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Bruk av genteknologi og lyskvalitet for å kontrollere strekningsvekst hos julestjerne (Euphorbia pulcherrima Willd ex Klotzsch)

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Dedication

This work is dedicated to the memory of my mother Rekatun Nesa and father late Amirul Islam

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Acknowledgements

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Abbreviations

ABA	Abscisic acid
At	*Arabidopsis thaliana*
B	Blue
CIM	Callus induction medium
CK	Cytokinin
DIF	Day and night temperature difference
DM	Dry matter
DW	Dry weight
EOD	End-of-day
FR	Far red
GA	Gibberellin
HPS	High pressure sodium lamps
IAA	Indole-3-acetic-acid
LED	Light emitting diode
LD	Long day
PCR	Polymerase chain reaction
PPS	Phytochrome photostationary state
qRT-PCR	Quantitative real time PCR
R	Red
RIM	Root induction medium
SD	Short day
SEIM	Somatic embryo induction medium
SHI	*SHORT INTERNODES*
TL	Transgenic line
WT	Wild type

Abstract

Poinsettia (*Euphorbia pulcherrima* Willd ex Klotzsch) is a non-food and non-feed, vegetatively propagated ornamental plant which is among the economically most important ornamentals worldwide. Desirable plant height is one of the most important traits in such species. To obtain compact plants, growers are regularly using chemical growth retardants such as CCC (chlormequat chloride) or alar/daminozide (dimethylaminosuccinamic acid), which inhibit the gibberellin (GA) biosynthesis, resulting in compact plants. However, growth retardants have negative impacts on the environment and human health among others by potentially being carcinogenic. Thus, it is highly desirable to further restrict their use.

This PhD project has explored the use of a plant genetic engineering approach and regulation of light quality by using light emitting diodes (LEDs) to control shoot elongation in poinsettia. The *SHORT INTERNODES* gene from *Arabidopsis thaliana* (*AtSHI*) was introduced into poinsettia by the use of an *Agrobacterium*-based transformation system (paper I). Light quality effects on plant morphology was investigated in greenhouse compartments and growth chambers by comparing use of traditional high pressure sodium (HPS) lamps as supplementary light with a combination of 80% red (R) and 20% blue (B) light from light emitting diodes (LED) (paper II). Also, the effect a 30 min end-of-day (EOD) treatment provided by R LED light was investigated in both light regimes (Paper II). To investigate the effect of manipulation of the phytochrome system on hormone physiology, and since the knowledge on hormone physiology in poinsettia was limited, the effects of EOD-R and EOD-FR on hormone levels were compared (paper III).

Three independent transgenic lines (TL1, TL2, TL3) harbouring *AtSHI* were identified by PCR, and stable integration was confirmed by Southern blot analysis (paper I). When grown under short (SD; 10 h) or long day (LD; 16 h) conditions all three transgenic lines showed reduced shoot elongation compared to untransformed wild type (WT) control plants. TL1

showed the shortest stems and internodes under SD with 52% and 49% reduction, respectively, compared to the WT. This correlated with the highest *AtSHI* expression, and a trend of 31% lower levels of indole-3 acetic acid (IAA) in TL1 compared to the WT.

All three cultivars tested ('Christmas Spirit', 'Christmas Eve' and 'Advent Red') showed reduced plant height (20-34%) under 20% B and 80% R provided by LED light compared to the traditionally used HPS lamps (5% B) (paper II). The phytochrome photostationary state (PPS) under the LED and HPS was very similar, indicating that B light receptors such as cryptochromes are important to control stem elongation of poinsettia. Furthermore, in 'Advent Red' exposure to EOD-R resulted in reduced stem extension by 13% when HPS was used as supplementary light (paper II). By contrast, under the B-R supplementary LED light, EOD-R did not reduce shoot elongation, suggesting that the light-receptor dependent inhibition of shoot elongation had already been saturated due to the high content of B and R. In the other cultivar tested, 'Christmas Eve', no effect of the EOD-R-treatment was observed under any of the two light regimes. This might be due to differences in phytochrome light receptors or their action.

Lower plants under EOD-R compared to EOD-FR correlated with lower levels of IAA, gibberellin (GA) and abscisic acid (21%, 28% and 19%, respectively) in shoot tips (paper III). The GA analyses revealed that the 13-hydroxylation pathway of GA biosynthesis is probably dominating over the non-13-hydroxylation pathway.

In conclusion, these results demonstrates the potential for using genetic engineering and exploiting light quality responses in order to reduce the use of plant growth retardants in greenhouse production of poinsettia. However, the difference in response to EOD-R in the tested cultivars emphasizes the importance of investigating light quality responses in different commercially grown cultivars.

Sammendrag

Julestjerne (poinsettia; *Euphorbia pulcherrima* Willd ex Klotzsch), som verken benyttes til fôr eller mat og formeres vegetativt, er blant de økonomisk viktigste potteplantekulturer i verden. Kontroll av plantehøyden er av avgjørende betydning i slike arter. For å oppnå kompakte planter benyttes regelmessig behandling med kjemiske veksthemmere som CCC (chlormequat chloride) eller alar/daminozide (dimethylaminosuccinamic acid). Disse hemmer gibberellin- (GA) biosyntesen. Kjemiske veksthemmere har imidlertid negative effekter på miljøet og human helse, blant annet ved å være potensielt kreftframkallende. Det er derfor sterkt ønskelig å begrense bruken av disse ytterlige.

Dette PhD-arbeidet har undersøkt muligheten for å benytte genteknologi og manipulering av lyskvalitet ved hjelp av lys-dioder (LED; fra engelsk «light emitting diodes») til å kontrollere strekningsveksten hos julestjerne. «*SHORT INTERNODES*»-genet fra vårskrinneblom (*Arabidopsis thaliana*) (*AtSHI*) ble satt inn i julestjerne ved hjelp av *Agrobacterium*-basert genteknologi (artikkel 1). Effekter av lyskvalitet på plantemorfologi ble undersøkt i veksthus og vekstkammere ved å sammenligne bruk av tradisjonelle høytrykksnatriumlamper (HPS; fra engelsk «high pressure sodium») som tilleggslys med bruk av LED-lys som ga en kombinasjon av 80% rødt (R) og 20% blått (B) lys (artikkel II). I tillegg ble effekten av en 30 minutters behandling med R LED-lys på slutten av dagen (EOD; fra engelsk «end of day») studert (artikkel II). For å undersøke effekten av manipulering av fytokromsystemet på hormonfysiologien, og siden kunnskapen om hormonfysiologien i julestjerne var begrenset, ble effektene av 30 minutter med EOD-R og EOD-mørkerødt (MR) på plantenes hormoninnhold sammenlignet (artikkel III).

Tre uavhengige transgene linjer (T1, T2, T3) med innsatt *AtSHI* ble identifisert ved hjelp av PCR og stabil integrering bekreftet ved hjelp av southern blot-analyse (artikkel I). Ved dyrking under kort (KD; 10 timer) og lang dag (16 timer) viste alle tre linjer redusert strekningsvekst sammenlignet med ikke-tranformerte villtype-kontrollplanter. TL1 hadde de

korteste stenglene og internodiene under KD med henholdsvis 52 % og 49 % reduksjon sammenlignet med villtypen. Dette korrelerte med det høyeste *AtSHI*-uttrykket og en tendens til lavere (31 %) nivåer av indol-3-eddiksyre (IAA) i TL1 sammenlignet med villtypen.

Alle de tre testede julestjernesortene ('Christmas Spirit', 'Christmas Eve' og 'Advent Red') viste redusert plantehøyde (20-34 %) under 20 % B and 80 % R fra LED, sammenlignet med de tradisjonelt brukte HPS lampene (5 % B) (artikkel II). Fytokromstatus var svært lik under LED og HPS og dette tyder på at B-lysreseptorer som kryptokromer er viktige i å kontrollere strekningsveksten i julestjerne. I 'Advent Red' førte EOD-R til 13 % reduksjon i stengelstrekningen når HPS ble brukt som tilleggslys i veksthuset, sammenlignet med bruk av kun HPS uten EOD-R (artikkel II). I motsetning til dette, ble strekningsveksten ikke redusert av EOD-R-eksponering under B-R-LED-lysbehandlingen. Dette kan tyde på at lysavhengig hemming av stengelstrekning i dette tilfellet allerede var mettet på grunn av det høye nivået av B og R-lys. I den andre testede julestjernesorten, 'Christmas Eve', ble det ikke observert noen effekt av EOD-R under noen av de to lysregimene. Forskjellen mellom sortene kan muligens skyldes forskjeller i fytokrom-lysreseptorer eller deres virking.

Lavere planter under EOD-R sammenlignet ned EOD-MR korrelerte med lavere nivåer av IAA, GA og abscisinsyre (henholdsvis 21 %, 28 % og 19 %) i skuddspissene (artikkel III). GA-analysene viste at 13-hydroksylerte gibberelliner forekom i større mengder enn ikke-13-hydroksylerte.

De oppnådde resultatene viser potensial for å benytte genmodifisering og utnytte responser på lyskvalitet for å redusere bruk av kjemiske veksthemmere i veksthusdyrking av julestjerne. Forskjellene i respons på EOD-R i ulike julestjernesorter understreker imidlertid betydningen av å undersøke lyskvalitetsresponser i forskjellige kommersielt dyrkede sorter.

List of papers

Paper I

M Ashraful Islam, Henrik Lütken, Sissel Haugslien, Dag-Ragnar Blystad, Sissel Torre, Jakub Rolcik, Søren K Rasmussen, Jorunn E Olsen, Jihong Liu Clarke. 2013. Overexpression of the *AtSHI* gene in poinsettia, *Euphorbia pulcherrima*, results in compact plants. PLoS ONE 8(1): doi:10.1371/journal.pone.0053377

Paper II

M Ashraful Islam, Goutam Kuwar, Jihong Liu Clarke, Dag-Ragnar Blystad, Hans Ragnar Gislerød, Jorunn E Olsen, Sissel Torre. 2012. Artificial light from light emitting diodes (LEDs) with a high portion of blue light results in shorter poinsettias compared to high pressure sodium (HPS) lamps. Scientia Horticulturae 147:136-143

Paper III

M Ashraful Islam, Danuše Tarkowská, Jihong Liu Clarke, Dag-Ragnar Blystad, Hans Ragnar Gislerød, Sissel Torre and Jorunn E Olsen. 2013. Impact of end-of-day red and far-red light on plant morphology and hormone physiology of poinsettia. (Manuscript)

1. Introduction

1.1. Poinsettia

Poinsettia, *Euphorbia pulcherrima* Willd ex Klotzsch, belongs to the large and diverse *Euphorbiaceae* family. The *Euphorbia* genus includes about 2000 species (Yang et al., 2012). The members of the *Euphorbiaceae* family are widely distributed all over the world except the arctic region. The species name *pulcherrima* means most beautiful. Poinsettia originates from Mexico and Central America and was introduced to the United States in 1825 by the first US Ambassador in Mexico, Dr. Joel Roberts Poinsett.

Each cyathium of poinsettia consists of a single female flower (attached in the cyathium center), which lacks sepals and petals. The female flower is surrounded by several male flowers and all are enclosed in a cup-shaped structure called cyathium (plural-cyathia). Bracts are modified leaves which contain red anthocyanin pigment. Breeding has also resulted in different bract colors such as purple, pink and white. In its natural environment, poinsettia is a shrub which grows to a height of more than 3 m (Figure 1) (Huang, 2007). Furthermore, poinsettia is tetraploid (4n) and has 11 different chromosomes (n). The genome size of poinsettia is 10 times larger than that of the dicotyledonous plant, *Arabidopsis thaliana*. It is documented that the genome size of a single chromosome set (n) in poinsettia is 1666 Mbp (1.7 pg), whereas in *Arabidopsis* the size is 162 Mbp (0.17 pg) (Bennett et al., 2000; Munster, 2006).

Poinsett collected poinsettia plants in hilly areas of Mexico and sent some plants to his home in Greenville, South Carolina. After that, it was distributed to various botanical gardens and growers. In the early 1900s poinsettia plants were grown in the field and sold as fresh cut flowers by Ecke in Southern California (Ecke et al., 1990). After recognition of the requirement

Figure 1: Poinsettia is a common landscape plant in the tropics (Huang 2007)

for a short photoperiod in flowering of poinsettia breeding greatly improved the quality of commercial cultivars (Garner and Allard, 1923; Ruehle, 1941). Controlled pot plant production and breeding programmes had been initiated across the U.S. in the mid-1950s and included among others the Pennsylvania State University, the University of Maryland, the USDA Research Center at Beltsville, Maryland, a number of commercial horticulture farms in California and Florida, in Hamburg, Germany, as well as Thormod Hegg and son in Lier, Norway. Now-a-days, new poinsettia cultivars are introduced by poinsettia breeder companies each year. Since the start in 1993, national poinsettia trial programs have been evaluating poinsettia cultivars in collaboration with poinsettia breeding companies (Dole et al., 2012).

In Norway, the first free branching cultivar 'Annette Hegg' was introduced in 1967 by the breeder Thormod Hegg (Taylor et al., 2011). The branching is caused by phytoplasma which are cell wall-less and unculturable bacteria (previously called mycoplasma like organisms-MLOs). In general, phytoplasma is causing symptoms such as virescence (green coloration of

non-green flower parts), phyllody, witches' broom (clustering of branches) appearance, stunting and decline (Hogenhout et al., 2008; Lee et al., 2000). However, in poinsettia phytoplasma infection mainly results in reduced apical dominance, by which more lateral shoots are formed. More branches and more flowers are thus formed (Lee et al., 1997). Such poinsettia plants are denoted free-branching poinsettia. In contrast, restricted-branching poinsettia produces few axillary shoots and flowers due to a strong apical dominance (Figure 2). To remove potential plant pathogens including *Poinsettia mosaic virus* (*PnMV*), poinsettia plants are traditionally exposed to heat treatment, meristem tissue culture or somatic embryogenic tissue culture (Dole et al., 1993; Rulz-Sifre, 1993). Such treatments results in loss of phytoplasma, and production of poinsettia with restricted-branching. In addition to resulting in the free-branching characteristics in poinsettia, phytoplasma acts as a basic growth retardant in modern poinsettia cultivars.

Figure 2: Poinsettia without (left) and with (right) phytoplasma (Lee et al, 1997)

Poinsettia is the largest pot plant culture in Norway with about 6 million plants sold annually with a market value of over 200 million NOK (Statistics of the Norwegian Growers' Association, 2010). It is also popular worldwide as potted plants for the Christmas market in different regions like North America, Europe, Asia and Australia and it represents an industry valued over $ 154 million (USDA, 2009).

Poinsettia is heterozygous and can be propagated by cuttings. Roots as well as vegetative growth can be developed in pots with high humidity and high temperature under long day (LD) conditions. To obtain desired plant height and branching as well as correct time of flowering, the cultures are started at late summer/early autumn. To induce flowering, plants are after having reached a certain size, transferred to short day (SD) conditions to induce flowering. Poinsettia plants are commonly available at the market in early November (Bævre, 1994; Odula, 2011). The appropriate time for marketing of poinsettia is after the development of red bracts (transition leaves) and cyathia. The upper green leaves become colored (bracts) due to accumulation of anthocyanin and loss of chlorophyll (Kannangara and Hansson, 1998).

1.2. Greenhouse production of poinsettia requires control of shoot elongation

In poinsettia as well as other ornamental plants control of shoot elongation in order to produce compact plants is one of the most important quality traits. Compact plants are more convenient to handle and transport compared to elongated plants. Besides, compact plants need less space in the production facilities (greenhouse culture) and reduces the costs of handling and transportation for retailers (Müller, 2011). Consequently, the production and the postharvest chain are then becoming more profitable.

Different tools for control of shoot elongation can be used such as growing plants with inherited traits making them compact, using diurnal temperature drops or lower day (DT) than night temperature (NT) (negative temperature difference; negative DIF) or manipulation with light quality by using different lamp types or photoselective films (further discussed below). Furthermore, genetic engineering might potentially be a useful tool in breeding towards cultivars showing compact growth. Due to the current high energy costs, energy saving in greenhouse production is as yet a very important focus point (Körner and Van Straten, 2008). Suppressed stem elongation under negative DIF compared to positive (higher DT than NT) and zero (constant temperature) DIF, is commonly expensive during warm areas and periods due to a need for cooling in order to obtain lower DT than NT. On the other hand, a temperature drop can commonly easily be obtained in Northern areas such as Norway by opening the greenhouse vents during the early morning. However, ventilation also typically results in greater energy consumption since it is commonly necessary to heat up the greenhouse again after temperature drop. Also, there is a limit to how much the temperature can be decreased without undesirable effects on quality parameters like shelf life. A temperature drop is also commonly difficult to obtain in warmer periods during the summer or early autumn (August to September) or in warmer areas of the world.

