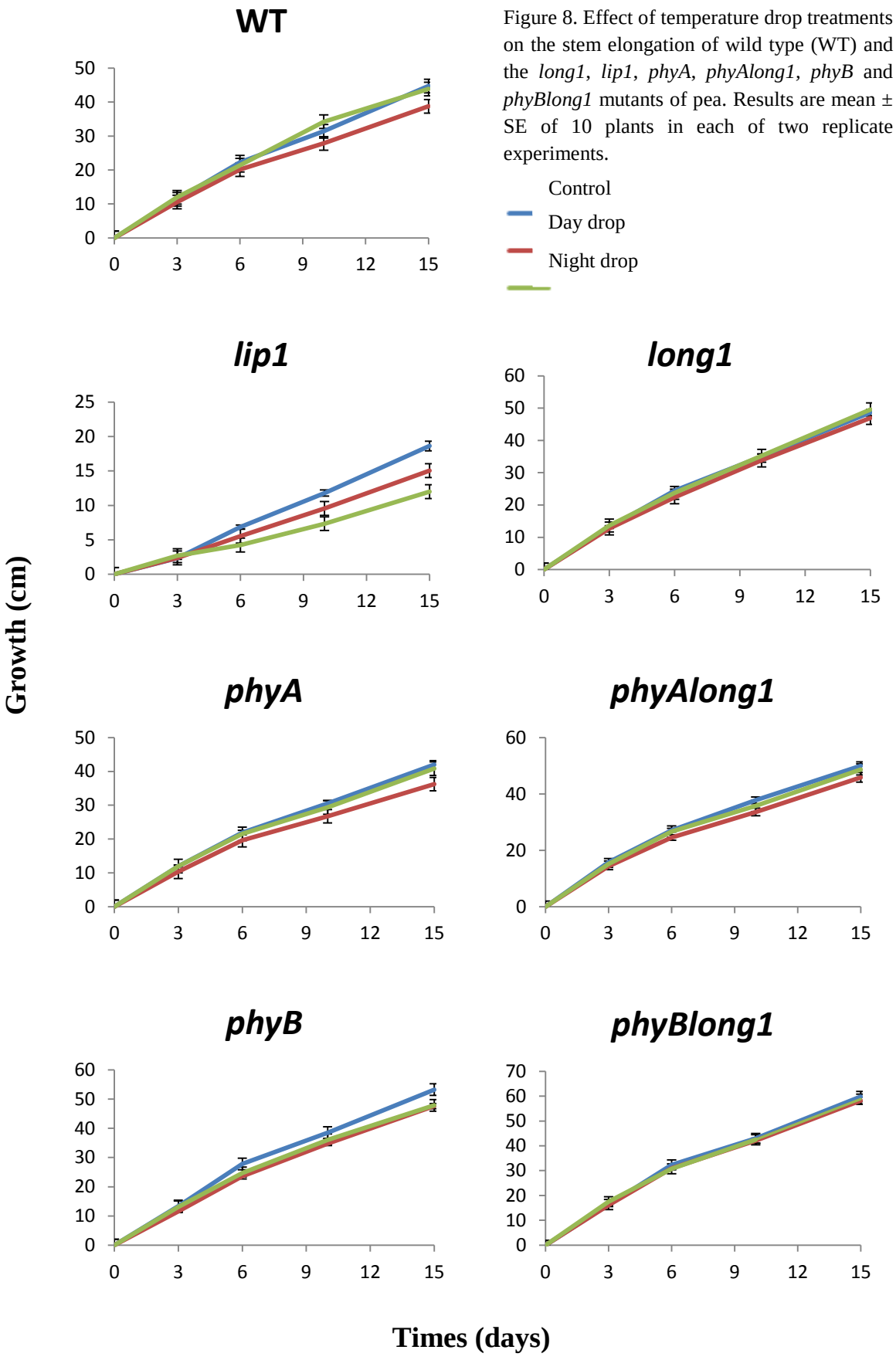


Figure 8. Effect of temperature drop treatments on the stem elongation of wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants of pea. Results are mean ± SE of 10 plants in each of two replicate experiments.

4.1.2. The effects of day and night temperature drops on number of leaves

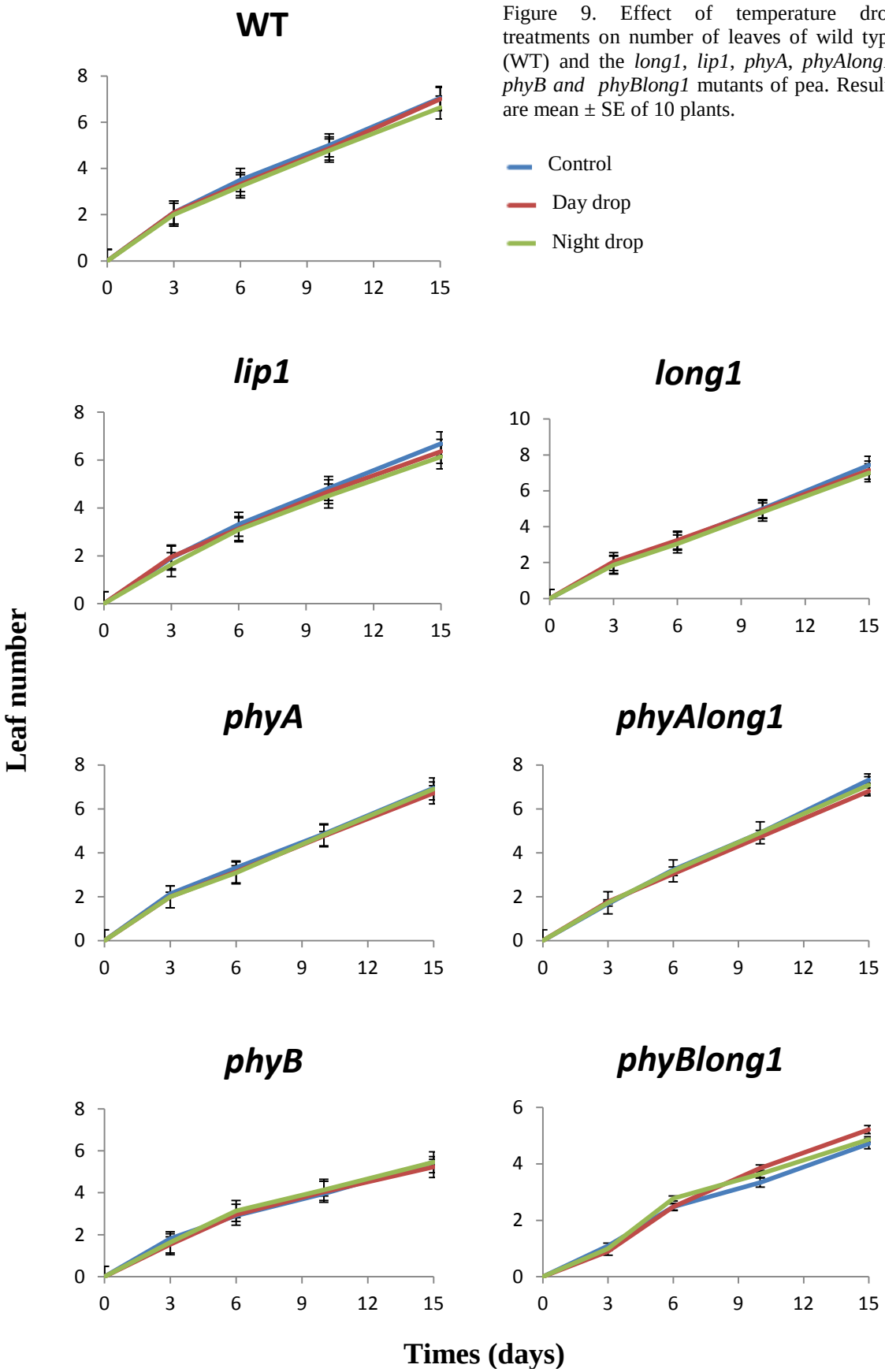
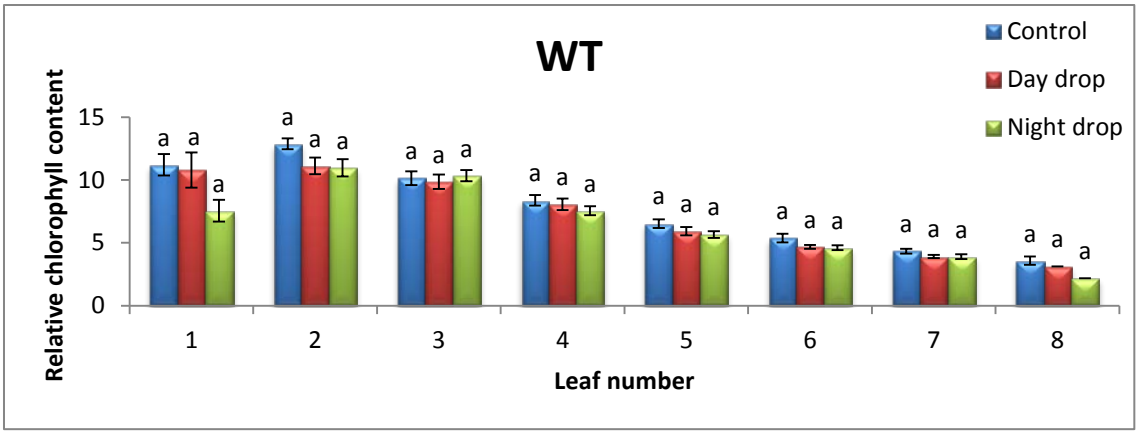


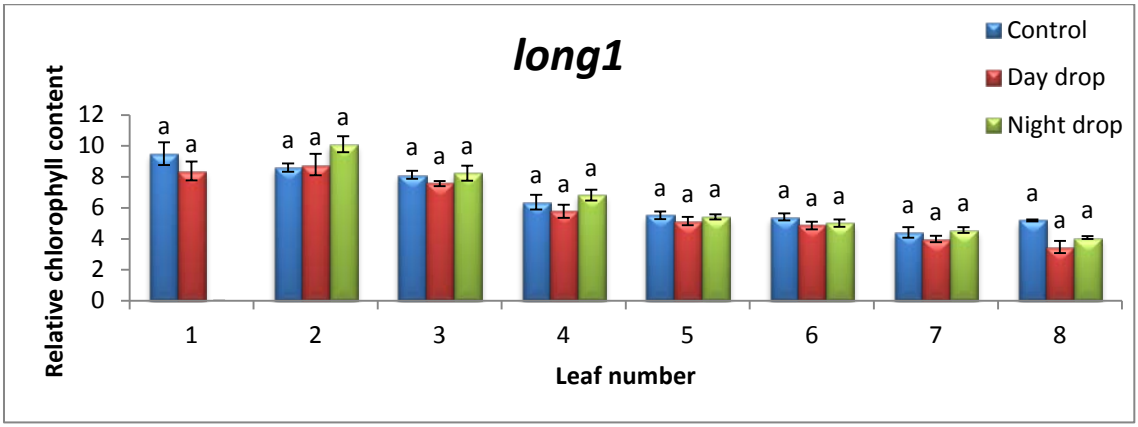
Figure 9. Effect of temperature drop treatments on number of leaves of wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB and phyBlong1* mutants of pea. Results are mean ± SE of 10 plants.

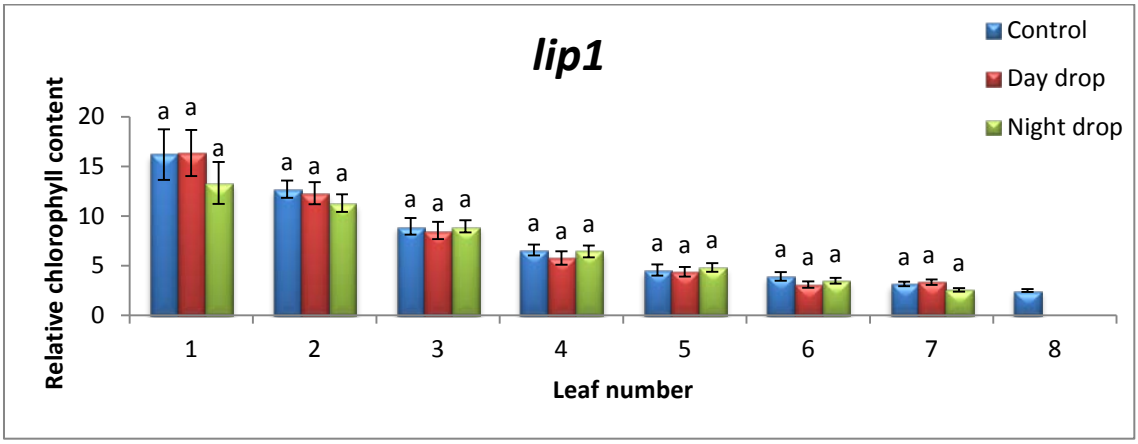
The leaf number at day 15 was very similar in all measured pants with around 7 leaves, except for *phyBlong1*, which then had about 2 leaves less (around 5 leaves). There were no significant differences in leaf number between day and night drop in any of the mutants (Figure 9).