Growers also routinely using chemical growth retardants such as chlormequat chloride (CCC), daminozide (dimethyl aminosuccinamic acid) or paclobutrazol. However, chemical control has negative impacts on human health due to toxicity and possible carcinogenic effects, as well as negative environmental impacts (De Castro et al., 2004; Sørensen and Danielsen, 2006; Yamada et al., 2001). Restrictions have thus limited their availability and utilization and it

is desirable to further restrict or phase out their use. Therefore, it is an important issue for the poinsettia industry to reduce or even avoid the use of such compounds.

In greenhouse culture of poinsettia as well as other ornamental plants e.g such as lilies, geraniums and chrysanthemum graphical tracking is a commonly used tool for height management (Currey and Lopez, 2010; Heins and Carlson, 1990; Lopez and Currey, 2011). The graphical tracking utilises a predicted static sigmoid curve to determine the progress toward a desired plant height. Then the actual growth is compared with the predicted curves. Climatic factors can be used to control the plant height according to the graphical tracking curve. In addition, in ornamental plants, desirable timing of flowering and numbers/sizes of flowers as well as post-harvest quality are also among the most important trait. Thus, it is of outmost importance that tools used to control shoot elongation do not affect flowering and keeping quality.

1.3. Status of genetic engineering in ornamentals including poinsettia

One of the main methods of plant nuclear transformation is *Agrobacterium*-mediated transformation and biolistic transformation. In the model species *Arabidopsis thaliana* *Agrobacterium*-mediated transformation is now generally done by floral dip. Many floriculture crops have been transformed with *Agrobacterium*-protocols through shoot regeneration from explants or somatic embryogenesis (Brand, 2006). Key regeneration factors such as explant type, sterilization of explants, explant pre-culture, wounding, developmental stage and medium as well as *Agrobacterium* strains, and cultivation conditions often need to be optimized. (Clarke et al., 2008; Sriskandarajah et al., 2004). Besides, transformation systems rely on the use of a selectable marker gene during regeneration, to select the transgenic cells as well as the

regenerated shoots. Different selective agents (e.g. antibiotic kanamycin, hygromycin, phosphinothrin, gelatin etc) are used depending on the crop. Use of promoter strategy is also an important issue, and commonly a promoter driving very high level of expression of the introduced genes is used. The constitutive cauliflower mosaic virus 35S (CaMV35S) promoter has been shown to function very well with stable expression levels also in ornamental species such as poinsettia, kalanchoe, carnation and rose. Some inducible or tissue specific promoters (such as alcohol- induced, floral abscission- or senescence specific promoters) have also been used for *Agrobacterium*-mediated transformation methods (Sriskandarajah et al., 2007; Topp et al., 2008; Zakizadeh et al., 2013). However, such promoters have been less used than the well-studied constitutive CaMV35S promoter.

Most common dicotyledonous floricultural crops can be transformed by *A. tumefasciens*. Although the *Agrobacterium*-mediated transformation method has been reported in some monocotyledonous crops, it is commonly not easy to apply in such species. Instead, the microprojectile bombardment (biolistic) method has been used as a gene delivery method in a number of monocotyledonous species such as lily and tulip (Chandler and Tanaka, 2007). In poinsettia, there are only a few reports describing genetic transformation; *Agrobacterium*-mediated transformation, a biolistic transformation approach as well as electrophoresis based transformation (Clarke et al., 2011; 2008; Islam et al., 2013; Smith et al., 2006; Vik et al., 2001).

In general, a novel trait in ornamental plants is mainly developed by classical breeding, a combination of crossing and selection, as well as mutational breeding. High ploidy levels, high chromosome number and incompatibility are important barriers for classical breeding (Müller, 2011; Petty et al., 2003). Since conventional plant breeding is time consuming, biotechnological approaches are highly interesting in order to increase the efficiency of breeding (Chandler and

Tanaka, 2007; Potera, 2007). Crop improvement can then be done by the available gene pool and even by using desirable genes from unrelated species or even organisms. There are many reports on genetic modification with respect to e.g. disease resistance, flower color, flower longevity, floral scent and plant growth habit. Genetic engineering is currently an important tool for ornamental plant breeding and to date transgenic ornamentals from over 30 genera have been produced by different transformation approaches (Nishihara and Nakatsuka, 2011). Some traits for example blue rose developments are only possible to achieve by genetic engineering (Tanaka et al., 2010). Flower color modification is the most applied genetic modification. Flavonoids, carotenoids and betalains are the major pigment classes that contribute to flower color. Now, genetically modified purple color carnations and blue roses are available commercially in the market in countries allowing sale of transgenic ornamentals. The reasons behind lack of genetically modified varieties in the market are mainly financial and regulatory barriers for commercialization.

Flowers breeders are continuously trying to develop resistance to diseases. Diseases (fungi, bacteria, viruses, viroids, phytoplasmas) cause significant losses and are difficult to control. Genetic modification strategies to improve the resistance to pathogens have been discussed by Hammond et al. (2006). Clarke et al. (2008) have developed a transgenic poinsettia with resistance to *Poinsettia mosaic virus (PnMV)* using hairpin RNA gene silencing. The transgenic PnMV-resistant poinsettia plants have been verified and evaluated under ordinary growing conditions in a growers' greenhouse. Such plants can be useful as virus-free, not transformed stock is difficult to keep free form re-infection. It can be noted that poinsettia is rapidly re-infected by PnMV, although no vector is known (Blystad and Fløistad, 2000; Siepen et al., 2005). Similarly, insect pests and mites are of importance for floriculture and cause major

costs for growers. A genetic modification strategy using the *Bt* (*Bacillus thuringensis*) genes have very little effects on the most prevalent pests of the floriculture industry (Christou et al., 2006). In the future, alternative insecticide genes (such as proteinase inhibitors and lectins) might be more efficient.

For the ornamental industry, vase life of flowers is very important. Leaf and flower senescence are mainly regulated by cytokinins and ethylene. The molecular aspects of ethylene biosynthesis and ethylene receptors are well understood. The 1-aminocyclopropene 1-carboxylic acid (ACC) synthase and ACC oxidase, which in sequence catalyze the final steps of ethylene biosynthesis, are key regulatory points. There are some reports on genetically modified (GM) plants showing reduced sensitivity to exogenous ethylene and delayed senescence due to expression of the cytokinin biosynthesis gene *ISOPENTENYL TRANSFERASE (IPT)* or the ethylene receptor gene (*ETR1-1)* from *A. thaliana* (Sriskandarajah et al., 2007; Zakizadeh et al., 2013).

As discussed above, compactness of the plants is an important trait in greenhouse-grown poinsettia. Biotechnology offers a prospect of isolating structural or regulatory genes which may have a profound effect on plant shape. GM ornamental plants harbouring such genes can then be used in breeding programs. The status of modification of plant morphology through genetic engineering is described below after discussion of general aspects of plant hormone physiology and their control of plant morphology.

1.4. Hormonal regulation of elongation growth

Hormones are naturally occurring organic substances in plants. They are chemical messengers active in very small amounts and may exert their effects either at the site of biosynthesis or in a different cell, organ or part of the plant. Hormones are produced in one cell

and modulate cellular processes in either the same or another cell by interacting with specific proteins that function as receptors which are linked to one or more signal transduction pathways (Taiz and Zeiger, 2010a). In addition to being controlled by intrinsic growth regulators such as hormones, plant growth and development are controlled by external signals which in turn affect the hormone physiology (Santner et al., 2009). The major classes of plant hormones are auxin, gibberellin (GA), cytokinin, abscisic acid (ABA), ethylene, brassinosteroid, salicylic acid, jasmonate and strigolactone. In addition, other compounds such as the peptid systemine, polyamines, nitric oxide and karrikins are also known to act as growth regulating substances. Only auxin, GA, cytokinin and ABA will be discussed here since these hormones were studied in the present work (biosynthesis/metabolism in figure 5, paper III).

1.4.1. Auxin

Auxin regulates cell division, cell expansion, cell differentiation, lateral root formation, flowering and tropic responses (Davies, 2004). Indole-3-acetic acid (IAA) has been recognized as a major auxin from the 1930s. In recent years it was demonstrated that IAA directly interacts with the F-box protein TIR1 (TRANSPORT INHIBITOR RESPONSE 1) and promotes the degradation of the aux/IAA transcriptional repressor to trigger diverse auxin-responsive genes (Dharmasiri et al., 2005; Kepinski and Leyser, 2005; Tan et al., 2007). Four biosynthesis pathways of IAA from tryptophan (trp) are well known in plants: the YUCCA (YUC) pathway, the indole-3-pyruvic acid (IPA) pathway, the indole-3-acetamide (IAM) pathway, and the indole-3-acetaldoxime (IAOx) pathway. Trp is the main precursor of IAA and indole-3-acetaldehyde is probably not a precursor of IAA in the IPA pathway (Mashiguchi et al., 2011; Sugawara et al., 2009; Woodward and Bartel, 2005; Zhao, 2010). In *A. thaliana* SHORT INTERNODES (SHI)

protein regulates the expression of YUC4 (Sohlberg et al., 2006). IAA mainly moves from the apical to the basal end of the shoot (basipetally) and from the basal to the apical end of the roots (acropetally). This type of unidirectional energy-demanding transport is denoted 'polar transport'. Auxin can be produced in shoots and roots. IAA causes cell extension by stimulating proton pumps in the plasmamembrane. The resulting acidification of the cell wall leads to cell wall loosening as a consequence of an effect of the lowered pH on expansin proteins. At acidic pH the expansins probably somehow weakens the hydrogen bonds between the polysaccharide components of the cell wall.

Generally, IAA acts as a positive regulator of photomorphogenesis. Phytochrome light receptors, which act as red (R) and far-red (FR) light sensors, may regulate the stem elongation by depleting IAA within the epidermis of plant. This reduces the growth of the stem. Increased IAA under low photosynthetic active radiation (PAR) and low R:FR ratio resulted in increased hypocotyl elongation in *A. thaliana* through increased activity of IAA-mediated gene expression (Steindler et al., 1999; Vandenbussche et al., 2003). DT and NT temperature differences has also been shown to affect the IAA levels in *A. thaliana* with reduced IAA levels correlating with reduced elongation under negative DIF compared to positive DIF (Thingnaes et al., 2003). Bioactive GAs and auxin positively regulate stem elongation and the level of the active GA has been shown to be affected by IAA in some plants. In pea removal of the apical bud (source of auxin) reduced the endogenous level of GA_1 and this was completely reversed after the application of IAA to the decapitated plant. A similar interaction of auxin and GA has been found in elongating parts of shoots and internodes also in tobacco, barley and *A. thaliana* (O'Neill and Ross, 2002; Ross et al., 2000; Wolbang et al., 2004; Wolbang and Ross, 2001).

1.4.2. Gibberellin

GAs are tetracyclic terpenoid compounds that are biosynthesized through complex pathways. Both GA biosynthesis and deactivation pathways are regulated by developmental, hormonal and environmental signals as documented by biochemical, genetic and genomic approaches (Yamaguchi, 2008). Plant growth and development, stem elongation, seed germination, leaf expansion, flower and seed development are all processes affected by biologically active GAs. Up to now, there are 136 GAs are identified (http://www.plant-hormones.info/gibberellins.htm); most of them are inactive precursors and only a few (basically GA_1, GA_3, GA_4 , GA_5, GA_6 and GA_7) are biological active (Hedden and Phillips, 2000; Yamaguchi, 2008). The biosynthesis of GA takes place in three different compartments of the cell; the plastid, the endoplasmic reticulum and the cytosol. Conversion of geranylgeranyl diphosphate to bioactive GA (GA_4 in *A. thaliana* and GA_1 in pea) needs different classes of enzymes. The late steps of the GA biosynthesis are in sequence catalyzed by GA 20-oxidase (GA20ox) and GA3-oxidase (GA3ox) enzyme activities, with GA3ox being responsible for production of the active GA_1 and GA_4. GA catabolism refers to the conversion of bioactive GAs to inactive products and is catalyzed by a type of 2-oxoglutarate dependant dioxygenase (2-ODD) named GA 2-oxidases (GA2ox).

An active GA signalling pathway requires expression of GA-induced genes and degradation of the DELLA repressors. DELLA proteins are a subtype of proteins belonging to the GRAS family of transcription factors. In *A. thaliana*, the most important DELLA proteins are repressor of GA (RGA), GA insensitive (GAI) as well RGL1, RGL2 and RGL3 (RGA-like) (Zentella et al., 2007). The known GA receptor, GIBBERELLIN INSENSITIVE DWARF 1 (GID1) can stabilize the interaction of DELLA proteins with the SLEEPY1 (SLY1) F-box

protein, a SCF type E3 ubiquitin ligase component that targets DELLA proteins for degradation through the 26S proteosome pathway (Depuydt and Hardtke, 2011). Lack of the DELLA domain in some mutant alleles makes the respective proteins hyperactive and insensitive to GA regulation (Peng et al., 1997; 1999; Silverstone et al., 1998).

Both the sensitivity of GA and GA levels are important in regulation of shoot elongation. GA biosynthesis is regulated by active phytochrome (phy). Decreased GA_1 levels in dark-grown pea seedlings after exposure to white light, R, FR or blue (B) light are mediated redundantly by phyA and cryptochrome 1 (cry 1) but not phyB (Ait-Ali et al., 1999; Foo et al., 2006; Gil and García-Martinez, 2001; Reid et al., 2002). It has been reported in pea that reduced elongation in response to reduced GA_1 level under B and R light is correlated with down-regulation of *PsGA3ox1* and up-regulation of *PsGA2ox2* (Foo et al., 2006; Reid et al., 2002). Similarly, in *A. thaliana* decreased elongation growth due to reduced GA_4 levels was associated with down-regulation of *AtGA20ox1*, *AtGA3ox1* and up-regulation of *AtGA2ox1* gene expression (Zhao et al., 2007). When *A. thaliana* plants were transferred from SD to LDs, an increase in the level of GA_{20}, GA_1 and GA_8 was observed and was probably due to enhanced activity of *GA20ox* (Xu et al., 1997). Also, GA_1 and GA_4 do apparently not reach the shoot apex in vegetative plants of *A. thaliana* due to deactivation by GA2ox at the base of shoot apex (King and Evans, 2003). However, GA_5 and GA_6 are more effective in promoting flowering compared to GA_1 and GA_4 and these compounds may thus act as floral stimuli (King and Evans, 2003). Furthermore, in *Lolium* selective deavtivation of GA below the shoot apex is critical to flowering but not to stem elongation (King et al., 2008). As described below in the section on thermoperiodism, thermoperiodic control of stem elongation in pea is associated with modulation of GA levels largely through altered activity of *PsGA2ox2* (Grindal et al., 1998a; Grindal et al., 1998b;

Stavang et al., 2007; 2005). Reduced elongation growth due to decreased GA_1 levels under a negative DIF or a diurnal temperature drop in the middle of the light phase is linked to substantially enhanced expression of *PsGA2ox2.* Also, in *A. thaliana* enhanced hypocotyl elongation in response to increased temperature was associated with increased transcript levels of *AtGA2ox1* (Stavang et al., 2009).

1.4.3. Cytokinin

Cytokinins (CKs) are plant hormones regulating the proliferation and differentiation of plant cells as well as various processes like delay of senescence and control of shoot/root balance. Naturally occurring CKs are derivatives of adenine containing an isoprenoid or aromatic side chain at the N^6 position. 2-isopentenyl adenine (2iP) and its hydroxylated forms zeatin (Z) and dihydrozeatin (DHZ) are examples of isoprenoid CKs. The two isomers of Z, *cis*Z (cZ) and *trans*Z (tZ) differ in the position of their terminal hydroxyl group in the isoprenoid side chain. tZ and iP generally exhibit the highest activity whereas cZ has a weak biological impact only (Gajdošová et al., 2011; Sakakibara, 2006). This occurs through either ethylene action or blocking the transportation of IAA. However, how endogenous CKs mediate photomorphogenesis is unclear.

1.4.4. Abscisic acid (ABA)

Like GAs, ABA is a class of metabolites known as isoprenoids, also called terpenoids. ABA plays important roles in control of cellular processes including seed development, dormancy, germination, vegetative growth and environmental stress responses (Xiong and Zhu, 2003). ABA promotes the closure of stomata to minimize transpirational water loss. A high level

of exogenous ABA inhibits plant growth under non-stressed conditions and increased ABA level under environmental stress conditions results in ABA-induced changes at the cellular and whole plant level (Xiong and Zhu, 2003). ABA biosynthesis occurs in roots, vascular tissue and stomata.

ABA is a 15 carbon atom formed from the methyl erythritol phosphate (MEP) pathway by cleavage of a C_{40} carotenoid precursor, followed by a two-step conversion of the intermediate xanthoxin to ABA via ABA–aldehyde (Nambara and Marion-Poll, 2005; Taylor et al., 2000). ABA is inactivated by two main pathways. Hydroxylation of the free ABA forms results in formation of phaseic acid (PA) which is further converted to dihydrophaseic acid (DPA) by the oxidation pathway. Alternatively, ABA can be the conjugated to monosaccharides (mostly with glucose) forming ABA-glucose ester (ABA-GE). It is considered that ABA-GE is a storage form of ABA, which can be stored in the vacuoles and released when ABA is needed (Dietz et al., 2000). ABA is well known as an important regulator of transpiration through its action on stomata function. High ABA levels under drought results in stomatal closure. In tomato (*Solanum lycopersicum*) ABA levels in different ABA mutants showed strong correlation with plant height (Nitsch et al., 2012). The ABA deficient double mutants *notabilis/flacca* (*not/flc)* in tomato had the lowest ABA levels and the lowest expression of ABA genes, resulting in smaller cell size and fruit size (Nitsch et al., 2012).

Phytochrome appears to be involved in change of endogenous ABA levels. The ABA content in mature plants *of Lemna gibba* and *A. thaliana* increased when transferred to darkness but was reduced when *L. gibba* was treated with R light (Weatherwax et al., 1996). Endogenous ABA levels in plants have mostly been investigated under stressful conditions. Under drought stress, the turgor pressure is reduced and this inhibits turgor-dependent activities like cell

expansion, resulting in reduced stem length. The relationship between light and ABA levels has not yet been clearly established (Kraepiel and Miginiac, 1997).

1.5. Genetic engineering for control of shoot elongation

As discussed above, commonly consumers desire short and compact plants with a high ornamental value and good keeping quality. Compacts plants are easier to transport compared to more elongated plants and tolerate mechanical handling better than longer plants and also need less space during production in greenhouses (Müller, 2011). In most cases modulation of plant height by molecular engineering approaches has aimed to reduce the endogenous bioactive GA content. Increasing the expression of *GA2ox* has been shown to increase the degradation of GA in a variety of species like *A. thaliana*, rice, wheat (*Triticum aestivum*), tobacco (*Nicotiana tabacum*) and Japanese plum (*Prunus salicina*) as well as species of *Citrus*, *Populus* and *Solanum* (Appleford et al., 2007; Busov et al., 2003; Curtis et al., 2000; Dijkstra et al., 2008; El-Sharkawy et al., 2012; Eriksson et al., 2000; Fagoaga et al., 2007; Huang et al., 1998; Radi et al., 2006; Sakamoto et al., 2003; Schomburg et al., 2003). Reducing the expression of *GA20ox* has been shown to suppress GA biosynthesis and result in reduced elongation growth (Coles et al., 1999; Hedden, 2003; Topp et al., 2008).

Also, increased expression of the regulatory *GA INSENSITIVE (GAI) DELLA* gene involved in GA signal transduction resulted in a dwarf or semi-dwarf phenotype in *A. thaliana,* apple (*Malus domestica*), chrysanthemum and petunia (Koorneef et al., 1985; Petty et al., 2003; Tanaka et al., 2005; Zhu et al., 2008). As discussed above (in the GA section) several genes encoding DELLA family proteins acting as transcriptional regulators, have been identified (Eckardt, 2002; Wen and Chang, 2002). Important well-known effects of DELLA proteins are

their functions as negative regulators of GA-responses. In dwarf plants obtained by suppression of the GA response, some abnormalities were observed such as delayed flowering in chrysanthemum and reduced rooting ability in apple (Petty et al., 2003; Zhu et al., 2008).

Increased expression of the cytokinin biosynthesis gene *IPT*, which encodes a rate-limiting step in cytokinin biosynthesis, in chrysanthemum resulted in reduced internode length and more flowers, although flowers were small and the flower formation was delayed (Khodakovskaya et al., 2009). Introduction of the *root loci* (*rol)* genes isolated from *Agrobacterium rhizogenes* are known to affect the levels of cytokinin and auxin, and thus provide another tool for modification of plant architecture (Giovannini et al., 1999). Infection with strains of *A. rhizogenes* gives 'hairy root' characteristics at the site of infection. The use of such modification techniques in different plants is reviewed by Lütken et al. (2012).

The Knotted-1 like homeobox (*KNOX*) gene family *is* divided into three classes and is mainly expressed in the meristematic tissue. Several of these homeotic genes have been functionally characterized and are used in molecular breeding towards compactness in ornamentals (Lütken et al., 2011). Increased expression of *KNOX* genes lead to increased cytokinin biosynthesis and negatively regulated GA biosynthesis through suppression of *GA20ox*'s (Leibfried et al., 2005). Also, auxin plays a significant role in down-regulation of *KNOX* expression during organ differentiation (Hay et al., 2006).

Furthermore, overexpression of the *SHI* from *A. thaliana* has previously been shown to result in dwarfed plants of the ornamental *Kalanchoe* (Lütken et al., 2010). Details of the transformation strategy with SHI family genes and their known characteristics are discussed below in the section of the SHI family. The above discussed strategies to control shoot elongation using genetic engineering are summarized in figure 3.

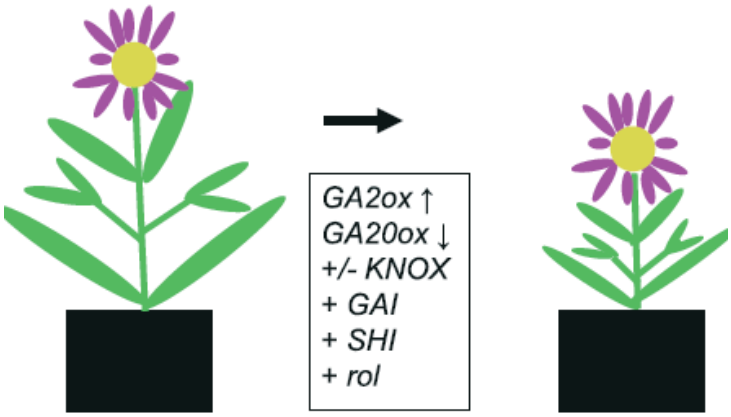

Figure 3: Genetic engineering strategies to control shoot elongation of plants. (+) indicate the upregulation and (-) indicate the downregulation of genes (Lütken et al., 2012). *GA2ox* : *GA 2-oxidase, Ga20ox* : *GA 20-oxidase*, *KNOX*: *KNOTTED-1 LIKE HOMEOBOX. GAI: GA INSENSTIVE. SHI: SHORT INTERNODES. ROL: ROOT LOCI.*

Considering the traditional plant breeding approaches are time consuming and rather inefficient for poinsettia due to its heterozygous genetic background. We introduced the *A. thaliana* gene *AtSHI* into the poinsettia genome controlled by the CaMV35S promoter through *Agrobacterium*-mediated transformation (Islam et al. 2013; paper I) using a method developed by Clarke et al. (2008) (figure 4). Compact plants were then obtained (Islam et al., 2013). Also, there is ongoing work with genetic engineering aiming at increasing *GA2ox* expression in poinsettia. Gateway cloning of *GA2ox2* from *Phaseolus coccineus* has been performed to develop the vector construction. Furthermore, development of marker-free transgenic poinsettia using ParA mediated site-specific excision technology is also ongoing. So far positive clones at the callus stage have been obtained for these constructs, but since more work is needed to obtain

transgenic plants, this work was not included in the present thesis. Molecular and physiological analysis will be performed after establishment of transgenic plants.

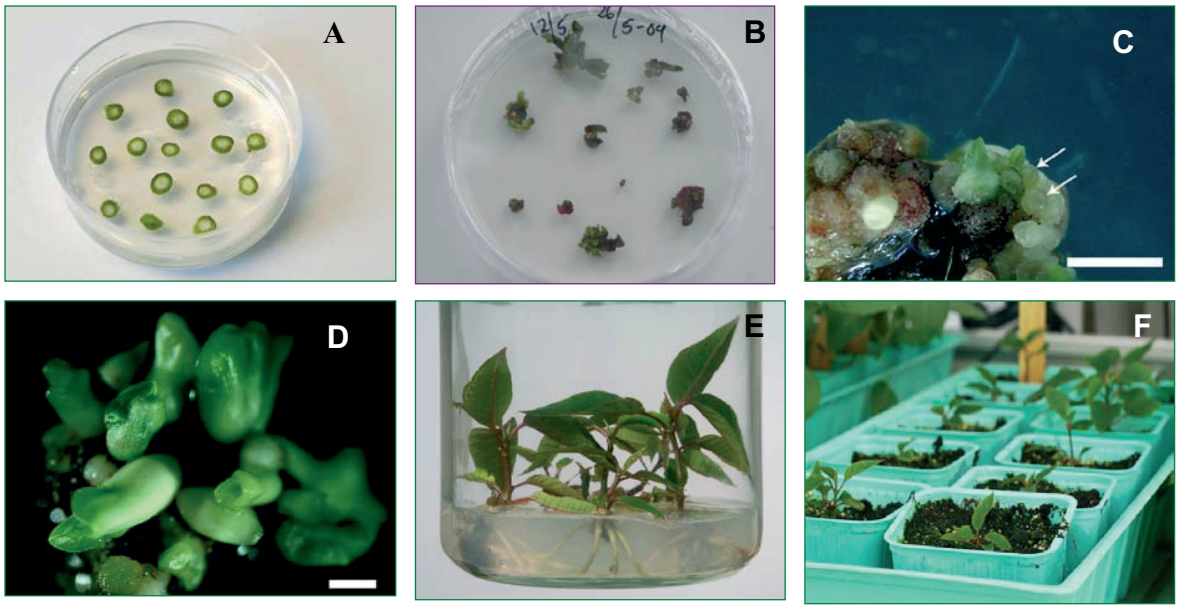

Figure 4: A: Stem explants of poinsettia growing in callus induction medium (CIM), B: callus growing in somatic embryo induction medium (SEIM), C: embryogenic structure and globular stage of somatic embryos D: cotyledonary stage of somatic embryos E: plantlets growing in root induction medium (RIM) F: regenerated plants established in soil (Source: Jihong Liu Clarke and Clarke et al., 2008).

1.6. The SHI family

The *SHORT INTERNODES (SHI)* gene was isolated from *A. thaliana* by Fridborg et al. (1999) and later shown to belong to the *SHI* gene family consisting of 10 members; *SHI, STYLISH 1 (STY1)* and *STY2, LATERAL ROOT PRIMORDIUM 1 (LRP1)* and *SHI-RELATED SEQUENCE 3* to *8 (SRS3* to *SRS8)* (Fridborg et al., 1999; Fridborg et al., 2001; Kuusk et al.,

2006; Zawaski et al., 2011). The *SHI* gene is a plant specific transcription factor which is not found in any organism outside the plant kingdom. *SHI*-homologs have been identified in different plants species like tomato (*Solanum lycopersicum*)), rice, soybean (*Glycine max*) and *Medicago truncatula* (Fridborg et al., 2001). In a phylogenetic analysis, *STY1* and *SHI* associated in one clade, *STY2* and *SRS4* in another clade and *SRS5* and *SRS7* in still another clade. *SRS3,* which resembled two poplar homologues (*Pt1* and *Pt2*), and *SRS6* and *LRP1* which resembled homologues in rice, *Populus*, tomato and the moss *Physcomitrella patens,* all grouped in a large clade, as supported by a high bootstrap value (Figure 5). *SRS8* was not included in this analysis, because it was considered as a pseudogene due to no amplification of *SRS8* cDNA. In contrast, the other genes of the *SHI* family were amplified from all tissue of *A. thaliana* (Kuusk et al., 2006).

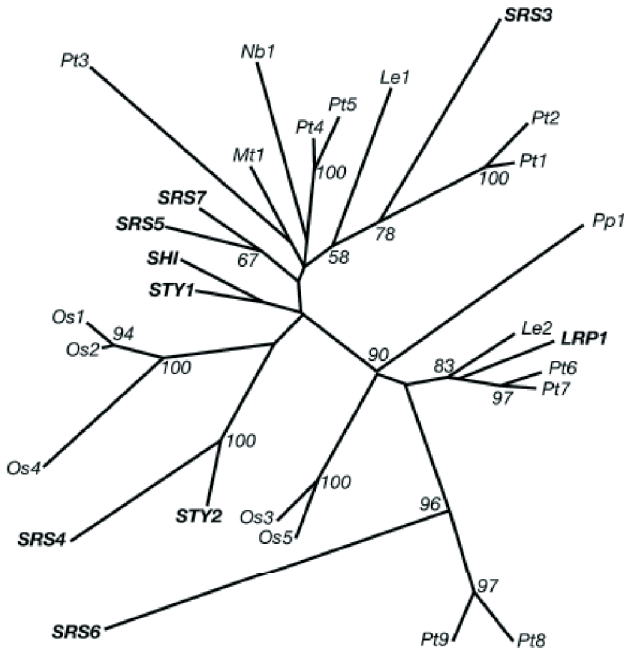

Figure 5: Phylogenetic inter-relationship of the *SHORT INTERNODES* (*SHI*) gene family; *Arabidopsis* genes are in bold. The tree is an unrooted phylogram on the basis of analysis of DNA sequences of the two conserved regions (zinc finger and IGGH domains). Branches lengths are drawn proportionally to evolutionary distance (Kuusk et al., 2006).

The *SHI* family-corresponding proteins have two highly conserved regions, a RING-like zinc finger motif positioned in the N-terminal end and an IGGH domain of unknown function in the C-terminal part of the protein (Fridborg et al., 2001; Kuusk et al., 2002; Kuusk et al., 2006). The zinc finger domain consists of one or several protein motifs. Each motif binds a zinc ion and the target molecules, which can be DNA, RNA, protein or lipids (Brown, 2005; Gamsjaeger et al., 2007; Hall, 2005; Klug, 1999). The binding of target molecules depends on the amino acid sequence of the zinc finger domain, the linker between the fingers, the number of fingers as well as the higher order structure. Both the zinc finger motif and the IGGH domain are unique to the SHI family protein. The sequence identity of the first region varies between 64% and 90% (Figure 6A). This motif is called the C3HC3H RING domain because the consensus arrangement of the RING domains of SHI, SRS1, SRS2, SRS4, SRS5 and SRS7 comprises Cys-X_2-Cys-X_7-Cys-X-His-X_2-Cys-X_2-Cys-X_7-Cys-X_2-His, a 31 amino acid residues (X is any amino acid residue). SRS3, SRS6 and LRP1 are lacking the first conserved His residue of the restricted C3HC3H RING consensus. The second conserved region in the C-terminal of the SHI family is called the IGGH domain due to four highly conserved residues within the region. In this domain of the SHI family a short acidic cluster is present (Figure 6B). In addition, in the SHI/STY family one or two Gln-rich regions and a nuclear localization signal are present (Eklund et al., 2010a). STY1 is the closest paralog of SHI having two identical domains in the N and C terminal (Figures 5 and 6).