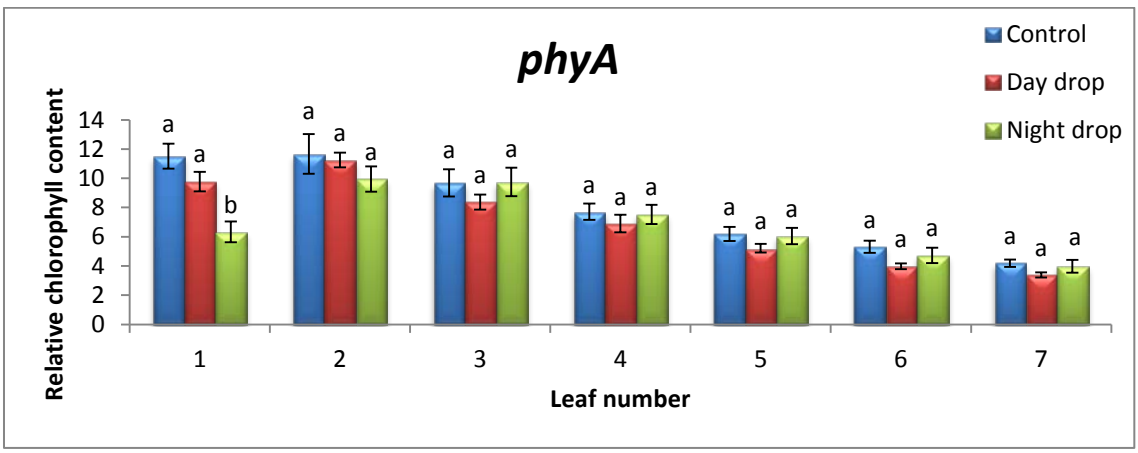
4.1.3. The effects of day and night temperature drops on chlorophyll content

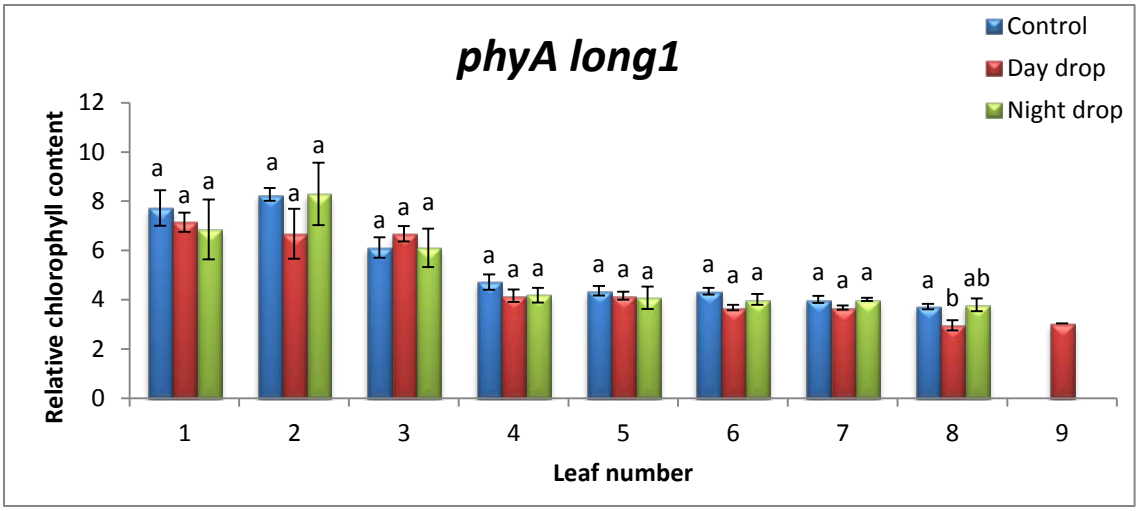
Relative chlorophyll content was estimated for each leaf, measuring from the bottom to the top, meaning from the oldest to the youngest leaf. As expected, the youngest leaves contained less chlorophyll than the older leaves (e.g. Stavang et al., 2009). Different plants appeared to respond slightly differently to the temperature drop treatments (Figure 10). However, there were no significant differences found in WT in any of the treatments, only a slight tendency of reduced chlorophyll content by night drop in the oldest and youngest leaf (leaf number 1 and 8). The situation in *lip1* resembled that of the WT with no significant differences in chlorophyll levels between the treatments, only trends of lower chlorophyll content in the oldest and youngest leaves (leaf 1 and 7) under night drop compared to the day drop and control. Also, in *long1* there was no significant difference in chlorophyll levels between the treatments, only a small trend of decreased chlorophyll levels in the youngest leaf under both night and day drop and possibly small trends of increased chlorophyll in leaf 2, 3 and 4. In *phyA* chlorophyll content was significantly reduced by night drop in leaf 1 but not in the youngest leaf (7). In several leaves of this mutant day drop appeared to result in slightly reduced chlorophyll levels in most of the other leaves. In *phyAlong1*, the situation was opposite for some leaves, day drop appeared to reduced chlorophyll content slightly in leaf 1 and 2, but increased it in leaf 4. The *phyB* and *phyBlong1* mutants were different from the other genotypes by having the lowest chlorophyll level in the oldest leaves. However, there were no significant different between different treatments in these mutants.

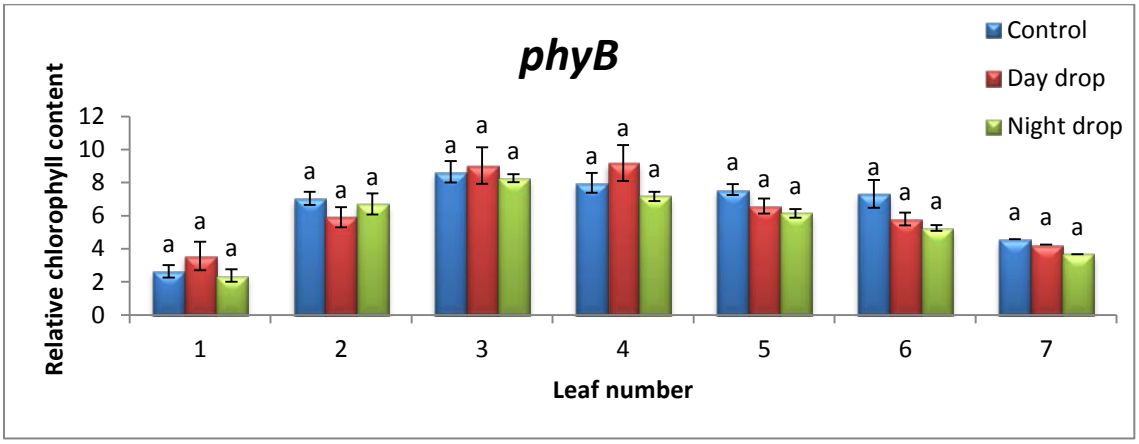

WT
Control
Day drop
Night drop
Relative chlorophyll content
Leaf number


long1
Control
Day drop
Night drop
Relative chlorophyll content
Leaf number


lip1
Control
Day drop
Night drop
Relative chlorophyll content
Leaf number


phyA
Control
Day drop
Night drop
Relative chlorophyll content
Leaf number





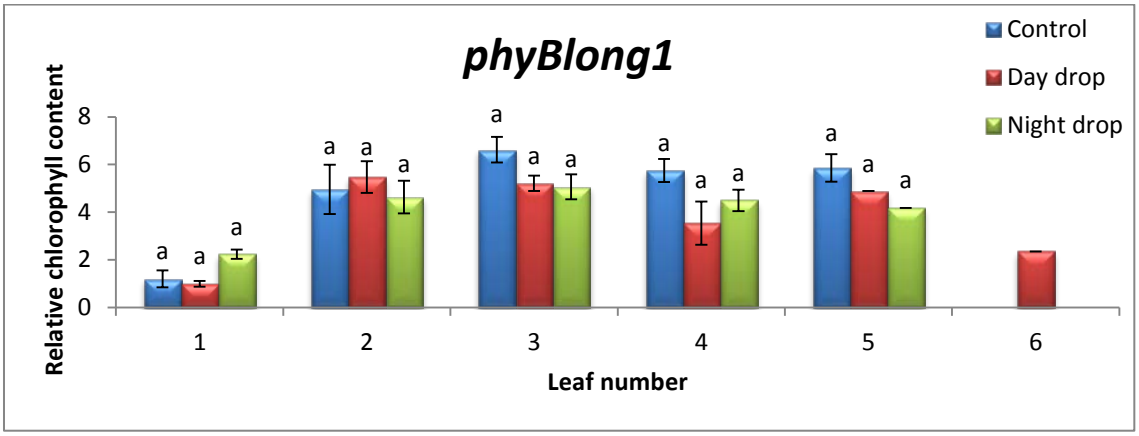
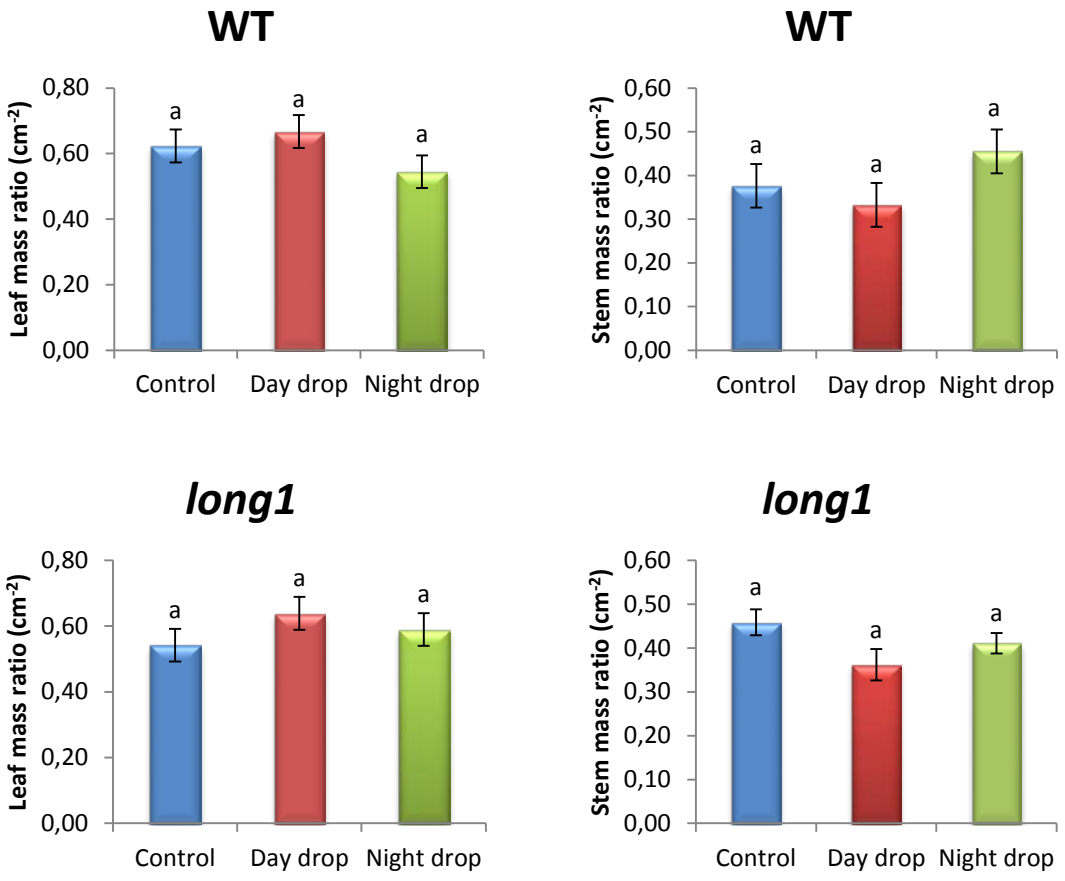


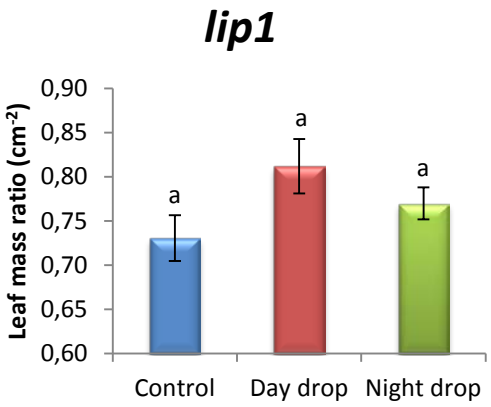
Figure 10. Effect of temperature drop treatments on chlorophyll content in wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants of pea from the oldest (1= lowest) to the youngest leaf (highest number). Results are mean ± SE of 4 plants in each of 2 replicate experiments. Within each leaf number different letters indicate significant differences and the same letters indicate no statistically significant difference ($p \leq 0.05$).