Fridborg et al. (1999) showed that the *SHI* gene in *A. thaliana* is involved in response to GA and identified the gene as a negative regulator of GA-induced cell elongation. Recently, it was reported that SHI family members act as DNA-binding transcription activators and that their

function appears to be primarily in maintaining auxin homeostasis in *A. thaliana* (Sohlberg et al., 2006; Ståldal et al., 2012; 2008). SHI accordingly is influencing plant growth and development

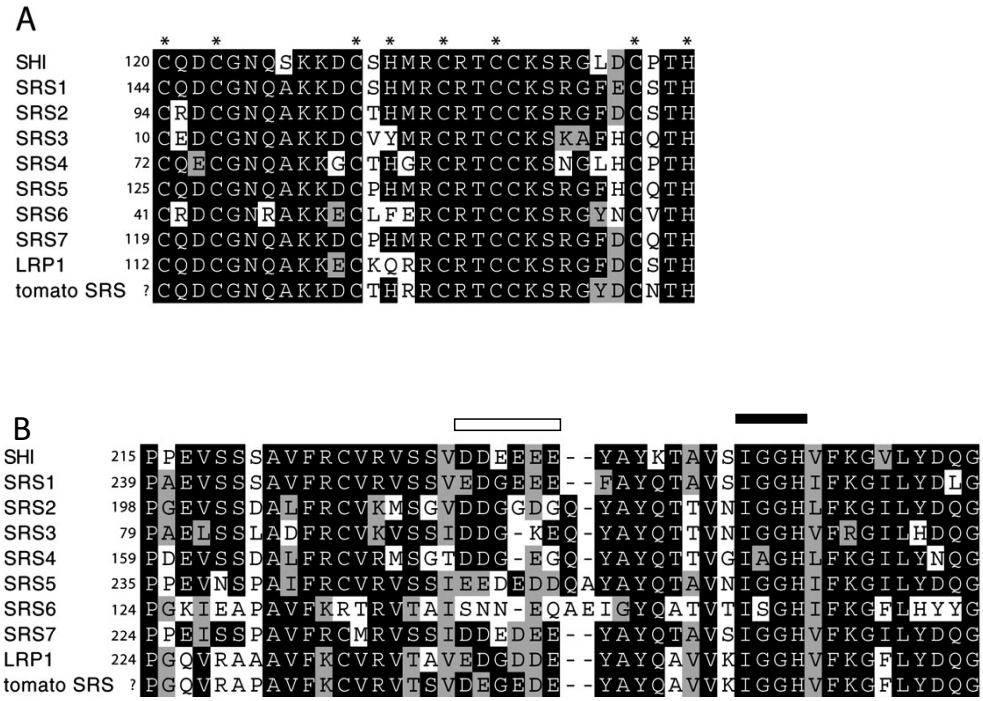

Figure 6: Sequence comparison of RING domains (A) and IGGH domains (B) of SHI-related proteins. Asterisks indicate the conserved Cys and His zinc ligand residues in the RING finger motif. In the IGGH domain white box indicate the acidic stretches and the black box are four IGGH residues. (Fridborg et al., 2001)

by affecting the phytohormones auxin and GA, which among others control shoot elongation in response to different stimuli (Fujita et al., 2008; Stavang et al., 2009; 2005; Yamaguchi, 2008; Zhao, 2010). Overexpression of *SHI* in *A. thaliana* resulted in dwarfed plants, but a normal, more elongated phenotype could be restored by application of auxin (Sohlberg et al., 2006; Ståldal et

al., 2008). Also, the *YUCCA4* (*YUC4*) auxin biosynthesis gene is induced by the SHI/STY family proteins. Furthermore, *SHI* overexpression in *A. thaliana* plants resulted in increased levels of the inactive GA_{34} compared to wild type (WT) plants (Fridborg et al., 1999). In *Brassica SHI*-related genes have been identified as negative regulators of GA-induced cell division (Hong et al., 2010). Moreover, in several plant species a correlation between auxin and GA has been observed. IAA application to the apical meristem has been shown to increase biosynthesis of bioactive GA (Frigerio et al., 2006; Ross et al., 2000; Wolbang et al., 2004). Also, levels of auxin and GA commonly follow each other such as during shoot elongation and short-day induced apical growth cessation in woody species, where levels of both hormones are high and low, respectively (Olsen et al., 1997a; b). In order to obtain compact plants, *AtSHI* represents a highly interesting target for a biotechnological approach. In an effort to apply genetic engineering in poinsettia compact plants without any effect on time to flowering were obtained by introduction of *AtSHI* (Islam et al., 2013) (paper I). Similarly, reduced shoot elongation was observed in *A. thaliana*, *Kalanchoe*, *Populus* and *P. patens* expressing *AtSHI* (Eklund et al., 2010b; Fridborg et al., 1999; Lütken et al., 2010; Zawaski et al., 2011). However, in *A. thaliana,* late flowering due to overexpression of *AtSHI* was observed.

1.7. Regulatory constraints and public concerns on genetic engineering

At present GM crop plants are grown over a large area in several countries and genetically modified grains are consumed globally (e.g. maize, soybean, cotton, canola, papaya). First genetically modified crops were commercialized in 1996 and then the cultivated area of GM crops were 1.7 million hectares, whereas it reached up to 170 million hectares in 2012 (James, 2012).This makes GM crops the fastest adopted crop technology in the recent era.

However, genetic engineering has not been actively practised by the floricultural breeding companies. A relatively small market for ornamental crops makes many costly breeding strategies less profitable for the breeders within the floricultural industries. Also, the cost for patents, license for patented methods and regulatory barriers might be the reason for lack of available cultivars in the market. Transgenic carnations have been are available in North America, Japan and Europe from 1997 and at present grown in Colombia, Ecuador and Australia (www.florigene.com.au). Thereafter, the transgenic rose variety 'Applause' was commercially released 2009 in Japan (www.suntorybluerose.com). In European countries, promotion of GM crops is very much restricted. However, in 2012 five EU countries (Spain, Portugal, the Czech Republic, Slovakia and Romania) grew a record of 129,071 hectares of transgenic Bt maize. This was a 13% area increase over 2011. However, some other countries like USA and China are relatively permissive in comparison, whereas Australia is somewhat in between (Lütken et al., 2012). Some GM floricultural crops in some environments, may be invasive and pose ecological risk (Auer, 2008). It can be mentioned that floricultural products are grown usually in closed cultivation in small areas. Thus, the potential environmental impact of genetically modified varieties is commonly limited to the production end of the supply chain. Besides, GM ornamentals are non-food, non-feed crops which are probably more uncomplicated to consumers compared to edible crops. It might be expected that more genetically modified cut flowers and pot flowers will be released and that public acceptance of genetic engineering will be increased further. Generally, some non-government organizations (NGOs) and some government organizations campaign to make GM free regions or to ban GM product crops. Thus, GM floricultural crops are also likely to be caught by this legislation. For example, the Moonseries™ carnation cannot be sold in the state of Tasmania, Australia due to Governmental legislation

banning of the genetically modified product in that state. Public perceptions on GM products are not very clear as they did not get any opportunity to choose whether or not they would purchase the GM products. Apparently there are mixed opinions on acceptability of GM food (according to surveying European reports). However, people are inclined to accept the GM food. (EU, 2006).

1.8. Light receptors and light quality responses for elongation growth

Generally, the B light absorbing cryptochromes and the R/FR absorbing phytochromes affect photomorphogenesis and floral transition in a variety of species but the effects varies among species (Barnes and Bugbee, 1991; Cosgrove, 1981; Guo et al., 1998; Imaizumi et al., 2003). In plants light acts as a source of energy which is essential for photosynthesis but also acts as a signal regulating growth and development. In the photosystems of the chloroplasts, light energy is converted to chemical energy in the so called light-reaction which involves passing of high-energy electrons through an electron transport chain consisting of series of compounds alternately acting as electron donors and electron receptors. Upon absorbtion of a photon (hv) by chlorophyll (chl), an electron in this pigment is transferred from its lowest-energy or ground state to a higher energy or excited state (chl*).

$$Chl + hv \rightarrow chl^*$$

Chlorophyll absorbance of B light results in excitation to a higher energy level compared to R light because of the higher energy of photons in the shorter B wavelength area. In photosynthesis, the B and R light are the most effectively absorbed, whereas green light is absorbed much less effectively (Bareja, 2011; Inada, 1976; McCree, 1972). In addition to the importance of B and R light in photosynthesis, these light qualities are also important in the

regulation of plant growth by light receptors in photomorphogenesis. In this respect, the B light (peak at 465 nm) absorbing cryptochromes and the R (peak at 660 nm) and FR (peak at 730 nm) light absorbing phytochrome light receptors are of particular importance.

Phytochrome exist as two photoconvertible isomers, the FR light absorbing form P_{fr} which is considered the active form and the R light absorbing inactive form P_r (Nagatani, 2004). Phytochrome is a soluble protein complex and act as a dimer where each part consists of a chromophore (light absorbing pigment molecule) and an apoprotein (polypeptide chain). Together the chromophore and apoprotein are called the holoprotein. The chromophore of phytochrome is phytochromobilin, which is a linear tetrapyrrole. The phytochrome apoprotein cannot absorb R or FR light or cannot form the holoprotein without being covalently linkage to phytochromobilin. The phytochromobilin is synthesized in plastids and it is exported to the cytosol where the apoprotein is attached through a thioether linkage. Most of the phytochrome pool moves to the cell nucleus to regulate gene expression which plays a role in photomorphogenesis (Castillon et al., 2007; Taiz and Zeiger, 2010b) (Figure 7). Once in the cell nucleus, P_{fr} is interacting with different phytochrome interacting factors (PIFs), which are proteins degraded by the 26S proteosome complex in response to a light signal (Castillon et al., 2007). In darkness or under low R:FR, a pool of phytochrome is excluded from the nucleus, enabling the accumulation of PIF proteins that promote elongation responses (Lorrain et al., 2008). In *A. thaliana* 7 members of the *PIF* gene family (*PIF*1 to 7) have been found, and PIFs are negatively regulating photomorphogenesis by activating expression of different genes (Castillon et al., 2007).

High pressure sodium (HPS) lamps are commonly used in greenhouses. They have a high emission of PAR and a high electrical efficiency but contain only 5% B light which is low

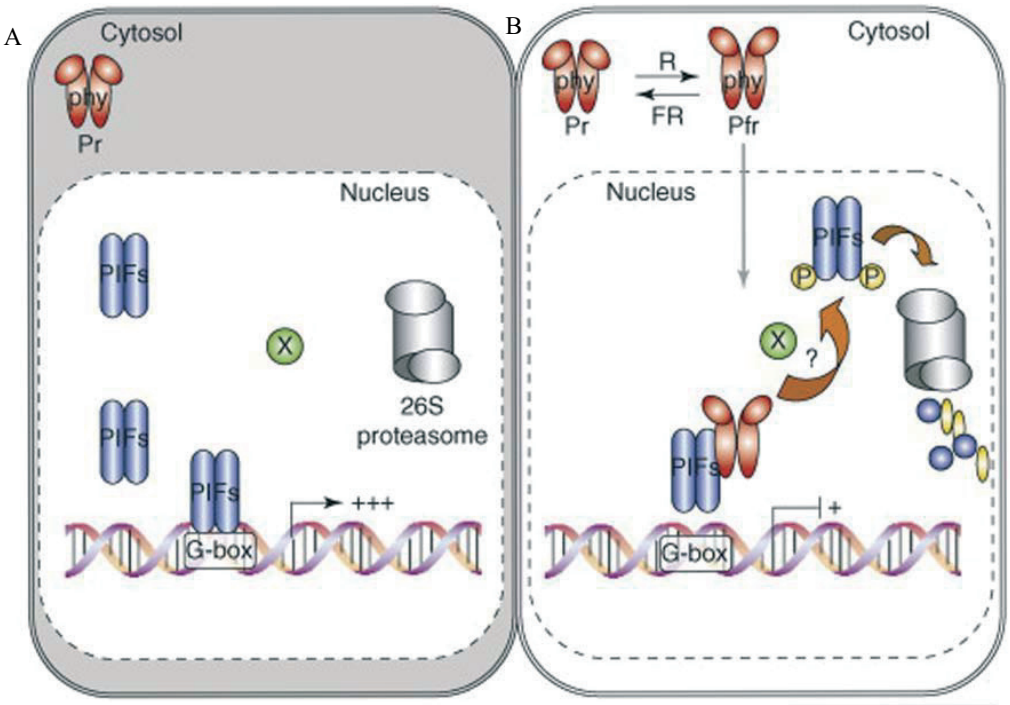

Figure 7: Model of PIF interaction in the phytochrome signalling pathway. A: In dark- PIFs negatively regulate photomorphogenesis by activating gene expression. Here phytochromes and PIFs are localized in the cytosol and nucleus, respectively. B: In light PIFs are interacting with P_{fr} in the nucleus. The phosphorylated forms of PIFs are degraded by 26 S proteosome and this result in relieving the negative regulation of photomorphogenesis. (Castillon et al., 2007)

compared to natural sun light (18% B). The R:FR-ratio during the light period or during relatively short periods at the end of the day (photoperiod) as well as the proportion of B light are known to affect stem elongation and morphology, but the effects vary among species (Blom et al., 1995; Decoteau et al., 1988; Gilbert et al., 1995; Imaizumi et al., 2003; Kasperbauer and Peaslee, 1973; Mata and Botto, 2009; Mohr, 1986; Olsen et al., 1997b).

Furthermore, plants are sensitive to shade by other plants and this is called the shade avoidance response (SAR). Shading increases stem extension due to decrease in the ratio of R:FR light or the ratio of P_{fr} to total phytochrome (P_{fr}:P_{total}). FR light is easier transmitted through the canopy than R light. It has been reported that a high R:FR ratio results in compact plants and that plants also are very sensitive to short periods (e.g. 10-60 minutes) of R or FR at the end of day (EOD) (Clifford et al., 2004; Hisamatsu et al., 2005; Ilias and Rajapakse, 2005; Mata and Botto, 2009; Symons and Reid, 2003). Flowering time was slightly delayed in poinsettia under high R:FR ratio in experiments where light quality was manipulated by using FR photoselective or transparent films (Clifford et al., 2004; Mata and Botto, 2009). Within a species the effect might vary with cultivar, like in the SD plant chrysanthemum the flowering was delayed under high R:FR ratio in 'Bright Golden Anne' but not in 'Spears' (McMahon, 1999; Rajapakse and Kelly, 1995). Generally, FR and B promote flowering through phytochrome A and cryptochromes 1 and 2 in *Arabidopsis* whereas R inhibits flowering through phytochromes B, D and E in pea (Aukerman et al., 1997; Devlin et al., 1998; Weller et al., 2001). In mitochondria and chloroplasts of *A. thaliana* CRY3 appears to have repair activity for UV–B lesions in single-stranded DNA and loop structures of duplex DNA, but apart from this its function remains elusive (Herbel et al., 2013).

Recently developed light emitting diodes (LEDs) can be used as a light source for greenhouse crop production and can be easily placed on the top of the plant canopy (Barta et al., 1992; Bula et al., 1991). Such LEDs can be used to provide a narrow-band light spectrum to the plants and have become an important tool in studies of light quality responses with respect to morphogenesis, photosynthesis, chlorophyll and anthocyanin synthesis in different plant species (Robin et al., 1994; Stutte, 2009; Tripathy and Brown, 1995).

In general, B light promotes stomata opening and photosynthetic efficiency more compared to other wavelengths (Goins et al., 1997; Sharkey and Raschke, 1981; Zeiger et al., 2002). Further, there are a substantial number of reports that B light is involved in suppression of internode growth and thus stem elongation due to inhibition of cell expansion or division (Appelgren, 1991; Dougher and Bugbee, 2004; Folta et al., 2003; Hoenecke et al., 1992; Terfa et al., 2012). However, there are also a number of reports that B light enhanced stem elongation in species like salvia, marigold and petunia compared to R light (Fukuda et al., 2011; Heo et al., 2002). Thus, the effect of B of shoot elongation varies with species. Clifford et al. (2004) suggested that removal of B light by using specific spectral filters does not affect poinsettia significantly. However, B light responses of poinsettia have generally been little studied. In paper II we showed that a high proportion of B light (20%) suppressed stem elongation in the poinsettia 'Christmas Spirit' and 'Christmas Eve' (Islam et al., 2012). Furthermore, B light has also been shown to affect flowering differently in different species. In LD plants like petunia and *Arabidopsis* flowering was enhanced by B compared to R light, whereas the day neutral plant (*Cyclamen persicum*) showed delayed flowering in B light (Eskins et al., 1989; Fukuda et al., 2011; Guo et al., 1998; Imaizumi et al., 2003). In the SD plants, *Xantthium pennsylvanicum* and *Lemna perpusilla* flowering was stimulated when B light was given continuously or when it replaced inductive darkness (Hillman, 1965; Withrow and Withrow, 1940). On the other hand, the flowering time of day-neutral pot roses as well as poinsettia, which is a SD plant, were unaffected when grown under LED with 20% B compared to the tradition HPS lamp with only 5% B (Islam et al., 2012; Terfa et al., 2012).