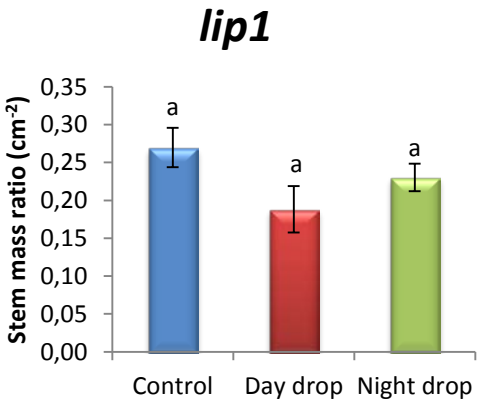
4.1.4. The effects of day and night temperature drops on leaf mass ratio and stem mass ratio

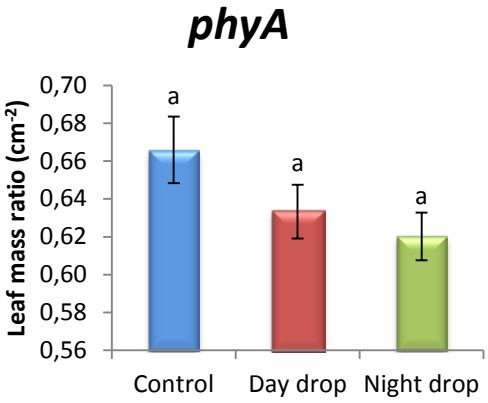
The leaf mass ratio and steam mass ratio were not significantly affected by the temperature drop treatments in neither the WT nor any of the mutants (Figure 11). However, there appeared to be slight (statistically insignificant) tendencies of increased leaf mass ratio and decreased stem mass ratio by day drop in WT, *long1* and *lip1*. In *phyBlong1* there appeared to be a slight tendency of increased stem mass ratio in night drop treatment.

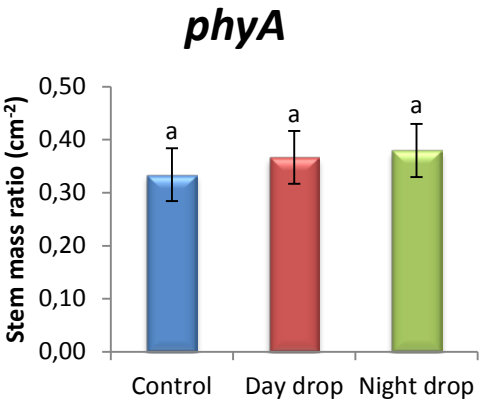
The different genotypes showed different resource allocation pattern. The *lip1* mutant seems to generally allocate more resources into leaves and less into stems compared to the WT. There is a tendency also of *phyB* allocating less resources into leaves and more into the stem and this is even more clear in the *phyBlong1* mutant. While the *long1*, *phyA* and *phyAlong1* mutants showed very similar pattern as seen in WT.

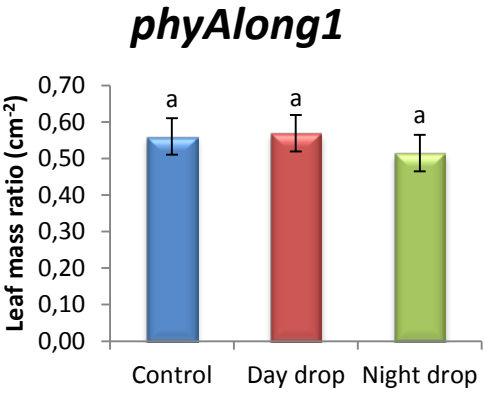


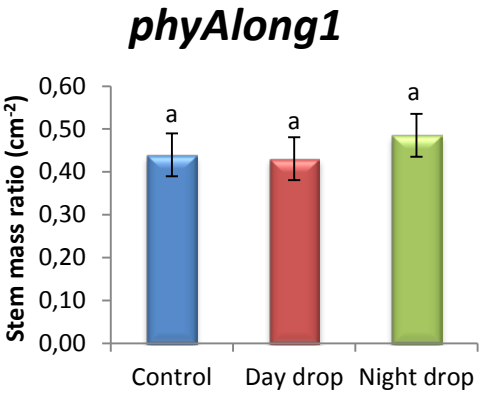

lip1
Leaf mass ratio (cm-2)
0,90
0,85
0,80
0,75
0,70
0,65
0,60
a
a
a
Control
Day drop
Night drop

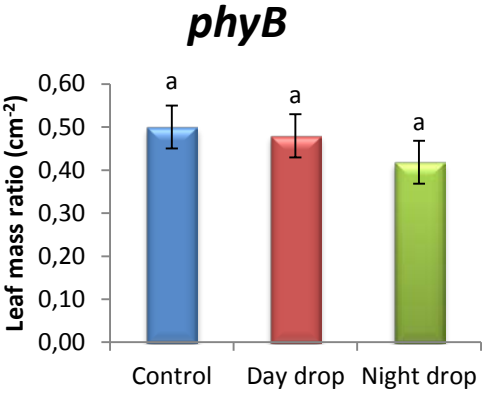

lip1
Stem mass ratio (cm-2)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
a
a
a
Control
Day drop
Night drop

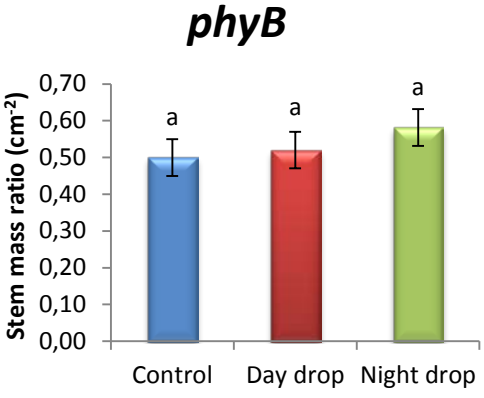

phyA
Leaf mass ratio (cm-2)
0,70
0,68
0,66
0,64
0,62
0,60
0,58
0,56
a
a
a
Control
Day drop
Night drop


phyA
Stem mass ratio (cm-2)
0,50
0,40
0,30
0,20
0,10
0,00
a
a
a
Control
Day drop
Night drop


phyAlong1
Leaf mass ratio (cm-2)
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
a
a
a
Control
Day drop
Night drop


phyAlong1
Stem mass ratio (cm-2)
0,60
0,50
0,40
0,30
0,20
0,10
0,00
a
a
a
Control
Day drop
Night drop


phyB
Leaf mass ratio (cm-2)
0,60
0,50
0,40
0,30
0,20
0,10
0,00
a
a
a
Control
Day drop
Night drop


phyB
Stem mass ratio (cm-2)
0,70
0,60
0,50
0,40
0,30
0,20
0,10
0,00
a
a
a
Control
Day drop
Night drop

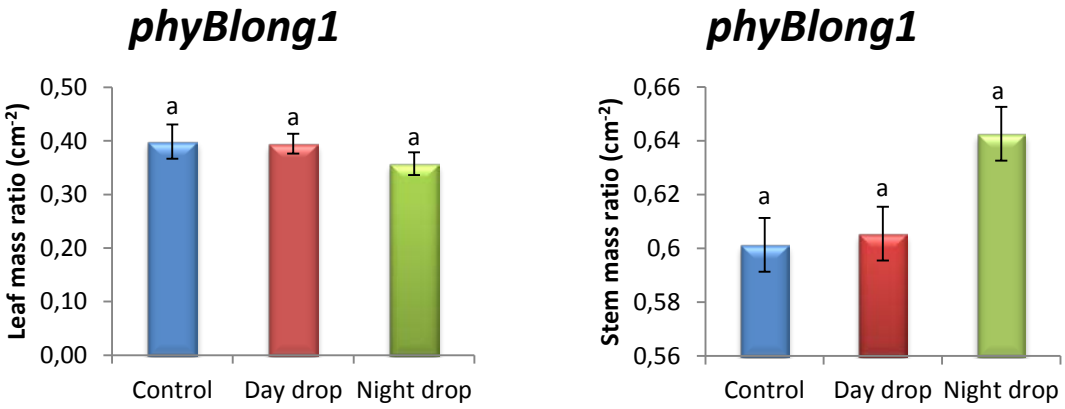


Figure 11. Effect of temperature drop treatments on leaf mass ratio and stem mass ratio in wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants in pea. Results are mean ± SE of 4 plants in each of 2 replicate experiments. Different letters within each staple graph indicate significant differences and the same latters indicate no statistically significant difference ($p \leq 0.05$).

4.1.5. The effects of day and night temperature drops on leaf area

The leaf area was generally not significantly affected by the temperature drop treatments in the different genotypes (Figure 12). The only exception was *phyBlong1* were a significantly larger leaf area was observed under night drop treatment. However, this was not observed in any of the other mutants.

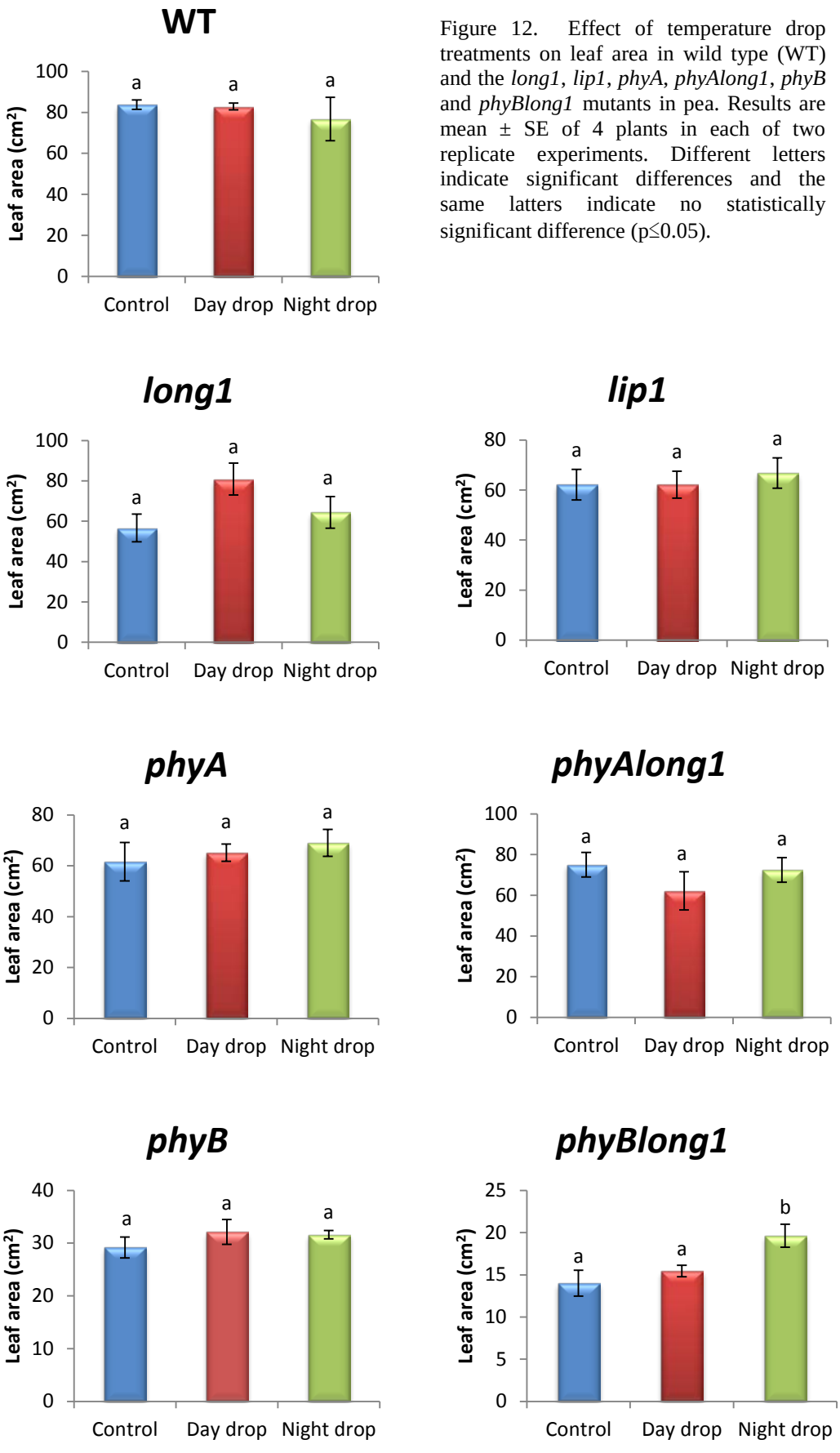


Figure 12. Effect of temperature drop treatments on leaf area in wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants in pea. Results are mean ± SE of 4 plants in each of two replicate experiments. Different letters indicate significant differences and the same latters indicate no statistically significant difference ($p \leq 0.05$).

4.2. Effects of increased irradiance and temperature drop in the day

In the second experiment the goal was to investigate the effects of interaction between temperature drop and irradiance. 10 plants of each of the WT and the *long1, lip1, phyA, phyAlong1, phyB and phyBlong1* mutants were subjected to following treatments:

1) Constant irradiance (50 μmol m^{-2} s^{-1}) and constant temperature (21°C)
2) Increased irradiance to 150 μmol m^{-2} s^{-1} for 4 h in the middle of the light period and constant temperature (21°C)
3) Increased irradiance to 150 μmol m^{-2} s^{-1} and temperature drop (from 21°C to 13°C) for 4 h in the middle of light period

In the results plants exposed to constant temperature and irradiance are referred to as control plants or simply control, while plants exposed to the constant temperature and increased irradiance are called increased irradiance, while increased irradiance and temperature drop are called increased irradiance + day drop. The same measurements were performed as in the first experiment.

4.2.1. The effects of increased irradiance and temperature drop in the day on stem elongation

In the WT increased irradiance in combination with the day drop decreased stem elongation significantly by about 45% at day 15 (Figure 13). In the *long1* mutant there were no significant differences between the two treatments and the control. Stem elongation was significantly reduced in *lip1*, *phyA* and *phyB* by about 60% at day 15 by increased irradiance + day drop compared to the control. In *phyBlong1* and *phyAlong1* there was no significant difference between the control and any of the two treatments at any time point.

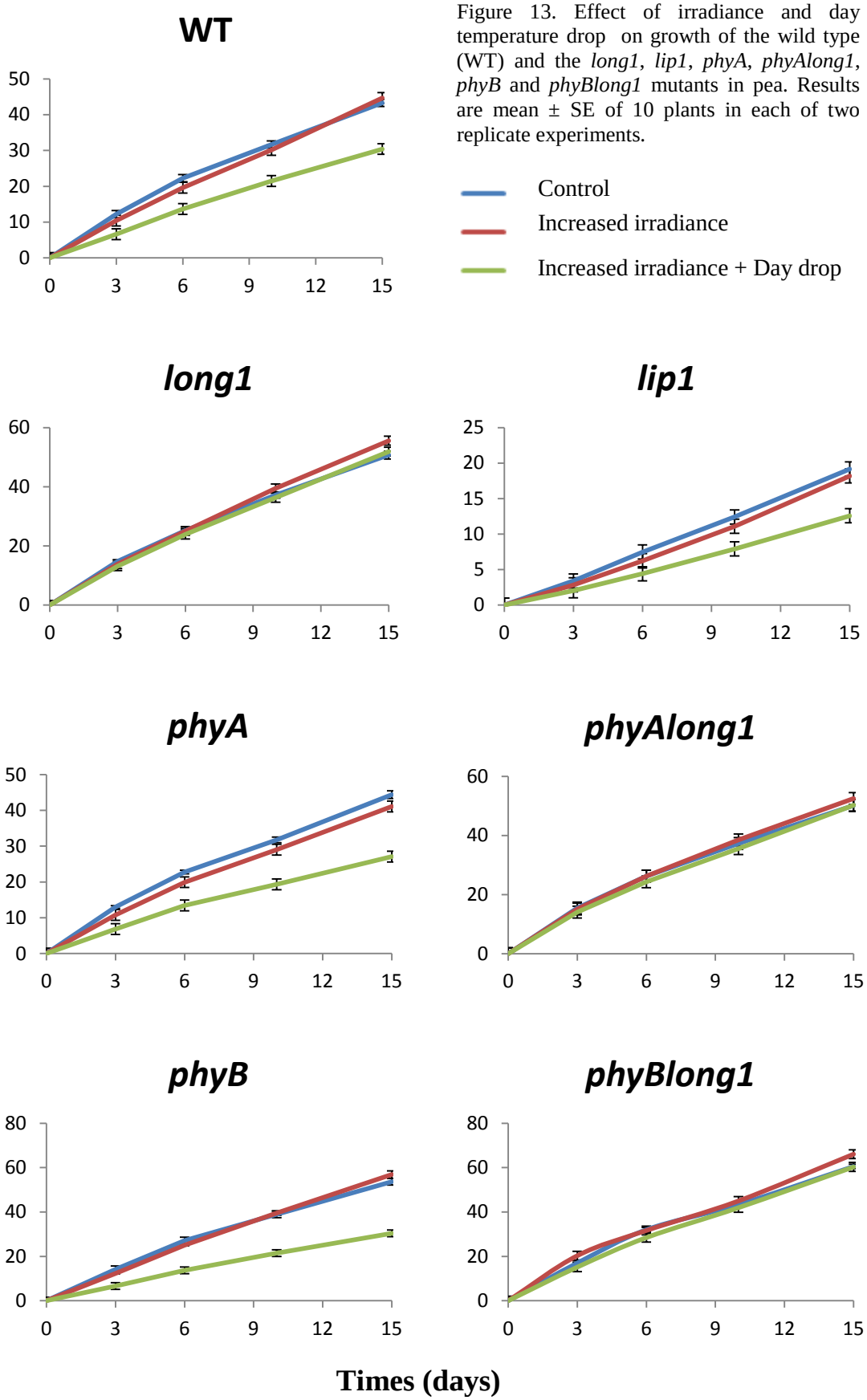


Figure 13. Effect of irradiance and day temperature drop on growth of the wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants in pea. Results are mean ± SE of 10 plants in each of two replicate experiments.

4.2.2. The effects of increased irradiance and temperature drop in the day on number of leaves

In the WT there was a significant trend of slightly increased numbers of leaves at day 15 under increased irradiance (around 8 leaves) compared to the control and the combined temperature drop and increased irradiance (around 6 leaves) (Figure 14). There were similar trends in the *lip1* and *phyA* mutants with significantly increased number of leaves only at day 15 in increased irradiance. Increased irradiance alone also significantly increased number of leaves in *phyB* at both days 10 and 15 in comparison with the control and increased irradiance + day drop. In *phyBlong1*, increased irradiance increased number of leaves significantly at day 6, 10 and 15, while increased irradiance + day drop were not different from the control (Figure 14). In the *long1 and phyAlong1* mutant there was no significant effect of the treatments on leaf number.

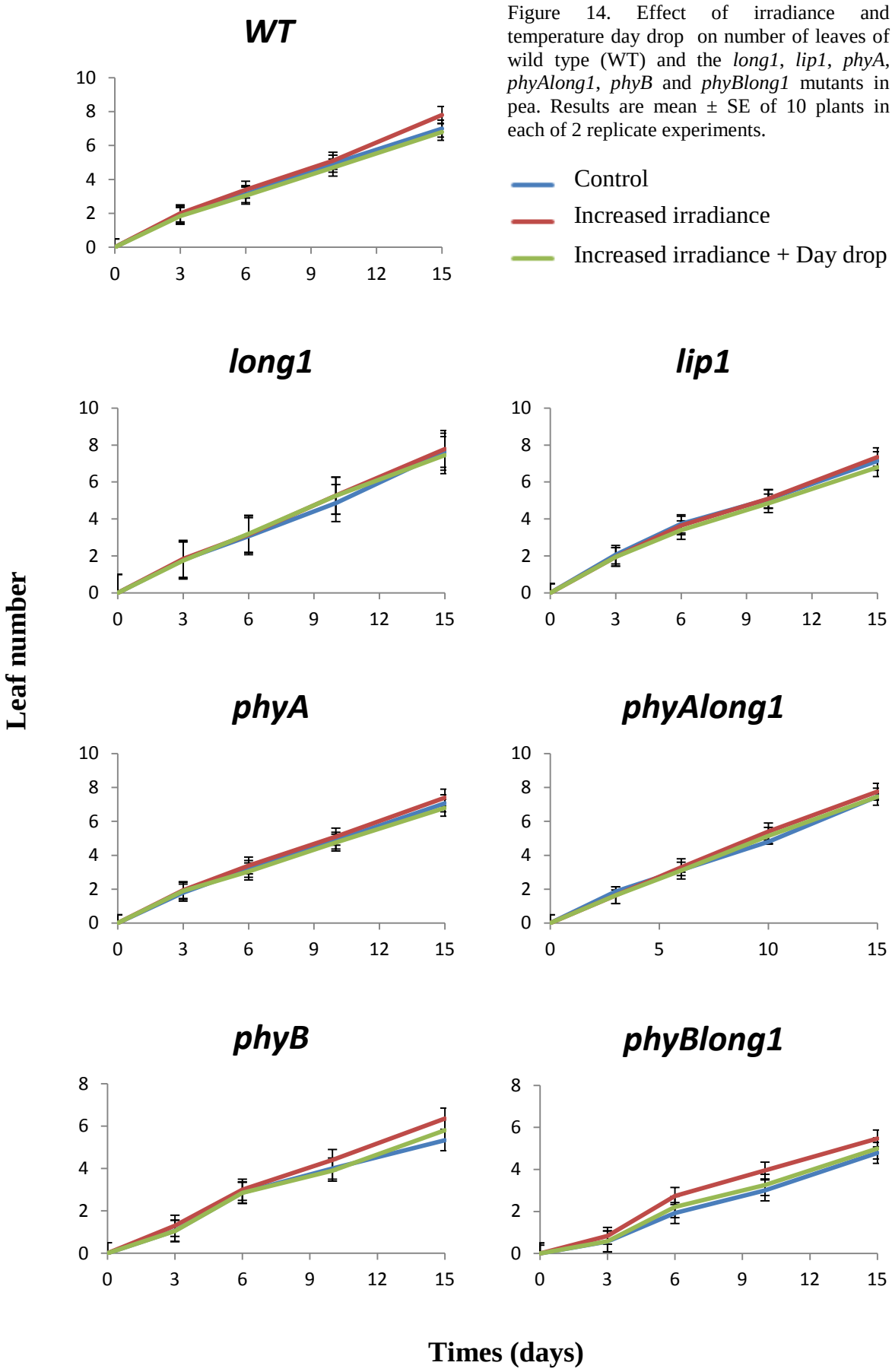
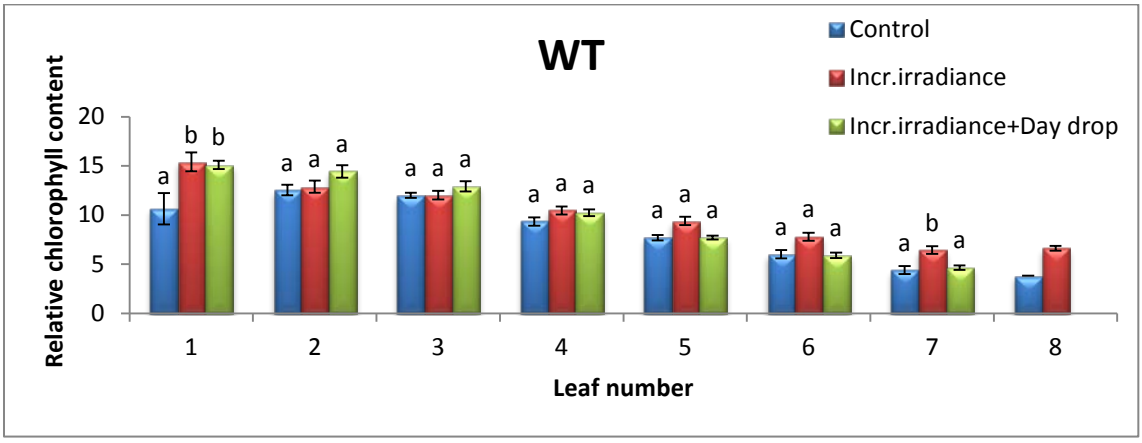
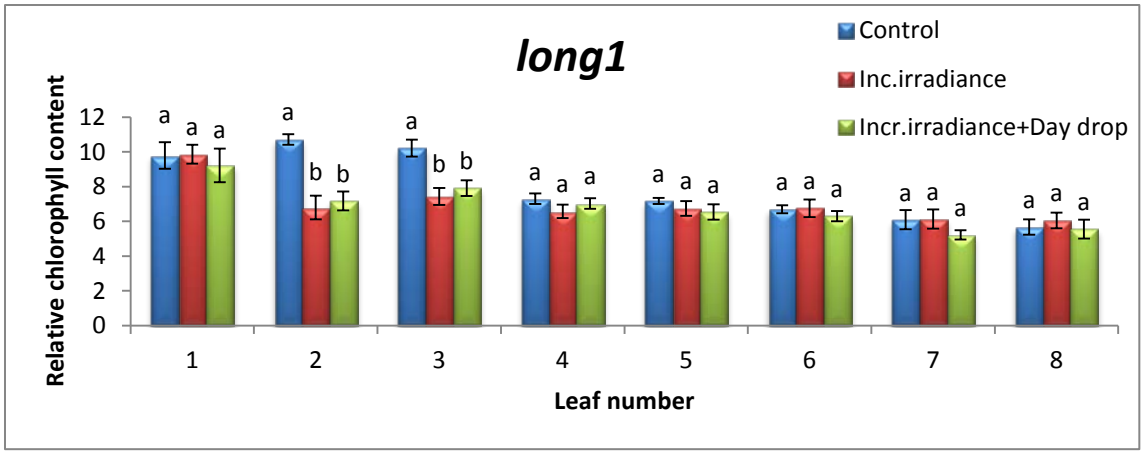