The action of the phytochrome system is complex. There are two different classes of phytochromes: type I (light -labile form) and type II (light stable form). There are also five

phytochrome genes (*PHY A* to *E*) in *A. thaliana*, three (*PHYA* to *C*) in rice (*Oryza sativa*), three (*PHYA, PHYB1* and *PHYB2*) in *Populus* and 3 in Norway spruce (*Picea abies*) (*PHYP* resembling *PHYB*, *PHYN* resembling *PHYA* and *PHYO* resembling *PHYC/PHYA*) (Basu et al., 2000; Dehesh et al., 1991; Howe et al., 1998; Mathews and Sharrock, 2008; Olsen, 2010; Sharrock and Quail, 1989). PhyA function throughout the life of a plant to sense R and FR light.

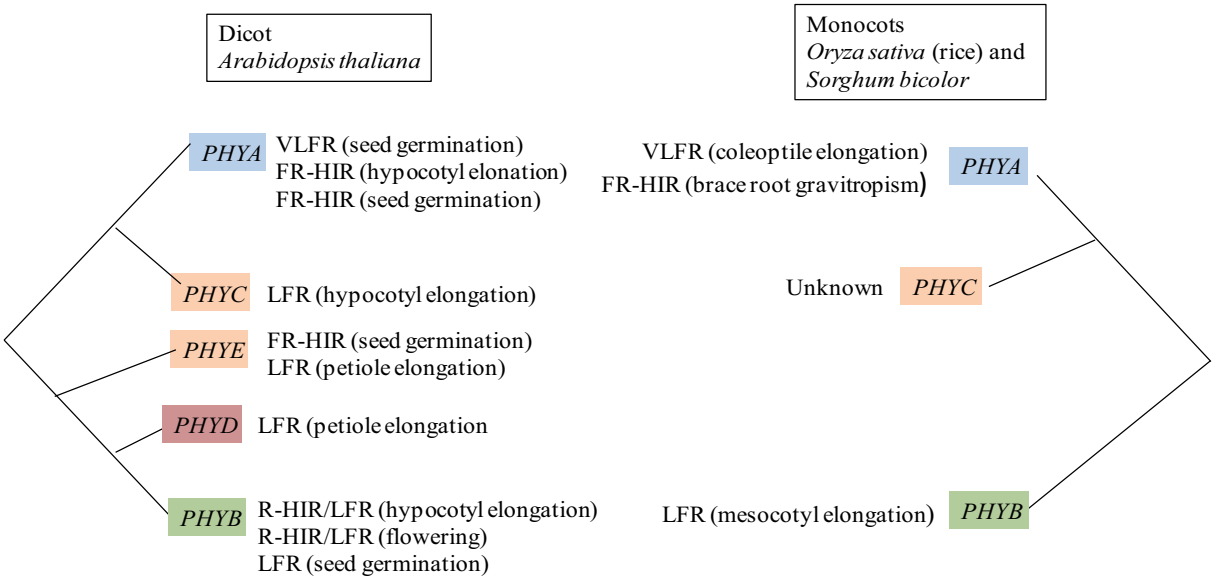

Figure 8: Figure shows a comparison of the phytochrome gene family function and structure between monocot and dicot plants.
VLFR: very low fluence response, LFR: low fluence response, HIR: high irradiance response- (µmol m^{-2} s^{-1}), FR: far red, R: Red (Taiz and Zeiger 2010b)

phyA, *phyB* and *phyC* are known to be involved in photoperiodic control of flowering in *A. thaliana* and rice (Takano et al., 2005; Valverde et al., 2004). In the shade avoidance response phyB, phyD and PhyE are involved in perception of the R:FR ratio (Devlin et al., 1999; Smith,

1995). *phyA phyB* double mutants are more elongated than the single mutant *phyB*. Furthermore, *phyB phyD* and *phyB phyE* double mutants are more elongated and show earlier flowering than the single mutant *phyB* (Devlin et al., 1998; 1999). A study with overexpression of *PHYC* indicates a role of phyC in extension growth of *A. thaliana* (Franklin et al., 2003). Modes of action of the phy family members are highly conserved and the responses may be distinguished by the irradiance level and light quality, as shown in figure 8.

1.9. Temperature and thermoperiodic control of shoot elongation

Temperature is an important factor for plant growth and development. Most processes are affected by temperature, ranging from enzymatically catalyzed reactions and membrane transport to physical and physiological process such as photosynthesis, respiration and biosynthesis (Stavang, 2005). For an enzymatic process to be initiated, the energy level must be above a threshold value. This energy level is denoted the activation energy, and the ambient temperature influences this threshold energy level as well as the rate of the reaction. Generally, the DT in nature is higher than the NT due to solar radiation that heats the ground. Plants show different responses to DT and NT, a phenomenon referred to as thermoperiodism (Went, 1944). Also, the difference between the DT and NT (DIF) and short daily temperature drop periods have been shown to affect plant morphogenesis (Erwin et al., 1989). A lower NT than DT is denoted a positive DIF whereas a higher NT than DT is referred to as negative DIF. Diurnal temperature alternation influence internode length, plant height, petiole and flower stem length, chlorophyll content, leaf and shoot orientation and flowering in several plant species (Myster and Moe, 1995; Xiong et al., 2011). In order to control stem elongation in several horticultural crops, temperature drop and negative DIF strategies are used in green houses as discussed briefly above (Erwin and

Heins, 1995; Myster and Moe, 1995). A daily temperature drop in the early morning or during e.g. the last 2 h of the night commonly suppresses the stem elongation, whereas the weakest sensitivity is observed if temperature drops at night (Grindal and Moe, 1994; Ueber and Hendriks, 1992). Suppressed stem elongation in negative DIF compared to positive DIF and constant temperature (zero DIF) has been observed in a variety of species (Myster and Moe, 1995).

Both a moderate temperature drop (e.g. 24 to 16^{o}C) and a large temperature drop (e.g. 24 to 8^{o}C) have been shown to reduce plant height of poinsettia but leaf chlorosis and delayed flowering were observed under the large temperature drop (Ueber and Hendriks, 1992). Moe et al. (1992) obtained reduced plant height in poinsettia under negative DIF (DT<NT) compared to the plants grown in positive DIF and zero DIF (constant temperature). However, the plants grown under negative DIF were late flowering.

The knowledge on the physiological and molecular background of the effects of DIF and drop treatments on elongation growth is still relatively limited. There are some reports that DIF affects cell length or both cell length and number of cells (Erwin et al., 1994; Strøm and Moe, 1997; Thingnaes et al., 2003). DIF also interacts with light quality, light quantity, photoperiod and growth retardants but not with drought stress (Myster and Moe, 1995; Patil and Moe, 2009). Interestingly, in pea (*Pisum sativum*) negative DIF greatly enhanced the expression of the GA deactivation gene *GA2ox2* compared to positive and no DIF (Stavang et al., 2005). Similarly, expression of *PsGA2ox2* was stimulated during a temperature drop in the middle of the light phase, but no effect on *PsGA2ox2* was observed during a temperature drop in the middle of the night (Stavang et al., 2007). Thus, *PsGA2ox2* is apparently a main gene mediating thermoperiodic control of stem elongation in pea (Stavang et al., 2005). Furthermore, in *A.*

thaliana grown in continuous light increased expression of *AtGA2ox1* was observed after increasing the temperature (Stavang et al., 2009). It also appears that a defective phyB makes plant unable to respond to different DIF regimes by modulation of elongation growth. Alternatively, phyB is necessary to complete transduction of the temperature signal. *phyB* mutants in cucumber and *A. thaliana* did not show any response to DIF treatments whereas the wild types showed reduced plant height under negative DIF (Patil et al., 2003; Thingnaes et al., 2008). Further, in *A. thaliana* it appears that only phyB, but not phyD and phyE are necessary for complete temperature signal transduction (Moe et al., 2003; Thingnaes et al., 2008).

2. Aim of the study

The main aims of the present study were to use genetic engineering and light quality responses as tools to control shoot elongation in poinsettia plants without affecting the flowering time and postharvest quality. The approaches used were selected due to the need for controlling shoot elongation in greenhouse culture of poinsettia.

The specific objectives were

1. To use *Agrobacterium*-mediated genetic transformation to introduce the *AtSHI* gene in poinsettia in order to regulate shoot elongation in poinsettia.
2. To evaluate the potential of using LED with a high proportion of B light (20%) in production of compact poinsettia plants without affecting flowering time and postharvest quality, all in comparison with traditional HPS lamps.
3. To evaluate the potential of using EOD treatment with R light to reduce shoot elongation in poinsettia, either in combination with LED light with a high proportion of B or HPS lamps.

4. To study the effect of EOD-R as compared to EOD-FR on hormone levels in poinsettia. The idea behind this was to evaluate how manipulation of the phytochrome system at the EOD affects hormone physiology.

3. Materials and methods

In this work, the different cultivars of poinsettia used were 'Millenium', 'Christmas Spirit', 'Christmas Eve' and 'Advent Red'. An efficient protocol for genetic engineering of poinsettia using the 'Millenium' was previously established by Clarke et al. (2008). This cultivar was shown to be efficient in producing somatic embryos and high transformation efficiency was obtained. In the present work the effect of introduction of the *AtSHI* gene on plant morphology and other traits was investigated (Paper I). 'Christmas Spirit', 'Christmas Eve' and 'Advent Red' were used to study the effect of an increased proportion of B light and EOD-R on plant morphology, time to flowering and post-harvest quality (paper II). To study the effects of manipulation of the phytochromes system by EOD R and EOD-FR light on hormone physiology 'Christmas Spirit' and 'Christmas Eve' were used (paper III). These cultivars are commonly used in commercial poinsettia culture in Norway and were supplied from G3 Ljones Gartneri AS, Tørvikbygd, Norway.

The *Agrobacterium*-mediated transformation of poinsettia with the *AtSHI* gene and the tissue culture steps involved (paper I) were performed according to Clarke et al. (2008). Internode explants were then sterilized with 70% ethanol, 1% NaOCl and rinsed with autoclaved water. The stem segments were then inoculated in an *Agrobacterium tumefasciens* suspension and transferred to callus induction medium (CIM) for co-cultivation. After that, the stem segments were transferred to CIM with antibiotic (kanamycin) selection and kept on this

medium for 10 days before being transferred to somatic embryo induction medium (SEIM) with the same antibiotics. After formation of shoots, these were transferred to root induction medium. The somatic embryo- and root induction media (for shoots or plantlets) were changed after every three weeks. The details of the media and antibiotics used have been described in the materials and methods of paper I.

In paper I, the transgenic lines were screened by polymerase chain reaction (PCR) analysis and Southern blot analysis was thereafter performed to investigate whether the *AtSHI* gene was stably integrated into the genome of poinsettia (Sambrook et al., 1989). Real time quantitative PCR (qRT-PCR) analysis was conducted with gene specific primers and 1x Power SYBR® green PCR master mix (Applied Biosystems, Warrington, UK). cDNA was synthesized from two microgram total RNAs from each transgenic line. The *AtSHI* gene expression was analysed by following the $2^{-\Delta\Delta CT}$ method and the *α-tubulin* gene was used as internal reference gene (Livak and Schmittgen, 2001). Phenotypic analyses of the *AtSHI* expressing plants were conducted in growth chambers under SD conditions of 10 h photoperiod as well as in a greenhouse compartment under LD conditions of 16 h photoperiod. Shoot elongation and other morphological traits, flowering and post-harvest quality were evaluated. Since *AtSHI* is thought to affect auxin homeostasis IAA levels were analysed in shoot tips of 3 replicate samples using liquid chromatography-mass spectrometry (LC-MS).

In paper II the effect of an increased proportion of B light (20%) provided by LED (Round LED-light 162W, VA–24150T, SoLa-co, Grimstad, Norway) on poinsettia plant morphology, flowering and post-harvest quality was investigated as compared to traditional HPS lamps providing 5% B light but with similar phytochrome photostationary state (PPS). The experiment was performed in greenhouse compartments as well as growth chambers.

Furthermore, the effect of 30 min of EOD R light on morphology and flowering were investigated in plants grown in green house under supplementary lighting with HPS or LED.

In paper III the effect of manipulation of the phytochrome system by exposure of poinsettia plants to 30 min of EOD-R and FR light on hormone levels was investigated. The levels of different plants hormones were then quantified in shoot tips. For each genotype and treatment, 6 samples consisting of three replicate shoot tips were harvested before flowering. The samples were freeze-dried and analysed for their content of auxin, GA and cytokinin and ABA (paper III). The endogenous levels of auxin, ABA and CKs were analysed in 3 samples by liquid chromatography (LC)-MS according to Chiwocha et al. (2003) and Abrams et al. (2003); and GAs in 3 other samples by an ultra performance LC (UPLC)-MS/MS method according to Urbanova et al. (2013).

In the light quality experiments a 10 h photoperiod was used with a PPFD of 100±20 µmol m^{-2} s^{-1}, RH 70±5 % corresponding to 0.7 kPa vapour pressure deficit (VPD), and a temperature of 21±2^{o}C.

4. Results and discussions

4.1. Control of shoot elongation in poinsettia by *AtSHI* overexpression

The work in this thesis has focused particularly on the control of shoot elongation of poinsettia using different tools. As discussed above, control of shoot elongation is among the main challenges in greenhouse culture of poinsettia. Since biotechnological breeding in many cases have resulted in targeted and efficient modulation of specific traits such, one approach was to utilize gene modification as a tool to control shoot elongation in poinsettia. Since overexpression of the *AtSHI* gene was earlier shown to affect shoot elongation in *Kalanchoe*,

Populus and *A. thaliana* (Fridborg et al., 1999; Lütken et al., 2010; Zawaski et al., 2011), this gene was introduced in poinsettia in the present work.

In paper I, the effect of constitutive expression of the *AtSHI* gene in poinsettia 'Millenium' on plant morphology as well as flowering and post-harvest quality was investigated. Three independent transgenic poinsettia lines showing stable integration of the *AtSHI* gene were obtained, as verified by PCR and Southern blot analysis. The *AtSHI* expression resulted in a significant reduction of shoot elongation compared to untransformed control plants under SD as well as LD conditions (Figures 5 and 6A, paper I). Under SD the transgenic line (TL) 1 showed the strongest shoot elongation reduction response (52%) compared to untransformed control plants, whereas the TL2 and TL3 plants were reduced in height by 49% and 30%, respectively. Thus, in SD conditions the TL1 and TL2 showed the largest reduction in height compared to the WT control plants. Consistent with this, real-time PCR analyses of SD grown plants revealed significantly higher transcript levels of the introduced *AtSHI* gene in TL1 and TL2 than in TL3. The height reduction under SD was more pronounced than in LD where the height reductions were 25%, 21% and 23% in TL1, TL2 and TL3, respectively. This can be explained by more vigorous elongation growth under the higher light sum of LDs (16 h). Such a reduction of shoot length was also observed in *Kalanchoe*, *Populus* and *A. thaliana* expressing the *AtSHI* gene (Fridborg et al., 1999; Lütken et al., 2010; Zawaski et al., 2011). The highest degree of internode length reduction was also observed in TL1, where internodes were 49% shorter than in the control plants. Thus, reduction in internode length corresponded to the reduction in plant height, demonstrating that the reduction in shoot length was due to reduction in internode lengths.

No significant difference was observed in petiole length in *AtSHI* overexpressing *Arabidopsis* and similar result was obtained in poinsettia. However, in poinsettia the bract sizes

and bract numbers were significantly reduced by *AtSHI* expression. Reduced bract size is not desirable for such an ornamental plant, and might represent an obstacle in using such plants in breeding. On the other hand, in addition to reduced height TL3 showed a trend of comparatively larger bract area and bract number than TL1 and TL2 (Figure 6A, 7C and 7D, paper I). TL3 might accordingly be particularly promising. The reduced height as well as reduced bract area and bract number might be due to an effect of *AtSHI* on hormone metabolism as discussed further below.

No significant difference was observed in the relative chlorophyll content among the transgenic and control plants. This is similar to in *AtSHI* overexpressing *Kalanchoe,* but in contrast to the situation in *A. thaliana*, which showed darker green leaves. This difference might be due to the growth habit and developmental stage of *Arabidopsis* compared to *Kalanchoe* and poinsettia.

4.2. Control of shoot elongation in poinsettia by light quality

Growth modulation by different light qualities has previously been studied to a limited extent only in poinsettia. The potential use of LED light in greenhouse cultures is currently subjected to considerable interest due to the possibility of targeted growth modulation while saving energy due to less heat generation by such light sources. Since B light has been shown to reduce shoot elongation in many, but not all species, we thus studied effects of an increased B light proportion provided by LED light (paper II). Specifically, the effect of an increased B light proportion provided by LED light with 20% B and 80% R light was investigated as compared to HPS lamps. Among the three cultivars (‘Christmas Spirit’, ‘Christmas Eve’ and ‘Advent red’) used in this study, ‘Christmas Spirit’ showed the highest reduction in shoot elongation (paper II).

‘Christmas Spirit’ and ‘Christmas Eve’ were tested under SD conditions in greenhouse compartments with natural and supplemental light as well as under controlled conditions in growth chambers. About 34% plant height reduction was observed in ‘Christmas Spirit’ both in greenhouse and growth chamber-grown plants. Compared to HPS, the LED-treatment resulted in 27% and 21% reduction in plant height of ‘Christmas Eve’ when grown in greenhouse compartments and growth chambers, respectively (Figure 2). ‘Advent Red’ showed 20% height reduction in greenhouse compartments.

For both light treatments the phytochrome photostationary state (PPS) was very similar at 0.89 in LED and 0.85 in HPS. This means that 89 and 85% of the phytochrome was in its active form (P_{fr}) in LED and HPS, respectively. Thus, in both cases a high percentage of phytochrome was in its active form. Therefore, the reduction of height under LED light was apparently due to high percentage of B light and not other aspects of the light (Sager et al., 1988; Stutte, 2009). Thus, cryptochrome photoreceptors, which are known to be important in B light control of shoot elongation in several species, might also very important also for stem elongation in poinsettia. The clear, reduced shoot elongation response to increased B light proportion is similar to what has been observed for other crops like as soybean, pepper and lettuce (Brown et al., 1995; Hoenecke et al., 1992; Schuerger et al., 1997; Wheeler et al., 1991). On the other hand, there are also reports showing very small or no effect of B light on shoot elongation (Clifford et al., 2004; Mortensen and Stromme, 1987). Some species such as *Petunia* even show a considerable promotion of stem extension in response to B light (Fukuda et al., 2011; 2012). This suggests that B light perception or signaling differs in different plant species. This might be due to differences in B light receptors (probably cryptochromes) or one or more factors in the downstream signaling.

Use of EOD treatments with low intensity light to control shoot elongation requires little energy and are thus of great interest in greenhouse production. In our study the Advent Red' showed significantly reduced shoot length in response to a 30 min EOD-R treatment (LED) when HPS light was used as supplemental light in greenhouse compartments (Figure 7, paper II). In contrast, when the LED with 20% B and 80% R light was used as supplemental light there was no significant effect of the EOD-R treatment in this cultivar, just a slight, insignificant tendency of reduction. 'Christmas Eve' did not show any significant response to EOD-R when either HPS or LED was used as supplemental light. The explanation for the general lack of effect of EOD-R in combination with LED may be that the light-receptor dependent inhibition of shoot elongation had already been saturated due to the high content of B (20%) and R (80%) of the LED light. The difference in response between the cultivars studied suggests that there might be differences in the phytochrome system or downstream signaling. Other workers have also reported suppressive effects on stem elongation of EOD-R in species like *Petunia*, *Chrysanthemum*, tobacco and soybean (Ilias and Rajapakse, 2005; Kasperbauer and Peaslee, 1973; Rajapakse et al., 1993).

Although effects of R:FR ratios have been studied to a limited extent only in poinsettia, previous studied using selective films absorbing FR or R light as well as the present one (Clifford et al., 2004) (paper II), indicate that morphology in this species is affected by the R:FR ratio. In paper III we aimed to investigate the effect of EOD-R and EOD-FR (provided by monochromatic LED) on hormone levels in order to evaluate the effect of manipulation of the phytochrome system at the EOD on hormone physiology. In this study (paper III), the shoot elongation of poinsettia was 54% and 34% shorter in the 'Christmas Spirit' and 'Christmas Eve' respectively, under 30 min of EOD-R light compared to EOD-FR light in growth chambers with

the main lighting provided by HPS (10 h) (Figure 1 and 2, Paper III). There was a positive correlation between internode length and stem elongation. Thus, the shorter plant height under R-EOD compared to EOD-FR treatment was apparently linked to reduced internode elongation rather than production of fewer internodes. Different sensitivity to EOD-R and EOD-FR in different poinsettia cultivars probably depends on differences in the phytochrome photoreceptors or their action (down-stream signaling).

4.3. Endogenous auxin and its correlation with shoot elongation in *AtSHI* expressing poinsettia

The degree of stem elongation is related to the extensibility of the cell wall and osmotic uptake of water. Osmotic uptake of water is not influenced by GA, rather GA is influencing the mechanical extensibility and the stress relaxation of cell walls by affecting the microtubuli which in turn affect cellulose microfibril orientation (Taylor and Cosgrove, 1989). When the cellulose microfibrils in response to GA get more perpendicular orientated relative the axis of extension, elongation is enhanced. GAs also promotes cell division in the subapical (rib) meristem (Hansen et al., 1999; Sachs, 1965). Auxin, another important hormone regulating stem growth, stimulates cell elongation through cell wall acidification, which results in cell wall loosening (Rayle and Cleland, 1992; Taiz and Zeiger, 2010a). CKs are well known as a regulator of cell proliferation and auxin is also involved in control of cell division in addition to the control of cell elongation (Depuydt and Hardtke, 2011).

As discussed above, in this study reduced stem extension was observed in the *AtSHI* expressing transgenic poinsettia plants (paper I). Among the three independent transgenic poinsettia lines showing stable integration of the *AtSHI* gene in poinsettia reduced shoot

elongation due to high *AtSHI* expression level was correlated with reduced plant height and reduced level of endogenous IAA in shoot tips (paper I). Sohlberg et al. (2006) reported that the SHI/STY family appears to control the developmental process of *A. thaliana* through regulation of auxin biosynthesis. IAA influences the biosynthesis of bioactive GA and inhibitors of auxin transport reduce the bioactive GA content in pea and *A. thaliana* (Frigerio et al., 2006; O'Neill and Ross, 2002; Ross, 1998; Ross et al., 2000). The reduction of shoot and internode lengths observed in the present study of poinsettia is consistent with the result obtained in the moss *P. patens* overexpressing *PpSHI* (Eklund et al., 2010b). The reduced bract size in the transgenic lines of poinsettia might also be due to the reduced level of auxin which might in turn also have affected the GA levels. An effect of auxin has been found in the expanding leaves and cotyledons in *Arabidopsis* (Ljung et al., 2002). In contrast to the results in poinsettia and *A. thaliana*, *AtSHI* expressing *Populus* did not show any change in auxin levels in spite of reduction in height, but cytokinin content was increased when the internode number was increased and petiole length was reduced (Zawaski et al., 2011).

4.4. Endogenous hormones and their correlation with shoot elongation in EOD-R and EOD-FR treated poinsettia

IAA as well as the total GA was significantly correlated with the stem elongation of poinsettia under EOD-R and EOD-FR treated plants (paper III). The lowest amounts of IAA and total GA were found in shoot tips of plants exposed to EOD-R, which as discussed above, resulted in reduced shoot elongation compared to EOD-FR light (Figure 6, Table 2, paper III). The analyses of individual GAs demonstrated that the early 13-hydroxylation pathway of the late GA biosynthesis is the dominating pathway in poinsettia with the non-13 hydroxylation as a

minor pathway (Table 2, Figure 5, paper III). Significantly increased amounts of total GAs, GA_{29}, and GA_8 as well as increased ratio of GA_1/GA_{20} and GA_6/GA_5 were found under EOD-FR compared to EOD-R light. However, there was no significant effect of the light quality treatments on the GA_8/GA_1 or GA_{29}/GA_{20} ratio, only slight insignificant trends of increased ratios in EOD-FR compared to R. On the other hand, the significantly lower GA_1/GA_{20} ratio in EOD-R compared to EOD-FR suggests that conversion of GA_{20} to GA_1 is lower under EOD-R. Furthermore, the slight but significant effect of the light qualities on the GA_6/GA_5 ratio suggests a more rapid conversion of GA_5 (which is a metabolite of GA_1) to GA_6. Thus it appears that the GA levels are regulated by light quality at several metabolic steps. Interestingly, poinsettia contained high levels of GA_3, similar to those of GA_1. This indicates that the side chain by which GA_3 if formed from $GA2_{20}$ via GA_5 (Sponsel and Hedden, 2004) is highly active in poinsettia. However, neither the GA_3 levels nor the GA_3/GA_5 ratios were significantly different in the EOD-R and FR treatments, indicating that this side chain is not affected by these light treatments. Similar trends of GA metabolites were found in other crops such as *Marah marcocarpus*, *Malus domestica and Zea mays* (Albone et al., 1990; Spray et al., 1996). Phytochrome light receptors, which percept R and FR light, are known to affect the levels of GAs and IAA. Increased amounts of GAs and IAA were previously e.g. found in cowpea and sunflower (*Helianthus annuus*) under FR as compared to R light (Behringer and Davies, 1992; Kurepin et al., 2007a, b).

In thermoperiodic studies reduced elongation under negative DIF and temperature drop in the middle of the light has been shown to be associated with increased GA inactivation in pea (Stavang et al., 2007; 2005). In *A. thaliana* increased temperature in plants grown in continuous light was shown to be linked to reduced inactivation of GA and increased biosynthesis of IAA.

Also, negative DIF treatment of *A. thaliana* was found to reduce IAA levels (Thingnaes et al., 2003). In contrast to when temperature is reduced during the day, there were, as discussed above, no clear indications of a significant effect of EOD-R on GA inactivation, only on GA biosynthesis. Thus, temperature alteration in the light phase and light quality apparently affect GA metabolism differently.

After the 30 min of EOD treatment in our study there was no significant difference in cytokinin level between the two light qualities, but the ABA content was higher under EOD-FR exposed plant compared to the EOD-R light treatment (Figure 7, paper III). This might suggest that ABA play some role in such light quality responses, like indicated in some earlier studies of sunflower and *L. gibba* (Kurepin et al., 2007b; Weatherwax et al., 1996). In contrast, there were no significant differences in the levels of different CKs metabolites in the present study (Table 3, paper III). This is consistent with previous studies showing no correlation between elongation and cytokinin levels (Kurepin et al., 2007a).

Leaf area and bract area were lower under R light compared to FR light (Figure 4, paper III). This might be due to effects of the reduced levels of GA and IAA. Previously, e.g. Kurepin et al. (2007b) , Olsen and Junttila (2002) and Martínez-García et al. (2000) have also demonstrated higher endogenous levels of GAs under low R:FR. In sunflower significantly increased levels of the inactive GA_8 and GA_{20} (the precursor of GA_1) were demonstrated.

4.5. Flowering and postharvest quality of poinsettia

Being used as an ornamental pot plant, not only shoot elongation but also flowering is an important feature in poinsettia. The time to appearance of visible cyathia and bracts depends upon SD and is observed after a certain time under such conditions. Generally, 'Millenium'

produces flowers more quickly compared to the 'Christmas Spirit' and 'Christmas Eve' under SD conditions. In the present studies 'Millenium' started to produce visible cyathia after 5 weeks of SD exposure whereas 'Christmas Spirit' and 'Christmas Eve' started to produce visible cyathia after 9 weeks under SD. No significant difference in flowering time was found among the transgenic *AtSHI* overexpression and non-transgenic control plants (paper I). Similarly, there was no difference in time to flowering in LED and HPS light-treated plants or the EOD treated plants (paper I, II and III). Generally, photoperiod, light quality and GA are well known as important factors which regulate flowering (Mouradov et al., 2002; Simpson and Dean, 2002). GA promotes flowering in LD-plants like *A. thaliana* and *Lolium* but inhibits flowering in SD-plants like rice (Eriksson et al., 2006; Izawa et al., 2002). Furthermore, the results in *AtSHI* overexpressing poinsettia with no effect on time to flowering was similar to those observed in *Kalanchoe* but in contrast to the situation in *AtSHI* overexpressing *A. thaliana*, which showed late flowering (Fridborg et al., 1999; Lütken et al., 2010),.

After completion of bract formation in poinsettia, plants (paper I and II) were transferred to a post-harvest room for testing their postharvest quality at a temperature of 21^{o}C, RH of 30-40% and an irradiance of 10 µmol m^{-2} s^{-1} for 12 h provided by fluorescent tubes. Postharvest-quality was tested in the plants ('Christmas Spirit' and 'Christmas Eve'-paper II) grown under LED and HPS light in greenhouse compartments as well as growth-chamber-grown transgenic *AtSHI* expressing plants compared to untransformed control plants ('Millenium' - paper I). No significant effect was found in time of flowering (paper I, II, III) and on the postharvest quality (bract necrosis, cyathia abscission or leaf dropping) of poinsettia from any experiment (paper I, II). The lack of effect of light quality on time to flowering is in contrast to an earlier study in which 'Freedom Red' poinsettia showed slightly delayed flowering time grown under a high

R:FR ratio under a photoselective film absorbing FR (Mata and Botto, 2009). The difference between this study and ours might indicate that different cultivars respond differently to light quality with respect to time to flowering. 'Lilo' and 'Starlight' poinsettia grown under negative DIF negative effects on postharvest quality was observed, i.e. all cyathia abscised (Moe et al., 1992). Since neither the light quality treatments tested in this work nor the overexpression of *AtSHI* appeared to affect post-harvest quality, such treatments might be a better strategy to modulate shoot elongation in poinsettia than using DIF regimes.

5. Conclusions

We have shown here that exploiting light quality responses and a genetic engineering strategy to control plant morphology in poinsettia constitute promising options in order to reduce the use of plant growth retardants in greenhouse production of poinsettia. Transgenic expression of *AtSHI* resulted in reduction of stem elongation without negative effects on flowering time and postharvest quality (paper I). The reduction in plant height was associated with reduced levels of IAA. Bract area was also decreased, but in one out of three transgenic lines obtained, bract area was somewhat larger than the two other lines. Since poinsettia is vegetatively propagated, the improved characteristics will be inherited, which is a clear advantage for the use of genetic engineering as a tool in poinsettia breeding. The present studies in paper II and III demonstrate that an increased proportion of B light and EOD-R provided by LEDs might be good tools to modulate shoot elongation without affecting time to flowering and post-harvest quality. However, the difference in response to EOD-R in the tested cultivars emphasizes the importance of investigating light quality responses in different commercially grown cultivars. We also characterized the hormone physiology linked to the reduced elongation under EOD-R as

compared to EOD-FR. Total GAs, IAA and ABA was higher under FR light compared to R light and different metabolites were correlated with shoot elongation. Thus, manipulation of the phytochrome system at the EOD provides a possibility to modulate the levels of the growth-promoting hormones GA and IAA.

6. Further perspectives

Exploiting transgenic poinsettia plants such as the *AtSHI* expressing ones reported here in breeding may be an interesting option for the future. Our study (paper I) demonstrates that poinsettia is amenable to improvement with respect to shoot elongation by gene modification. Other strategies such as alteration of expression of specific hormone metabolism genes such as e.g. *GA20ox* and *GA2ox* are also highly interesting in poinsettia, and studies on using *GA2ox* are ongoing. Also, breeders can potentially improve poinsettia productivity; ensure disease and insect resistance through identification of quantitative trait locus (QTL) in combination with genetic modification. To avoid any concern related to ecosystems and the public demand, molecular biologists have been trying to eliminate the selectable (antibiotic resistance) marker genes in transgenic plants. Further efforts in this respect are highly desirable also in poinsettia, and such an attempt is ongoing. In vegetatively propagated plants like poinsettia, a site specific recombination system can be used to eliminate the selectable marker gene (Schaart et al., 2004).