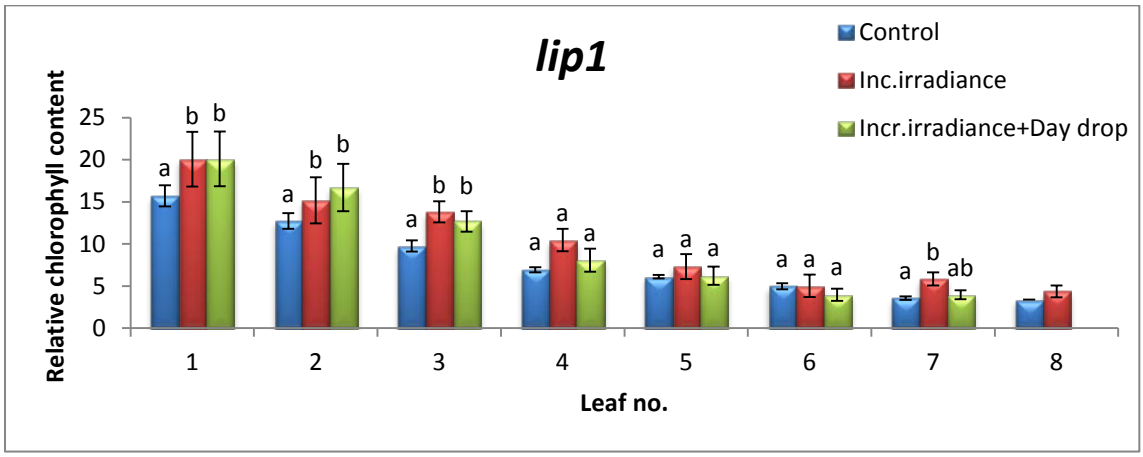
Figure 14. Effect of irradiance and temperature day drop on number of leaves of wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants in pea. Results are mean ± SE of 10 plants in each of 2 replicate experiments.

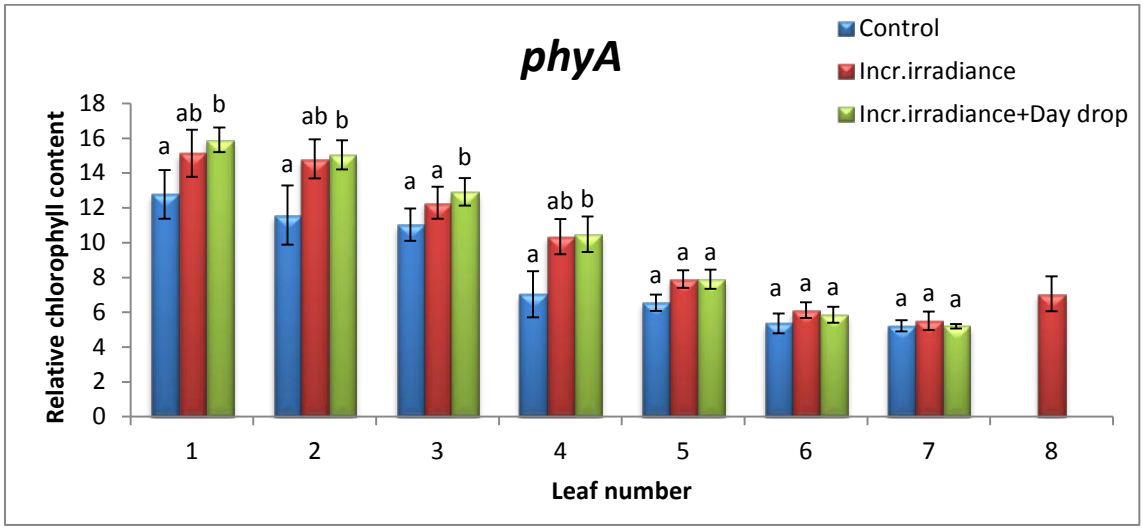
4.2.3. The effects of increased irradiance and temperature drop in the day on chlorophyll content

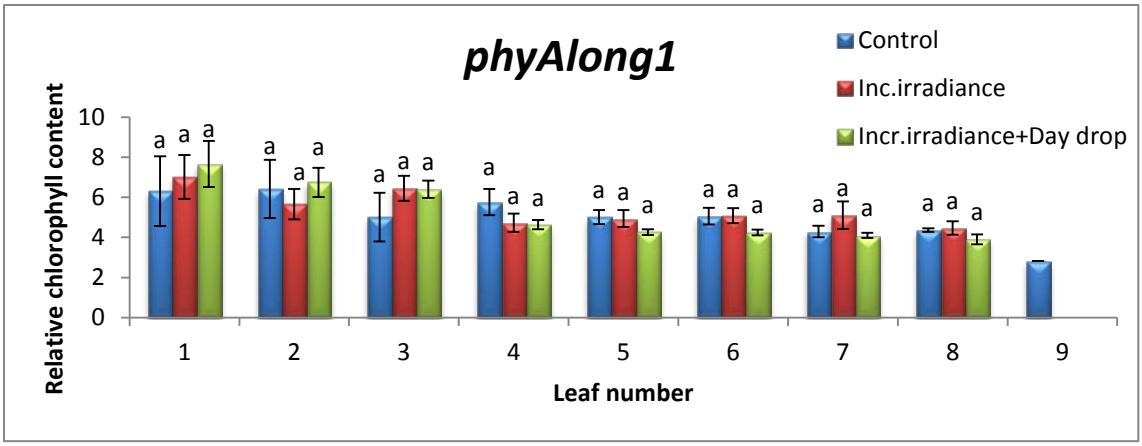
The results showed that there were several significant differences in chlorophyll levels between the different treatments in the different genotypes (Figure 15). In WT both increased irradiance and increased irradiance + day drop significantly increased chlorophyll level in leaf number 1 (oldest leaf) for approximately 45% and 42% respectively compared to the control. Also, increased irradiance alone significantly increased chlorophyll level in the leaf number 7 (young leaf) for about 46%. A similar pattern was observed in the *lip1* mutant. In addition, in this mutant both treatments had significant effect on leaves number 2 and 3. On the other hand there was no treatment effect found in leaf number 1 in the *long1* mutant, but both treatments significantly reduced the chlorophyll content in leaves number 2 and 3 for approximately 37% in leaf number 2 and 23% in leaf number 3. In the *phyA* mutant significantly increased chlorophyll content was observed in the first 4 (oldest) leaves in irradiance + day drop as compared to the control. The increase was approximately 20% in each of the 3 first leaves and 50% in the fourth leaf. There were no significant differences found in *phyAlong1* mutant between any of the treatments. On the other hand, both treatments significantly increased the chlorophyll level significantly in all measured leaves in *phyB* mutant for about 100% in leaves number 1, 2 and 5 and about 40% in leaves number 3, 4 and 6. In *phyBlong1* both treatments had a significant effect on leaf number 5, where chlorophyll was increased in comparison to the control for approximately 200%. As observed in experiment 1, the *phyB* and *phyBlong1* mutants appeared to have the lowest chlorophyll content in the oldest leaf.

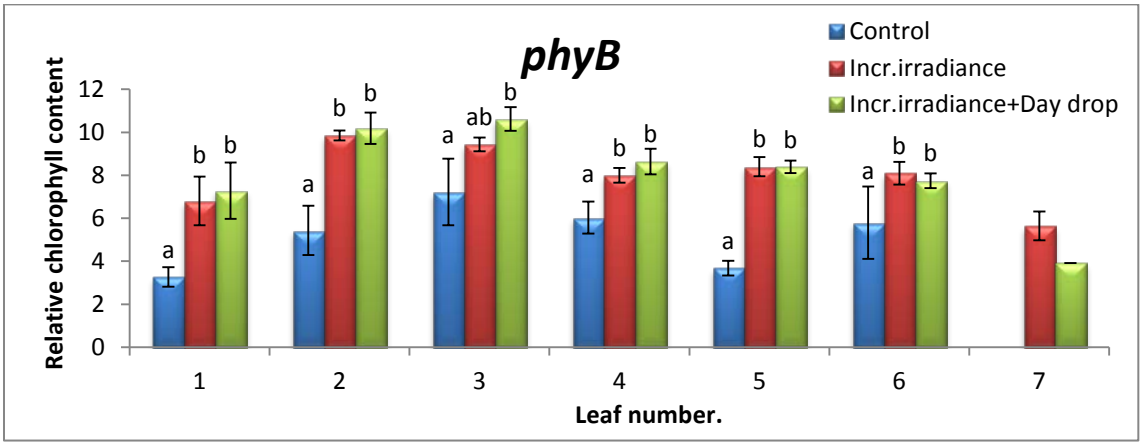



long1
Control
Inc.irradiance
Incr.irradiance+Day drop
Relative chlorophyll content
Leaf number


lip1
Control
Inc.irradiance
Incr.irradiance+Day drop
Relative chlorophyll content
Leaf no.


phyA
Control
Incr.irradiance
Incr.irradiance+Day drop
Relative chlorophyll content
Leaf number





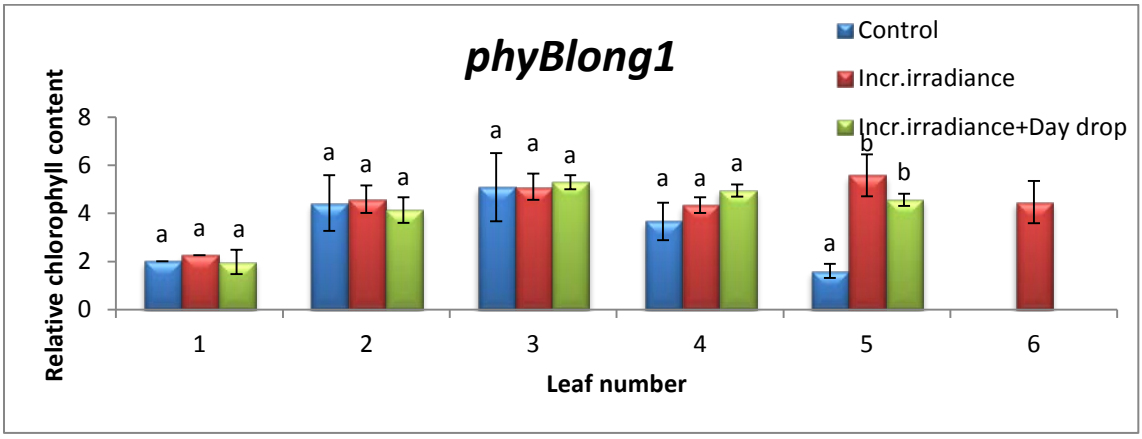
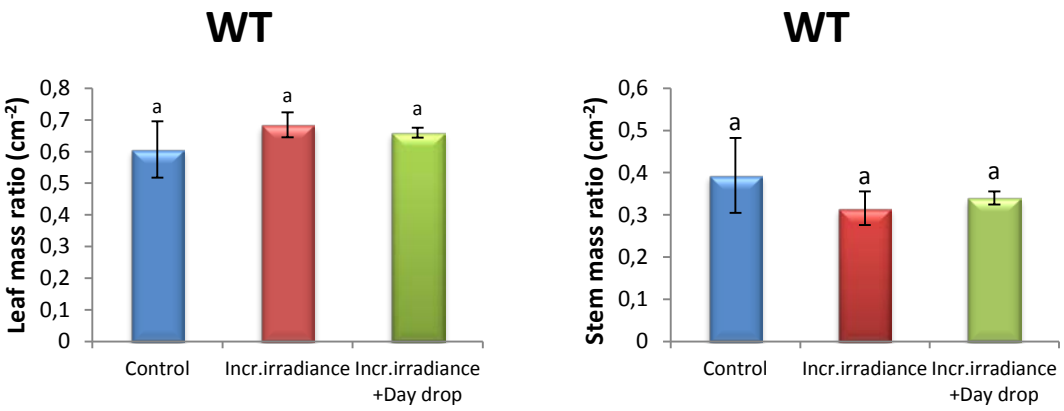


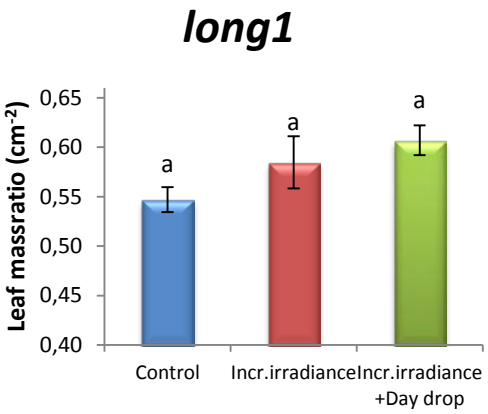
Figure 15. Effect of irradiance and temperature day drop on chlorophyll content in the wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB and phyBlong1* mutants of pea from the lowest (1) to the youngest leaf (highest number). Results are mean ± SE of 4 plants in each of 2 replicate experiments. Within a leaf different letters indicate significant differences and the same latters indicate no statistically significant difference ($p \leq 0.05$).