Since the effect of light qualities on elongation growth varied among different cultivars in the present and previous studies, it is desirable to investigate the shoot elongation response in different cultivars which are grown commercially. In this respect, also the effects of interaction between light quality, irradiance and temperature would be of considerable interest. Furthermore, the effect of UV-B radiation, which is known to reduce shoot elongation in a variety of species is also of interest in greenhouse culture of poinsettia (Jansen, 2002). The use of either UV-

transparent cladding material or short duration of UV-B radiation from fluorescent UV-B tubes have been shown to be promising in this respect (Torre et al., 2012). However, more detailed investigations of these strategies are necessary to evaluate the usefulness of such treatments in different commercially grown poinsettia cultivars. Finally, basic knowledge on light receptors and regulation of hormone physiology in poinsettia is scarce and merits further investigation.

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Paper I

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Overexpression of the *AtSHI* Gene in Poinsettia, *Euphorbia pulcherrima*, Results in Compact Plants

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Abstract

Euphorbia pulcherrima, poinsettia, is a non-food and non-feed vegetatively propagated ornamental plant. Appropriate plant height is one of the most important traits in poinsettia production and is commonly achieved by application of chemical growth retardants. To produce compact poinsettia plants with desirable height and reduce the utilization of growth retardants, the *Arabidopsis SHORT INTERNODE* (*AtSHI*) gene controlled by the cauliflower mosaic virus *35S* promoter was introduced into poinsettia by *Agrobacterium*-mediated transformation. Three independent transgenic lines were produced and stable integration of transgene was verified by PCR and Southern blot analysis. Reduced plant height (21–52%) and internode lengths (31–49%) were obtained in the transgenic lines compared to control plants. This correlates positively with the *AtSHI* transcript levels, with the highest levels in the most dwarfed transgenic line (TL1). The indole-3-acetic acid (IAA) content appeared lower (11–31% reduction) in the transgenic lines compared to the wild type (WT) controls, with the lowest level (31% reduction) in TL1. Total internode numbers, bract numbers and bract area were significantly reduced in all transgenic lines in comparison with the WT controls. Only TL1 showed significantly lower plant diameter, total leaf area and total dry weight, whereas none of the *AtSHI* expressing lines showed altered timing of flower initiation, cyathia abscission or bract necrosis. This study demonstrated that introduction of the *AtSHI* gene into poinsettia by genetic engineering can be an effective approach in controlling plant height without negatively affecting flowering time. This can help to reduce or avoid the use of toxic growth retardants of environmental and human health concern. This is the first report that *AtSHI* gene was overexpressed in poinsettia and transgenic poinsettia plants with compact growth were produced.

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Introduction

The ornamental industry is one of the fastest growing industries worldwide, especially in Japan and China. Global production of ornamental potted plants and cut flowers comprises about 50 billion €, corresponding to an estimated global consumption between 100 and 150 billion € [1,2]. The market for cut flowers and potted ornamental plants is not only determined by producers' choices but also by a continuously growing demand for novelties and high quality [3,4]. Compaction of plants is one of the most important traits in many ornamental potted plants, e.g. poinsettia [2].

Euphorbia pulcherrima Willd. Ex Klotzsch, poinsettia, is a non-food, non-feed and vegetatively propagated ornamental plant, known as a contemporary symbol of Christmas in many parts of the world [5]. It is a short day plant and flowering is initiated when the day length is shorter than a critical length [6]. Generally, poinsettia has an elongated natural growth habit. Dwarf characteristics can be obtained either by directly using dwarf cultivars or by grafting cultivars on dwarf rootstocks [7]. Similarly, spraying with growth retardants such as CCC (chlormequat chloride) or alar (dimethylaminosuccinamic acid), that among others inhibit the biosynthesis of the plant hormone gibberellin (GA), results in compact ornamental potted plants [8]. However, growth retardants are expensive, time consuming to apply and have negative impact on human health as well as the environment. Moreover, the growth regulators will likely be banned in EU countries in the near future [9–11].

In the poinsettia industry, alternative strategies like manipulation of temperature, light quality and light duration have previously been tested to control elongation growth of poinsettias [12–15]. In northern areas short term diurnal temperature drops, obtained by opening vents in the morning, are commonly used to reduce shoot elongation. However, in warmer periods and warmer areas of the world this is not applicable. Furthermore, in poinsettia phytoplasma is introduced to induce free-branching and this can also result in compact growth [16]. However, the phytoplasma is lost upon exposure of the plant materials to heat treatment as well as meristematic and somatic embryogenesis tissue culture, which is commonly used to obtain disease free plants by removing pathogens such as the poinsettia mosaic virus (PnMV) [16].

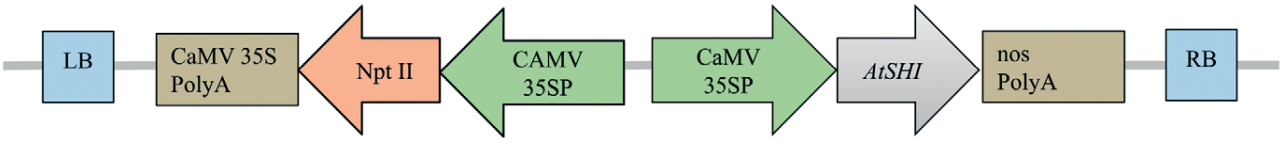

Figure 1. Gene construct: pKanIntron-35S-*SHI* used for *Agrobacterium*-mediated transformation of poinsettia.
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Genetic engineering is increasingly adopted as an important alternative to conventional breeding [1,17]. Recently, it was shown that introduction of the *Arabidopsis thaliana SHORT INTERNODES (SHI)* gene into *Kalanchoë* and *Populus* resulted in dwarfed growth without any morphological abnormalities [18,19]. However, in *Populus* the dwarfing effect on the stem was only very weak, although the internode and petiole lengths were significantly reduced. On the other hand, overexpression of *GA2-oxidase* genes, which control GA inactivation, resulted in dwarfed plants with delayed flowering time in *Solanum* and *Arabidopsis* [20,21]. Also, antisense silencing of the GA biosynthesis gene *GA20-oxidase* resulted in smaller leaves, delayed flowering time and reduced fertility in *Arabidopsis* [8]. Overexpression of the *Arabidopsis* GA signalling gene *GIBBERELLIN INSENSITIVE* (*GAI*) in apple and *Chrysanthemum* reduced plant height, but was correlated with reduced rooting ability and delayed flowering in the respective species [7,22]. In light of these observations, introduction of the *AtSHI* gene to poinsettia might be highly interesting as a means to control elongation growth without introducing undesired morphological or developmental changes. *SHI* gene is a plant specific transcription factor belonging to the *SHI* gene family, and it has been identified in different plants species like tomato, rice, soybean and *Medicago truncatula* [23]. The *Arabidopsis SHI* gene family consists of 10 members; *SHI, STYLISH 1 (STY1)* and *STY2, LATERAL ROOT PRIMORDIUM 1 (LRP1)* and *SHI-RELATED SEQUENCE 3* to *8 (SRS3* to *SRS8)* [18,23–25]. These corresponding proteins have two highly conserved regions, a RING-like zinc finger motif positioned in the N-terminal end and an IGGH domain of unknown function in the C-terminal part of the protein [23,25,26].

All genes could be amplified from all tissues of *Arabidopsis*, except *SRS8*, indicating it might be a pseudogene [25]. STY1 is the closest paralog of SHI having two identical domains in the N and C terminal. The *SHI* family genes have both diverse and redundant functions in plant growth and are involved in shoot apical region development as well as flower and leaf development [23,25,26]. In these respects *SHI/STY*-related genes appear important in gynoecium development, vascular formation and organ identity in floral whorls two and three [25]. It has been documented that *SHI* and *STY* are expressed in the apical region of the developing gynoecium [25,27–29]. Overexpression of *SRS7* conferred dwarfed growth in *Arabidopsis* with small and curled leaves, anther dehiscence and abnormal floral development [30].

SHI family members act as DNA binding transcription activators and may act on plant growth and development by affecting phytohormones like auxin and GA, which among other things control shoot elongation in response to different stimuli [31–35]. Plants overexpressing *SHI* are dwarfed, but a normal, more elongated phenotype can be restored by application of auxin [27,28]. Also, the *YUCCA4* (*YUC4*) auxin biosynthesis gene is induced by the SHI/STY family proteins. However, SHI and STY appear to differ slightly in function as shown by lower affinity of SHI than STY1 to the *YUC4* promoter in the yeast two-hybrid system [36]. Application of indole-3-acetic acid (IAA) to the apical meristem has been shown to increase biosynthesis of bioactive GA [37–39]. *SHI* overexpressing *Arabidopsis* plants show increased levels of the inactive GA_{34} compared to wild type plants [24]. Furthermore, in *Brassica SHI*-related genes have been identified as negative regulators of GA-induced cell division [40].

In this study, we report for the first time successful use of genetic engineering as a tool to control elongation growth in poinsettia, which is among the economically most important potted ornamental plants worldwide. Compact growth was obtained by overexpressing the *AtSHI* gene by using a recently developed *Agrobacterium*-mediated transformation method for poinsettia [41]. Apart from the desired dwarfed growth habit, no developmental abnormalities were scored and flowering time was unaffected.

Materials and Methods

Plant Materials

Poinsettia (*Euphorbia pulcherimma* Willd. ex Klotzsch) cv. Millenium cuttings were grown in the greenhouse under 16 h photoperiod provided by high pressure sodium (HPS) lamps (400W, GAN 4-550, Gavita, Superagro, Andebu, Norway) at 21±2°C with an average relative air humidity (RH) 70±5%. For *Agrobacterium tumefaciens*-mediated transformation 5–15 mm long internode stem explants were excised from 8–10 weeks old poinsettia plants. The explants were surface sterilized with 70% ethanol (1 min), 1% NaOCl (10 min) and then rinsed thoroughly three times with sterile deionized and autoclaved water for 3, 10 and 20 min. After sterilization, stem segments (1–1.5 mm thickness) were excised and utilized for *Agrobacterium* transformation.

Transformation, Selection and Plant Regeneration

Plasmid vector pAt35S:SHI was constructed and introduced into *Agrobacterium tumefaciens* strain GV3850 as previously described in details by Lütken et al. [19]. A brief schematic drawing of the *SHI* gene expression cassette is shown in Figure 1. The *Agrobacterium* culture and subsequent transformation were carried out basically as described by Clarke et al. [41]. The stem segments were inoculated in the *Agrobacterium* suspension for 10 min with gentle shaking. The stem segments were then blotted on sterile filter paper and transferred to callus induction medium (CIM) (MS medium supplemented with 0.2 mg l^{-1} BAP, 0.2 mg l^{-1} CPA and 30 g l^{-1} sucrose) for co-cultivation up to 72 h in the dark at 24°C. After co-cultivation, the explants were transferred to the CIM medium with antibiotic selection containing 10 mg l^{-1} kanamycin and 400 mg l^{-1} claforan (Aventis Pharma Ltd, Norway) for about 10 days. The embryogenic calli were subsequently transferred after every three weeks to somatic embryo induction medium (SEIM) (MS medium contains 0.3 mg l^{-1} NAA, 0.15 mg l^{-1} 2ip and 30 g l^{-1} sucrose) supplemented with antibiotics 25 mg l^{-1} kanamycin and 400 mg l^{-1} claforan. Shoots and plantlets which derived from the somatic embryos were transferred to root induction (RI) medium (1/2 strength MS with 20 g l^{-1} sucrose) with or without hormones (1 mg l^{-1} IAA or IBA). The pH was 5.7–5.8 in all MS media. Regenerated plants were transferred to soil and grown in a greenhouse once the root system was developed.

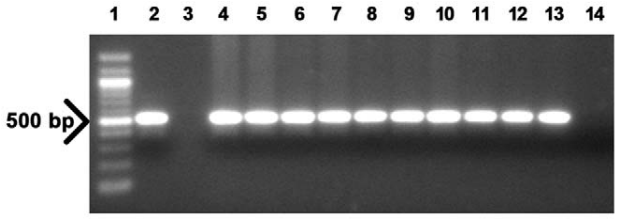

Figure 2. PCR analysis of poinsettia transformed with the *AtSHI* gene (genomic DNA was extracted from leaves). Lane 1:100 bp marker, lane 2: plasmid control, lane 3: blank, lanes 4–11: eight plants from independent transgenic lines 1 (TL1) (individuals 1–8), lane 12: TL2, lane 13: TL3 and lane 14: WT control line. The arrow indicates the 500 bp band.
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Light conditions provided by fluorescent tubes (Philips Master TL-D Super 58W/840, Eindhoven, The Netherlands) for *in vitro* cultures were 23 μmol m^{-2} s^{-1} for callus induction and 30 μmol m^{-2} s^{-1} for the SEIM and RI media, respectively, under a 16 h photoperiod at 24°C.

PCR Analysis

Genomic DNA was isolated from the putative transgenic poinsettia plants using the DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. PCR reactions (20 μl) were performed using 100 ng DNA, 0.2 μM of each primer, and 2xHotStarTaq Mastermix and supplied water from HotStarTaq® Plus Master Mix Kit (Qiagen GmbH, Hilden, Germany). Primer sequences used to amplify a fragment of approximately 500 bp for the *AtSHI* gene (At5g66350) were 5′-ACTCTAACGCTGACGGTGGA-3′ (forward) and 5′-TGCTGACCGGTAGAAAGCTG-3′ (reverse). PCR amplification was performed in a C1000^{TM} thermal cycler (BIO-RAD, Singapore) using the following conditions: 15 min at 95°C (1 cycle) followed by 30 s at 95°C, 45 s at 56°C, and 1 min at 72°C (35 cycles) with a final extension of 7 min at 72°C (1 cycle). PCR products were analysed by ultraviolet light after electrophoresis on 0.8% (W/V) agarose gels.

Southern Blot Analysis

Total genomic DNA was isolated from the leaves of the WT control plants and the PCR positive transgenic poinsettia lines using the DNeasy Plant Maxi Kit (Qiagen GmbH, Hilden, Germany). Southern blot analysis was performed according to Sambrook et al. [42]. Ten micrograms of genomic DNA were digested with *Hind*III for 5 h and separated on 1% (W/V) TBE agarose gel overnight at 37 V, and subsequently transferred onto Genescreen Plus(^{TM}) Hybridization Transfer membrane (NEF 988001 PK, Boston, MA, USA). The 500 bp PCR product representing the coding region of the *AtSHI* gene as described above was used as a probe for hybridization. Membranes were hybridized with the ^{32}P-labelled probe overnight at 65°C. After hybridization the membranes were washed and then exposed to film as described by Clarke et al. [41].

RNA Isolation and cDNA Synthesis

Young leaves of transgenic poinsettia and control plants were harvested for RNA extraction with E.Z.N.A Plant RNA Mini Kit (Omega bio-tek, Norcross, GA, USA) according to the manufacturer's instructions. RNAs were subsequently treated with Turbo DNA-*free*^{TM} kit (Ambion Inc., Austin, TX, USA) to eliminate DNA contamination. RNA quality and quantity were evaluated using agarose gel electrophoresis and Nanodrop 2000 Spectrophotometer (Wilmington, Delaware, USA), respectively. Two micrograms total RNA from each sample was used to synthesize cDNA in a 20 μl reaction using the cDNA SuperScript® VILO^{TM} synthesis kit from Invitrogen (Carlsbad, CA, USA) according to the manufacturer's instructions.

Real-Time Quantitative PCR

Real- time quantitative PCR analysis was performed in a 25 μl reaction volume, using 2.5 μl twentyfold diluted cDNA as a template with 12.5 μl of 1x Power SYBR® green PCR master mix (Applied Biosystems, Warrington, UK) and 0.4 μM of each primer. Primers were designed using Primer3 online software and sequences are listed in Table 1.

Reactions were conducted in an Applied Biosystems 7900HT Real-time PCR system combined with SDS 2.3 version software (Applied Biosystems, Singapore). The PCR conditions were as follows: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, 60°C for 1 min. Dissociation curve analysis was carried out to verify the specificity of the PCR amplification. PCR efficiencies were calculated from a dilution series of genomic DNA of transgenic plants for each primer pair of target gene and endogenous gene by following the equation $E = 10^{-1/slope}$. There were three replications for all samples including the control samples except the TL2, and a no template control was included for each primer pair. The transcript level was estimated by threshold cycle (C_t) values of each sample. The differences of C_t values between the endogenous α–tubulin and the target gene were normalized using the formula $2^{-\Delta\Delta Ct}$ [43]. The relative transcript levels (fold-differences) of the genes were converted to scale $\log_{10}$ values.