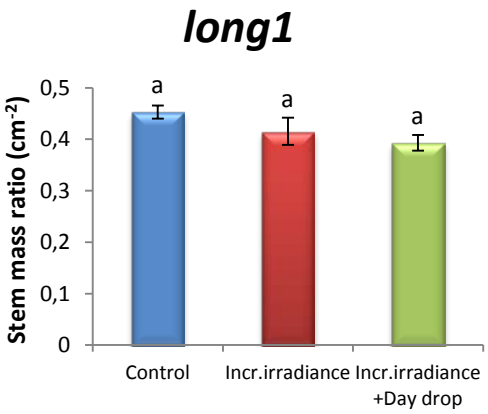
4.2.4. The effects of increased irradiance and temperature drop in the day on leaf mass ratio and stem mass ratio

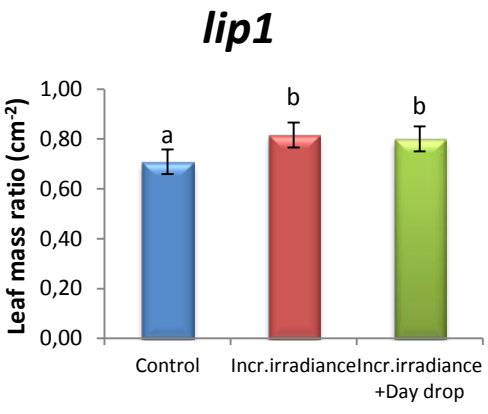
In general a similar pattern for the leaf and stem mass ratio was seen in all measured mutants. Increased irradiance and irradiance + day drop increased the leaf mass ratio area while at the same time stem mass ratio was decreased (Figure 16). Even though the same patterns were conserved between different mutants, the only statistically significant results ($p \leq 0.05$) were found *in lip1* and *phyA* when they were compared to the control. In these two mutants, increased irradiance and increased irradiance + day drop significantly increased the leaf mass ratio while stem mass ratio was significantly decreased. In *phyAlong1*, significant difference was found between increased irradiance and the control for both the leaf mass ratio, which was increased and steam mass ratio, which was decreased. In *phyBlong1* increased irradiance significantly affected only leaf mass ratio but not the stem mass ratio.

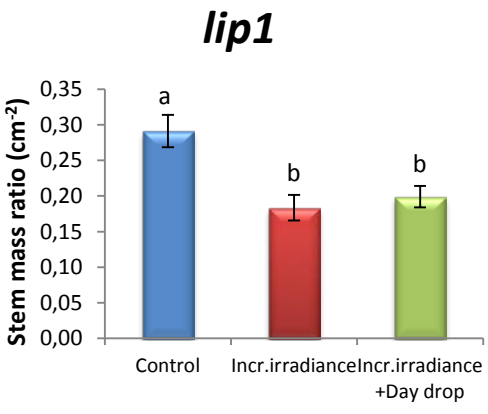
The different genotypes showed different resource allocation pattern. WT, *lip1*, *long1* and *phyA* mutants seem to generally allocate more resources into leaves and less into stem. *PhyB* and p*hyAlong1* allocated similar amounts into leaves and stem, while *phyBlong1* showed a tendency in allocating less resource into leaves and more into stem.

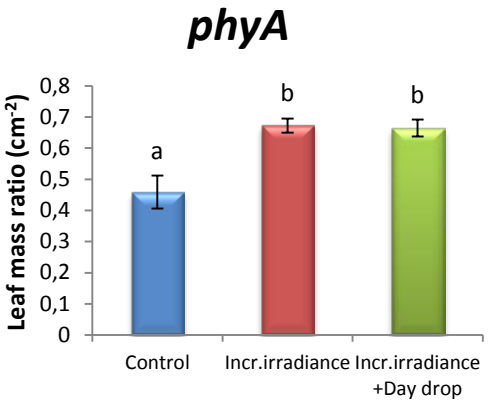


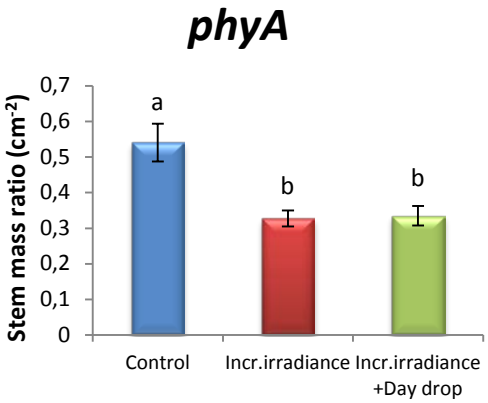

long1
Leaf massratio (cm-2)
0,65
0,60
0,55
0,50
0,45
0,40
a
a
a
Control
Incr.irradiance
Incr.irradiance +Day drop


long1
Stem mass ratio (cm-2)
0,5
0,4
0,3
0,2
0,1
0
a
a
a
Control
Incr.irradiance
Incr.irradiance +Day drop


lip1
Leaf mass ratio (cm-2)
1,00
0,80
0,60
0,40
0,20
0,00
a
b
b
Control
Incr.irradiance
Incr.irradiance +Day drop


lip1
Stem mass ratio (cm-2)
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
a
b
b
Control
Incr.irradiance
Incr.irradiance +Day drop


phyA
Leaf mass ratio (cm-2)
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
b
b
Control
Incr.irradiance
Incr.irradiance +Day drop


phyA
Stem mass ratio (cm-2)
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
a
b
b
Control
Incr.irradiance
Incr.irradiance +Day drop

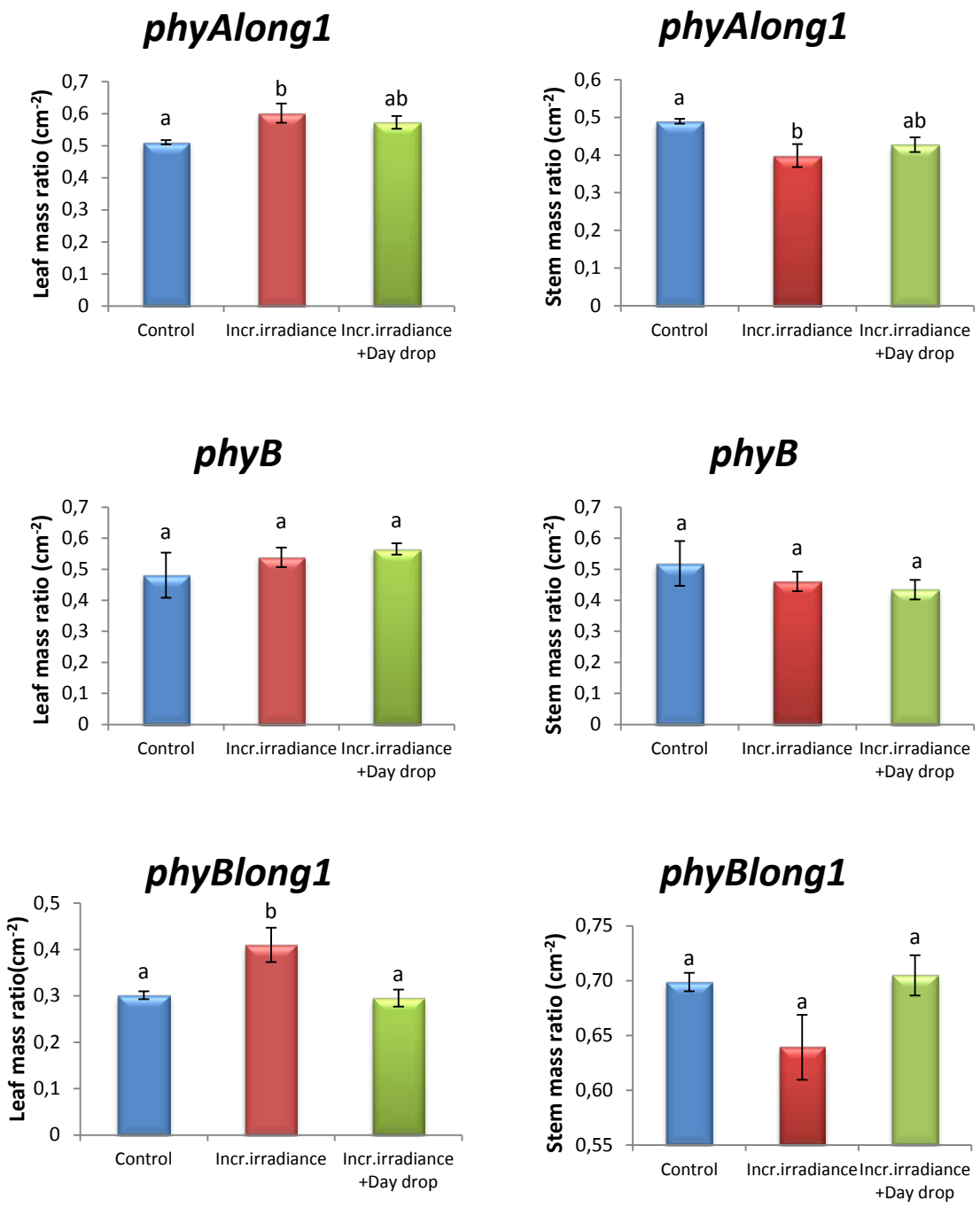


Figure 16. Effect of irradiance and temperature day drop on leaf mass ratio and stem mass ratio in wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants in pea. Results are mean ± SE of 4 plants in each of 2 replicate experiments. Different letters within each staple graph indicate significant differences and the same latters indicate no statistically significant difference ($p \leq 0.05$).

4.2.5. The effects of increased irradiance and temperature drop in the day on leaf area

In the WT and the *phyA* mutant, increased irradiance significantly increased leaf area both compared to the control (approximately 60%) and increased irradiance + day drop (Figure 17). In these genotypes the leaf area under increased irradiance + day drop was also significantly larger than that of the control (approximately 30%). In the *long1*, *phyB* and *phyBlong1* mutants both increased irradiance (40%, 70%, 60%, respectively) and increased irradiance + day drop (50%, 100%, 90%, respectively) led to significantly bigger leaf area than the control, both with a similar difference relative to the control. In the *phyAlong1* and *lip1* mutants there were no significant differences between any of the treatments, only slight trends resembling those of WT and *phyA* with increased leaf area in the increased irradiance treatment.

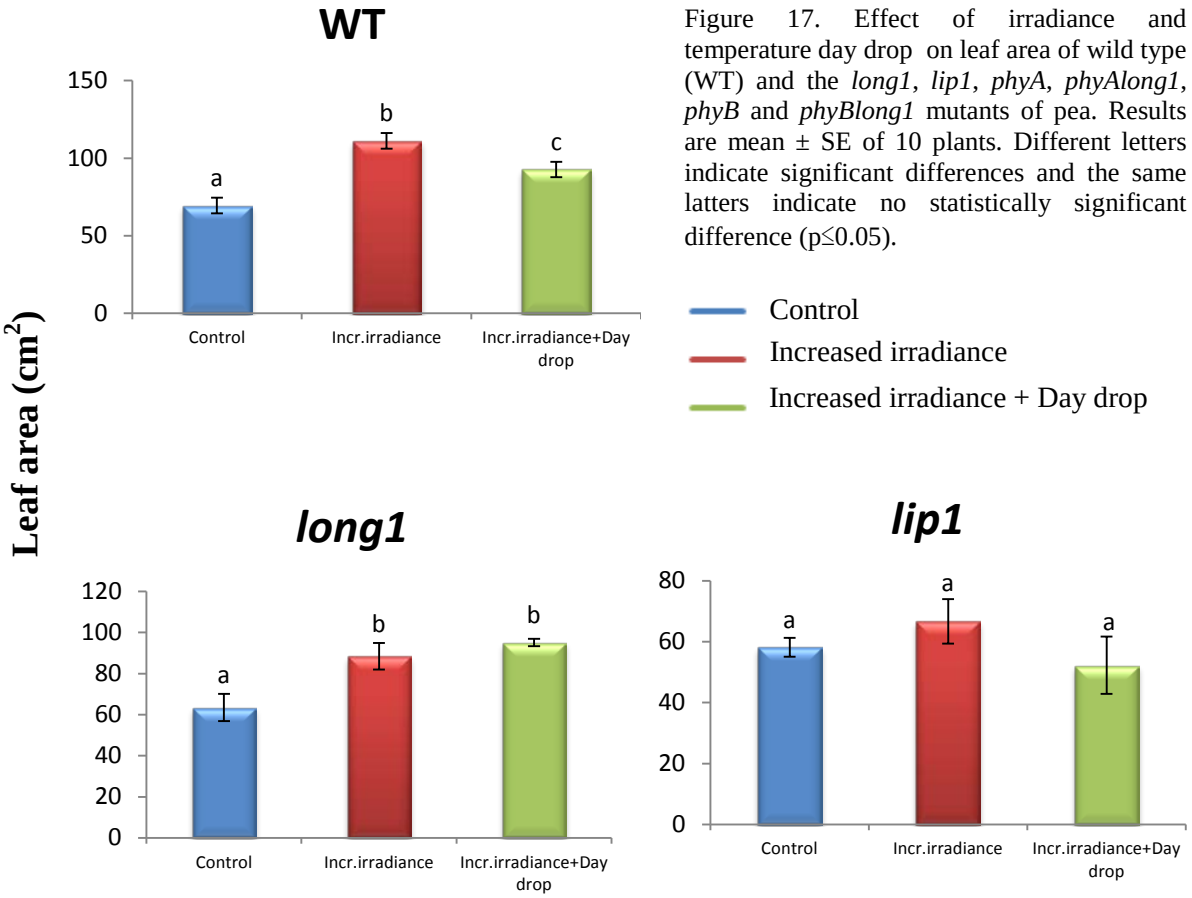
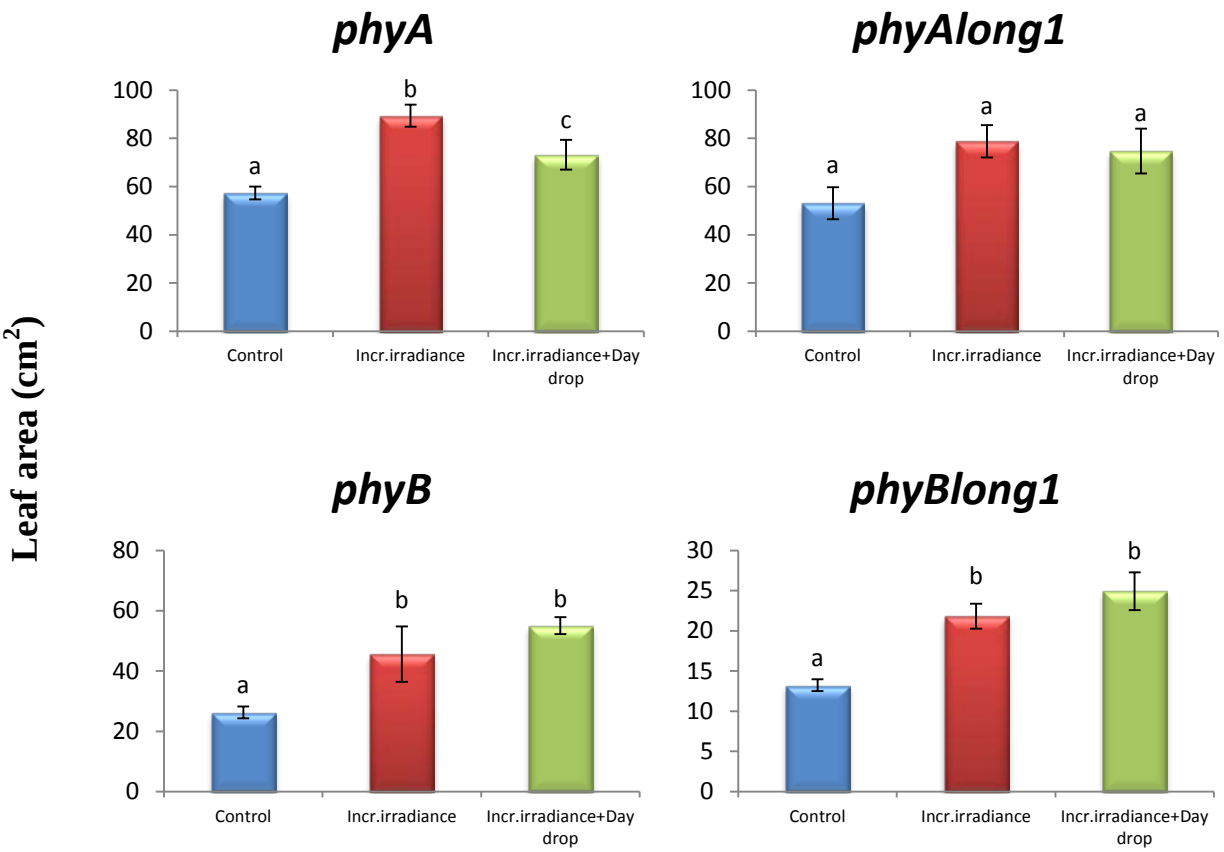


Figure 17. Effect of irradiance and temperature day drop on leaf area of wild type (WT) and the *long1*, *lip1*, *phyA*, *phyAlong1*, *phyB* and *phyBlong1* mutants of pea. Results are mean ± SE of 10 plants. Different letters indicate significant differences and the same latters indicate no statistically significant difference ($p \leq 0.05$).


phyA
phyAlong1
phyB
phyBlong1
Leaf area (cm2)
Control
Incr.irradiance
Incr.irradiance+Day drop
a
b
c

5. DISCUSSION

5.1. Effects of temperature drops during day and night

In order to investigate the effects of day and night temperature drops on pea morphology, WT pea as well as several pea mutants were exposed to temperature drops from 21 to 13°C either at the middle of the day or at the middle of the night. Different morphological parameters were tested, including stem elongation, leaf number, chlorophyll content, stem mass ration and leaf mass ratio, as well as leaf area.

5.1.1. Effects of temperature drop on stem elongation

Stem length of WT showed the greatest reduction (15% after 15 days) when the temperature was reduced during the day (day drop) compared to constant temperature and night drop (Figure 8). Our results are in agreement with several previous experiments. Although an accumulated effect during an extended period similar to ours was not reported. Stavang et al. (2007) reported 50% reduction of the stem elongation rate in pea during a 2 h temperature drop from 21°C to 13°C in daytime. Furthermore, similar results were observed in *Begonia hiemalis* where plants treated with a temperature drop of 3-6°C in the light period were shorter compared to plants grown at constant temperature (Grindal et al., 1994). In contrast, an equivalent 2 h temperature increase in the light period resulted in higher plants. In the morning, 2 h of temperature drop from 24°C to 8°C reduced stem elongation in poinsettia with more than 50% (Ueber and Hendriks, 1992). These results all show that stem elongation in many species is sensitive to a short period of temperature drop. It has been suggested by Ueber and Hendriks (1992) that in order to further increase the inhibitory effect on stem elongation in practical greenhouse culture an extended period of temperature drop should be applied. It could be speculated that a temperature drop in the middle of the light period is perceived by the plant as more stressful to the growth than a temperature drop in the dark.

The exact mechanisms behind the temperature drops effects on stem elongation are still not known. However, many studies have suggested that GAs are the mediators of temperature effects on plant elongation growth. In the short term temperature drop experiment in pea discussed above, the larger effect of a 2 h temperature drop on the shoot elongation rate in the middle of the day than in the middle of the night is linked to reduced levels of active GA_1 and increased expression of the GA-inactivation gene *GA2ox2* (Stavang et al., 2007). In addition,

it has been reported that an extended period of lower DT than NT (negative DIF) greatly decreased the GA_1 level in several studied plants (Jensen et al., 1996; Grindal et al., 1998; Stavang et al., 2005). In pea this was shown to be due to substantially increased mRNA level of *PsGA2ox2* (Stavang et al., 2005). In light of these studies (Stavang et al., 2005; 2007), the reduction in stem elongation observed in our study under a temperature drop in light was probably due to decreased level of bioactive GA as a result of increased GA-deactivation. In addition to GA, other hormones such as auxin and brassinosteroid might be involved in the process of stem elongation. Effects of increased temperature and exposure to different DT and NT on auxin metabolism have been shown in *A. thaliana* (Gray et al., 1998; Thingnæs et al., 2003; Stavang et al., 2009). Seedlings grown at 29°C had higher levels of auxin and transcripts of auxin biosynthesis genes in comparison to seedlings grown at 20°C (Gray et al., 1998; Stavang et al., 2009). Similarly, plants grown under positive DIF had higher auxin levels compared to plants under negative DIF (Thingnæs et al., 2003). Stavang et al. (2009) also demonstrated that presence of brassinosteroid is required for enhanced elongation growth in *A. thaliana* seedlings in response to increased temperature. Thus, a temperature drop in our experiment might have resulted in changes in the metabolism of GAs, auxin and/or brassinosteroid.

To shed light on other parts of the signaling mechanism underlying the thermoperiodic control of plant morphology, we have also studied the effects of temperature drop on several pea mutants mutated in known photomorphogenesis-related genes. The *A. thaliana* HY5 and COP1 ortologs in pea, LONG1 and LIP1, respectively, which act downstream of phytochromes, have been shown to be central photomorphogenesis components affecting elongation growth. In *A. thaliana* HY5 has been shown to be present in the day but not in the night due to a COP1-dependent HY5 degradation in the dark (Osterlund et al., 2000). Interestingly, our results showed no significant differences between day and night drop and the control on stem elongation in the *long1* mutant (Figure 8). Thus, in contrast to the WT, due to the lack of *LONG1* this mutant is apparently not able to perceive or respond to a temperature drop in the light phase. This is consistent with an important role of *LONG1* in thermoperiodic control of shoot elongation. Since a temperature drop in light (and negative DIF) in contrast to a night drop (and positive DIF) acts in reducing shoot elongation in the WT but not in the *long1* mutant, it appears that the effect of reduced temperature in light is linked to an action through HY5, which is present in light and not in darkness.

On basis on knowledge from *A. thaliana* it could be hypothesized that pea plants mutated in *LIP1* are not able to down-regulate *LONG1* in the dark and should accordingly have similar levels of *LONG1* in light and darkness. Since, LONG1 on basis of the results with the *long1* mutant here (Figure 8), appears to be an important player in thermoperiodic control of shoot elongation, we expected that both day and night drop treatments would inhibit elongation growth in the *lip1* mutant. Our results showed that this was indeed the case for the *lip1* mutant with both day and night drops inhibiting elongation growth relative to the control exposed to constant temperature (Figure 8). However, elongation growth was more reduced in the night drop (60%) than in the day drop (23%), although we expected that both treatments should result in a similar degree of inhibition of shoot elongation due to similar levels of *LONG1* in the day and night. Since we did not measure LONG1 levels in the WT and *lip1* mutant in pea, we cannot exclude that there are some differences in LONG1 levels in light and darkness. It might be that the *lip1* mutant is leaky and is still producing some *LIP1* that results in a small difference in LONG1 levels in the day and night. If so, this might explain a difference in degree of inhibition of shoot elongation in the *lip1* mutant between day and night drop. However, the response should then probably have been opposite to what was observed, with more inhibition by a day than a night drop. Thus, until further investigated, the stronger inhibition by a night drop than a day drop remains unexplained.

Furthermore, our study showed that the *long1* mutant is longer than the WT, while the *lip1* mutant is much shorter. This is in agreement with the study of Weller at al. (2009). These authors analyzed GA levels and transcript levels of GA metabolism genes in the WT and in both *long1* and *lip1* mutants as compared to the WT and concluded that the *long1* mutant is long due to higher GA_1 levels and reduced GA-inactivation by *GA2ox2* (compared to the WT). They also showed that the *lip1* mutant is short since it contained low levels of GA_1 due to increased GA inactivation as a consequence of increased *GA2ox2* expression.

Weller at al. (2009) suggested that the reduced height during a temperature drop in the day observed in the WT could be due to LONG1 enhancing *GA2ox2* gene expression. The authors also showed that LONG1 had to be present in order for light to be able to inhibit shoot elongation. Our results nicely demonstrate that when LONG1 is lacking like in the *long1* mutant a day drop will not affect shoot elongation. This suggests that the inhibition of shoot elongation by day drop in the WT in our study might be explained by an effect of *LONG1* on increasing the activity of *GA2ox2* and thus reducing the GA_1 level.

Furthermore, to examine the role of phytochromes in the temperature control of stem growth in pea, we conducted experiments with *phyA*, *phyAlong1*, *phyB* and *phyBlong1* (Figure 8). In control of flowering in *A. thaliana* the function of phyB has been shown to be temperature dependent (Halliday et al., 2003). Both in control of flowering and seed germination, the functional relationships between different phytochromes appear to be dependent on temperature (Halliday and Whitelam, 2003; Heschel et al., 2007). It has also been demonstrated that the phytochrome photoequilibrium is sensitive to temperature (Borthwick et al., 1952). In the present study, the *phyA* mutant responded similarly to the WT with inhibition of shoot elongation in response to a temperature drop during the day in contrast to a night drop. This indicates that phyA is not required for a thermoperiodic response. The *phyB* lacking plants were slender and longer than the WT and the *phyA* mutants (Figure 8). Similar results were reported in the study of Weller et al. (1995). The *phyB* mutant showed a similar response to a temperature drop during the day as compared to the WT and the *phyA* mutant. However, in contrast to WT and *phyA*, the *phyB* mutant showed a similarly reduced shoot elongation in response to a temperature drop treatment both during the night and day (Figure 8). Thus, it appears that phyB must be present for the pea plants to be able to distinguish between a temperature drop treatment in the day and the night. It is well known that COP1/LIP1 and HY5/LONG1 act downstream of phytochromes (Weller et al., 2009 and references therein), so it could be speculated that the *phyB* mutant does not show a normal LIP1-mediated degradation of LONG1 during the night. Thingnæs et al. (2008) detected that a negative DIF reduced stem and petiole elongation less than in the WT in *A. thaliana* genotypes lacking *phyB*. A similar result was observed also in a *phyB* mutant in cucumber (Xiong et al., 2002). These as well as other results suggested that phyB is needed for a complete thermoperiodic control of elongation growth in *A. thaliana* and in cucumber and appear somehow to act as a temperature sensor (Mahat, 2010 master thesis). In our study of pea it appears that phyB is needed to show a normal differential response to day and night temperature. In addition, the p*hyAlong1* and *phyBlong1* mutants behaved in a similar way as the *long1* mutant, again indicating that *LONG1* must be present for a proper thermoperiodic response.

5.1.2. Effects of temperature drop on leaf number, leaf area, leaf mass ratio and stem mass ratio

Our result did not show any significant difference in leaf number between WT and different mutants after any of the tested treatments (Figure 9). This is similar to previous observations from several studies showing that thermoperiod does not affect leaf number (e.g. Myster and Moe, 1995; Grindal et al., 1998; Stavang et al., 2005; Stavang et al., 2010). This means that in practical greenhouse growing temperature drops and negative DIF can be used to reduce shoot elongation without a delay in production time. There are several studies indicating that reduced leaf area in plants grown under negative DIF was caused by reduced amounts of active GA (Grindal et al., 1998; Stavang et al., 2010). In addition both *phyB* and *phyBlong1* mutants had much lower leaf area compared to WT (Figure 12). This suggests that phyB is an important light receptor involved in control of leaf area. The *phyB* mutant actually looks like a WT plant growing under heavy shade; elongated with reduced leaf area. The *PHYB* gene is shown to be important in the so-called shade-avoidance response (Smith and Whitelam, 1997), suggesting its importance for the plants to be able to adjust their growth according to whether they are growing in light or shade. Our results showed that temperature drop treatments did not have significant effect on the leaf mass ratio nor stem mass ratio (Figure 11).

5.1.3. Effects of temperature drop on chlorophyll content

Previous studies reported that 2 h-temperature drop during the day has a tendency of lowering the total concentrations of chlorophyll (chlorophyll a and chlorophyll b) in lemon balm *(Melissa officinalis)* and basil *(Ocimum basilicum)* compared to a 2 h temperature increase, while the effect was opposite in a more cold tolerant species like pansy *(Viola wittrockiana)* (Vågen et al., 2003). In addition, several studies showed that a negative DIF in a 12 h photoperiod reduced the level of chlorophyll in pea (Stavang et al., 2010). Our results did not show a consistent, significant effect of temperature drop on chlorophyll content, suggesting that chlorophyll level is not much affected by 4 h of temperature drop either during the day or during the night in WT or in any of the pea mutants (Figure 10).

5.2. Effects of increased irradiance

In order to investigate the effects of interaction between temperature drop and irradiance on pea morphology, WT pea as well as the *long1, lip1, phyA, phyAlong1, phyB and phyBlong1* mutants were subjected to either constant irradiance (50 μmol m^{-2} s^{-1}) and constant

temperature (21°C), increased irradiance to 150 μmol m^{-2} s^{-1} for 4 h in the middle of the light period and constant temperature (21°C), increased irradiance to 150 μmol m^{-2} s^{-1} and temperature drop (from 21°C to 13°C) for 4 h in the middle of light period. The same growth parameters as in the temperature drop experiment were measured.

5.2.1. Effects of increased irradiance on stem elongation

WT pea plants subjected to a temperature drop and increased irradiance in the middle of the day showed a reduction in growth rate by 45% (Figure 13) compared to constant irradiance and constant temperature. In the present experiments, the application of only increased irradiance had substantially less effect as compared to when irradiance was increased with day drop. Increased temperature and light exert positive and negative effects on the stability of PIF4 and PIF5, respectively, in *Arabidopsis* (De Lucas et al., 2008; Stavang et al., 2009). This might well explain the strong effect the combined treatments had on stem growth. It can be hypothesized that a temperature drop during the day might diminishes the level of these transcription factors and an irradiance increase might also lower the level, making the total effect larger than the contributions each treatment had per se.

Several earlier experiments have shown that light has major impact in reducing the growth rate of plants and that DIF or temperature drop treatments can be modified by light quality. Moe and Heins, (1990) suggested that the DIF response is phytochrome mediated. Kurepin et al. (2007) reported lower stem length in sunflower plants (*Helianthus annuus)*, grown under an irradiance of 421 μmol m^{-2} s^{-1} in comparison to 3 times lower irradiance. Experiments with the prairie and the alpine ecotypes of *Stellaria longipes* showed similar results (Kurepin et al., 2006). Both ecotypes had higher stem growth when exposed to lower irradiances. In pea, a 50% and a 90% reduction of the irradiance increased the average stem length to approximately 150% and 300% of the value for control plants, respectively (Gawronska et al., 1995). Plants grown in the dark exhibited an almost five-fold increase in the average stem length compared to control plants. On the other hand, our results show the highest effects of increased irradiance in combination with a temperature drop.

As already mentioned, GA, together with other hormones contributes to the growing process in plants. O'Neill et al. (2000) found that the level of bioactive GA in pea (GA_1) declined when the plants were transferred from dark to light (during de-etiolation). The decrease was limited to a period between 4 and 72 h after the transfer to light, and after 120 h, the level of

GA_1 was comparable to light-grown plants. The level of GA_{20} did not decrease significantly when the plants where transferred from dark to light, but the level of GA_8 did increase substantially (O'Neill et al., 2000). Thus, it appeared that the reduction in GA_1 was due to increased catabolism of GA_1 to GA_8 rather than a decrease in GA precursors such as GA_{20}. A GA deactivation enzyme in pea, *PsGA2ox2*, which catalyzes the step converting GA_1 to GA_8, is up-regulated after light exposure of dark-grown plants (Reid et al., 2002). The level of bioactive GA is also reduced during de-etiolation in other species such as *Arabidopsis* and barley *(Hordeum vulgare)* (Symons et al., 2008). Our experiment demonstrates that increased irradiance in combination with temperature drop in the photoperiod affects the growth of the pea mutants as well. The *lip1*, *phyA* and *phyB* mutants showed similar response to the WT, but with even more (about 20%) growth reduction. On the other hand, we registered the lack of treatment response in the *long1* mutant. Our results suggest that the *LONG1* gene appears to be very important in the inhibited elongation growth in response to increased irradiance since the plants that are mutated in this gene do not respond to increased irradiance (Figure 13). Similar results were reported in the study of Weller et al. (2009). The authors suggested that LONG1 somehow (probably indirectly) enhances *GA2ox2* gene expression which results in inactivation of GAs.

5.2.2. Effects of increased irradiance on leaf number, leaf area, leaf mass ratio and stem mass ratio

Our results showed a tendency of increased leaf number in WT as well as the *lip1*, *phyA*, *phyB* and *phyBlong1* mutants with increased irradiance, with the strongest effect found in the *phyB* and *phyBlong1* mutants (Figure 14). In addition increased irradiance increased leaf area in all measured plants (Figure 17). Taking together our results show the importance of irradiance in order to stimulate photosynthesis in WT as well as in all measured mutants. This effect appears to be counteracted by a temperature drop.

Trends of increased leaf mass ratio and decreased stem mass ratio under conditions with increased irradiance (Figure 16) suggest that the resource allocation pattern is affected by the irradiance, with slightly more resources allocated to the leaves compared to the stem. Thus, plants appear to favor building up leaves as the main photosynthetic part. Furthermore, in the longest mutants, the *phyB*, *phyBlong1* and *long1* stem mass ratio is somewhat increased relative to the leaf mass ratio. In the short mutant, *lip1*, the situation is opposite with more resources allocated to the leaves compared to the stem (higher leaf mass ratio and lower stem

mass ratio compared to the WT). This finding suggests that these genes affect the resource allocation.

5.2.3 Effects of increased irradiance on chlorophyll content

The change in sensitivity to a short temperature change during the photoperiod to affect chlorophyll formation in study of (Stavang et al., 2009) suggested that phytochrome is also involved in thermomorphogenesis in pea. Our results showed that both increased irradiance and increased irradiance + day drop significantly affected chlorophyll content much more than in the first experiment where irradiance was constant (Figure 15). Both treatments increased chlorophyll content significantly in WT and in all mutants except in *phyAlong1*. However the biggest effects were found in the *phyB* and *phyBlong1* mutants. Our results suggest that increased irradiance plays a big role in chlorophyll content. Very similar results were observed in leaf area, being increased in most of the plants after increased irradiance treatment.

6. CONCLUSION

In conclusion, our results showed that the temperature drop of 4 h in the middle of the day had significant effect on reduction of stem length in WT pea as well as in *lip1* and *phyA* mutants. Since there was no effect of a temperature drop in the night compared to the control in neither the WT nor *phyA* mutants, we conclude that *PHYA* is not important in thermoperiodic control of shoot elongation. The data for the *long1* and *lip1* mutants together suggest that a normal thermoperiodic control of shoot elongation with decreased shoot elongation in response to a day drop but not to a night drop (like seen in the WT) is linked to HY5/LONG1 being present in higher amounts in the day than in the dark. The same effect of a day and night drop in the *phyB* mutant also suggests that *PHYB* is the important PHY-light receptor in this process.

Furthermore, it was observed that the combination of increased irradiance and temperature drop had largest reduction effect in stem elongation in WT pea and pea mutants. The combination might be stressful to the pants, which might reduce the stem growth i.e., due to the induced expression of *GA2ox2* (Gonnet, 2009 master thesis). In addition temperature drop treatments did not significantly affects the other growth parameters in WT or any of the

mutants, while both increased irradiance and increased irradiance + day drop had several significant effects.

Taken together, signaling linked to response to light and response to a temperature drop in the light phase have much in common and the same signaling components are very important (*PHYB*, *LIP1* and *LONG1*). Thus, it appears that photomorphogenesis and thermomorphogenesis at least partly, share a common signaling pathway with respect to response to light and response to a temperature decrease during the light.

7. SUGGESTIONS FOR FUTURE RESEARCH

To confirm the significance of *LONG1* as a main signaling factor also in thermoperiodic responses, and *LIP1* in regulating the *LONG1* content in this respect, it would be very important to analyze the mRNA and protein levels of LONG1 and LIP1 in plants exposed to temperature drop treatments during the day and the night as well as in a situation with increased irradiance during the day drop. Furthermore, in this respect also the levels of GAs and GA metabolism genes should be analysed in the WT and the different mutants to confirm an action of *LONG1* on GA-inactivation in plants exposed to day drop alone and in combination with increased irradiance.

It could be interesting also to do experiments with a higher degree of drop and a larger irradiance increase and observe if the growth inhibition is enhanced. From a practical purpose, this could help us to produce plants with even more reduced size in both an economically and an environmentally friendly way.

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