Phenotypical Analysis of Transgenic Lines (*AtSHI*) Compared to Control Plants

The independent transgenic lines, TL1–6 from TL1, TL2, TL3 and control plants were vegetatively propagated. After root formation, the plants were potted in 13 cm plastic pot filled with *Sphagnum* peat (Veksttorv, Ullensaker Almenning, Nordkisa, Norway). The plants were kept at 21±2°C, 70±5% RH in the greenhouse under a 16 h photoperiod (8 h darkness) at an irradiance of 150±25 μmol m^{-2} s^{-1} provided by HPS lamps (400W, GAN 4–550). The plants were pinched over 3–4 leaves allowing three shoots to grow per plant. Four plants from each transgenic line including WT control plants were transferred to growth chambers (controlled environment) without any natural light. Light was provided by General Electric company, Fairfield, CT, USA at an irradiance of 100±20 μmol m^{-2} s^{-1} (measured by LI-COR Quantum/Radiometer/Photometer, Model LI-250, Lincoln, Nebraska, USA) under a 10 h photoperiod for flowering. The temperature was 21°C and the RH was 70±5%.

The plants were watered daily during the growth period with commercial nutrient solutions. The side shoots developed during the growth period were removed and counted and only three shoots were allowed to grow per plant. The length of these three shoots was measured from the base of the stem to the shoot apical meristem every fourth day until flowering. At the end of the experimental period (after flowering) petiole length of four mature leaves was measured. The number of leaves and bracts (namely the transition leaves which formed red color more than 40%) were counted and the average internode lengths were calculated by dividing the shoot height by the number of leaves and bracts. Relative chlorophyll content was measured from the middle leaf of the three side shoots on each plant by a chlorophyll content meter (Model CL-01, Hansatech instruments, Norfolk, England). Leaf

Table 1. Primers for real-time PCR expression analysis of *AtSHI* in poinsettia.

Target gene	Forward primer (5′-3′)	Reverse primer (5′-3′)	Product size
AtSHI	AGCTATGGCAACACCCAAAC	ATCCAGCCTTTGTTGCTGTT	71
α-tubulin	TGGAGCTCTCTTTGCTTCAA	CCAACAAAGCTGCATAGCAA	81

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and bract areas were measured by an area meter (Model 3100 area meter, LI-COR, Lincoln, Nebraska, USA). Specific leaf and bract area (SLA and SBA, respectively) were each determined from area/dry weight. After recording the fresh weight, the dry weight was recorded after drying at 65°C until a constant mass was reached. The number of days until visible bracts and cyathia was counted. At the selling stage plants were moved to a postharvest test room to compare differences in cyathia abscission and bract necrosis. The climate in the test room was 21°C, 30–40% RH and an irradiance of 10 μmol m^{-2} s^{-1} was provided 12 h daily by fluorescent tubes (Philips Master TL-D 58W/830).

Three replicate experiments in growth chambers were carried out in which phenotypic observations were performed, samples for auxin analysis collected and postharvest quality tested. In addition to the growth chamber experiments, two to four plants from each line were grown in the greenhouse with conditions as described above for evaluation of the morphological performance of transgenic plants under long day conditions (16 h). Both short and long day treatment experiments were conducted during November 2011 through January 2012.

Auxin Measurements

In the growth chamber experiment, elongating shoot tips were harvested after three weeks of starting short day conditions and immediately placed in liquid N_2. The samples were freeze-dried using a freeze dryer machine (Heto Holten A/S, Allerød, Denmark). For each genotype, 3 replicate samples, each containing 3 shoot tips (elongating parts of the stem) were used for auxin analysis. The frozen plant materials were ground in a mortar and extracted for 5 min with 1 ml cold phosphate buffer (50 mM, pH 7.0) containing 0.2% sodium diethyldithiocarbomate. ^{15}N and $^{2}H_5$-labeled internal standards of indole -3-acetic acid (IAA) and IAA metabolites were obtained from OlChemlm (Olomouc, Czech Republic). The measurements were performed in duplicate. The samples were processed according to Pěnčík et al. [44] using high performance liquid chromatography (HPLC) coupled to a tandem triple-quadrupole mass spectrometer (MS/MS).

Statistical Analysis

Different growth parameters of transgenic and WT control plants were subjected to analysis of variance (General Linear Model procedure) and Tukey's pair wise comparison test ($p \leq 0.05$) using Minitab version 16 (Minitab Inc., State College, PA, USA).

Results

PCR Screening and Southern Blot Analysis of Transgenic Poinsettia Plants

To generate stable compact growth transgenic poinsettia plants by overexpressing the *AtSHI* gene, *Agrobacterium*-mediated transformation experiments were carried out using stem segment explants. After selection and regeneration through somatic embryogenesis, regenerated poinsettia plants were obtained and established in the greenhouse. Using *AtSHI* specific primers, genomic DNA of putative transgenic poinsettia plants was screened by PCR analyses for presence of *AtSHI*. Three independent transgenic lines were confirmed, one plant was selected for each line except the transgenic line one (TL1) of which eight plants from the same clone were used (Figure 2). Transgenic lines were further analyzed by Southern blot hybridization. Results of Southern blot analysis confirmed the stable integration of transgene into the poinsettia genome (Figure 3). Of the three transgenic lines analyzed, line TL1 (with three individual transgenic plants) showed single copy of the transgene whereas TL2 and TL3 with one transgenic plant each showed two copies of the transgene integration (lanes 2–4, 5 and 6 in Figure 3). Lane 7 is the WT negative control, whereas lane 1 is the positive control.

AtSHI Expression in the Transgenic Lines

AtSHI transcript levels were analysed by real-time quantitative PCR in *AtSHI*-expressing transgenic poinsettia lines and the WT controls. Three transgenic lines (TL) were analyzed. Of these, two plants (TL1–4 and TL1–6) were from TL1, while TL2 and TL3 with one plant each were included for the quantitative real time PCR. *AtSHI* transcript levels varied among the transgenic lines as shown in Figure 4. The highest relative levels of transcript were found in TL1–6, whereas the lowest expression was found in TL3.

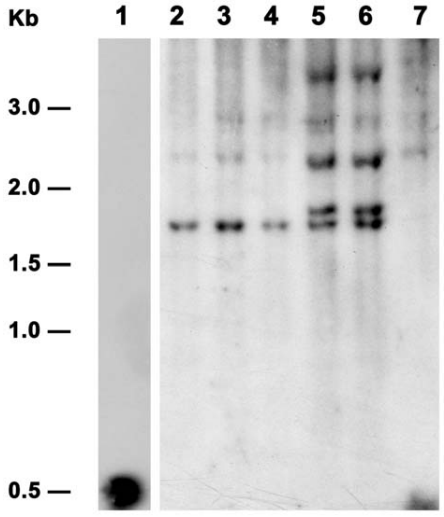

Figure 3. Southern blot analysis of PCR positive transgenic poinsettia lines overexpressing the *AtSHI* gene. *Hind*III-digested total genomic DNAs were hybridized with a *AtSHI* probe (the 500 bp PCR product). Lane 1: positive control, the 500 bp PCR product; lane 2–4: TL1–3, TL1–4, TL1–6 of the transgenic line one (TL1); lane 5: TL2; lane 6: TL3, lane 7: WT control.
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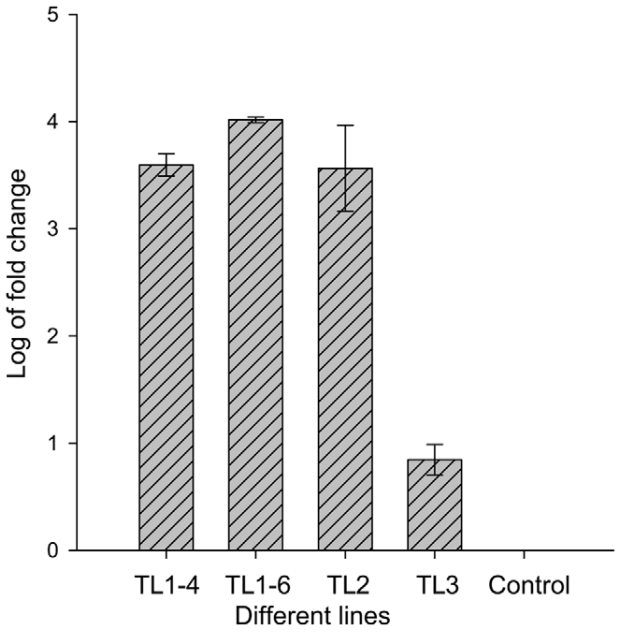

Figure 4. Quantitative real-time PCR analysis of *AtSHI* transgene in different transgenic lines of poinsettia. Two microgram total RNAs from transgenic poinsettia lines and the endogenous control, α-tubulin gene were used for synthesize cDNAs prior the real-time qPCR analysis. Values are means of three technical replications except TL2 (n = 2). Data were analyzed using the $2^{-\triangle\triangle C}{}_T$ method and represented as $\log_{10}$ values. Vertical bars represent the ± SE (standard error).
doi:10.1371/journal.pone.0053377.g004

Reduced Growth Elongation without Change in Flowering Time and Keeping Quality in *AtSHI* Expressing Transgenic Lines

Over-expression of *AtSHI* in poinsettia significantly reduced plant height compared to WT control plants. When grown under short day (SD) conditions, the TL1 transgenic line showed the strongest height reduction response (52%) whereas the TL2 and TL3 plants were reduced in height by 49% and 30%, respectively, compared to WT control plants (Figures 5A, 6A). Under long day (LD) conditions, the height reduction was 25%, 21% and 23% in TL1, TL2 and TL3, respectively, compared to control plants (Figure 5B). No differences were observed in leaf color or leaf shape, and there was no significant difference in petiole length (Figure 6 B, C).

As investigated under SD conditions, overexpression of the *AtSHI* gene significantly reduced the internode lengths and the internode number compared to WT control plants (Figure 7A, B). On average, internode lengths were significantly reduced by 49%, 41% and 31% and the internode number by 32%, 41% and 33% for TL1, TL2 and TL3, respectively. The transgenic plants had significantly lower bract number and reduced bract area as shown in Figure 7C and D. The average bract number was reduced by 44%, 50% and 40% and the bract area by 68%, 62% and 47% for TL1, TL2 and TL3, respectively. In TL1 the total dry weight of stems, leaves and bracts, specific leaf area, total leaf area and shoot diameter differed significantly from the WT control plants (Table 2). No significant difference in relative chlorophyll content was found between the transgenic and control plants (Table 2).

Bract formation started after four weeks when plants were kept under SD conditions and visible cyathia were observed after 5 weeks. No significant difference in time to initiation of flowering was observed between the transgenic and control plants. The development of bract color formation appeared a bit faster in control plants compared to transgenic plants. Bract necrosis was not observed and no significant differences in cyathia abscission or keeping quality between the transgenic plants and control plants were observed (data not shown). In the postharvest room, the cyathia abscission started after three weeks and one week later about 90% of the bracts were abscised from all plants (data not shown).

Reduced Height and Internode Lengths Correlate with Reduced IAA Levels

To investigate the mechanism of the reduced plant height and internode lengths in *AtSHI* overexpressed plants, the levels of IAA were investigated in shoot tips of transgenic poinsettia lines as well as the WT control plants. In the transgenic plants the IAA levels showed a reduction of 11–31% compared to the WT control plants as shown in Table 3. The lowest IAA levels (31% less) were recorded in the transgenic line TL1.

Discussion

In this study we have demonstrated that the over-expression of the *AtSHI* gene is an efficient tool to reduce plant height in the economically highly important ornamental plant poinsettia. PCR analysis verified the *AtSHI*-expressing transgenic poinsettia lines, while Southern blot analysis further confirmed the transgene integration into the poinsettia genome. With the vegetatively propagated nature of poinsettia, the desired compact growth characteristic in the transgenic poinsettia lines over-expression of the *AtSHI* gene will be maintained and propagated by cuttings, a clear advantage over sexually propagated plants. Recently, a dwarfing effect of this gene was shown also in *Kalanchoë* and *Populus* [18,19]. *SHI* gene has among others been suggested to act to control contents of plant hormones involved in control of shoot elongation [27,31,36,45].

AtSHI Expression in Poinsettia Results in Reduced Internode Elongation

AtSHI gene expression resulted in a significant reduction of shoot height compared with the control plants under SD as well as LD conditions (Figures 5, 6A). The strongest shoot length reductions were observed in TL1, which compared to control plants showed 52% and 25% reduction under SD and LD, respectively. In all three transgenic lines, the reduction in shoot lengths was more pronounced under SD compared to LD. This could be ascribed to a generally more vigorous height growth under the higher light sum of the LDs of 16 h. Under LD plants were vegetatively growing whereas under SD conditions shoot elongation ceased due to floral induction. The overexpression of *AtSHI* in poinsettia is comparable with observations made in *Populus*, *Kalanchoë* and *Arabidopsis* [18,19] where it also significantly reduced the height. In this study, the largest reduction in internode length in transgenic poinsettia was 49% compared to WT control plants.

In our current study, the number of internodes was reduced in the *AtSHI* overexpressed transgenic poinsettia plants. Such information is available from neither *Kalanchoë* nor *Arabidopsis*, which is a rosette plant and does not have elongated internodes, but the result is in contrast to the results from *Populus* [18,19]. On the other hand, the petiole length was reduced in the *Populus* [18,19]. In poinsettia, bract number and bract area were reduced significantly in the transgenic plants compared to the control

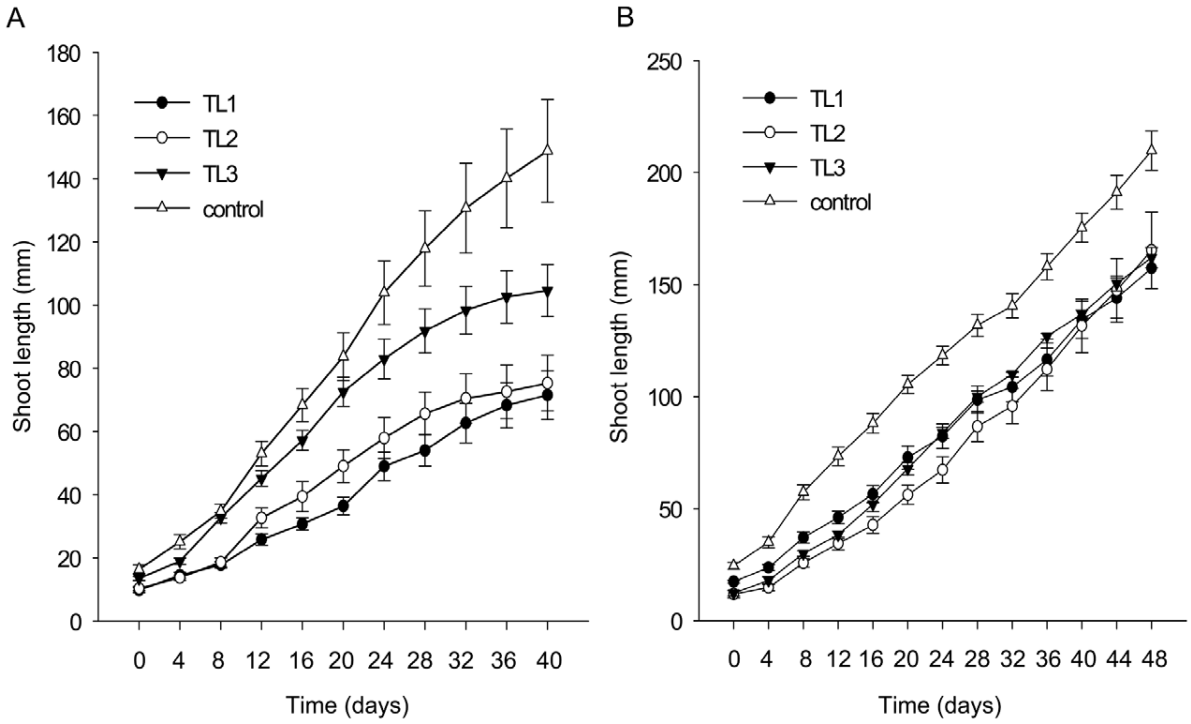

Figure 5. Height comparison among the different transgenic lines (TL) of *AtSHI* overexpressing poinsettia and untransformed control plants grown under short day (10 h) (A) and long day conditions (16 h) (B). Vertical bars represent the ± SE (standard error), n = 11–12 and 6–12 in A and B, respectively.
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plants. A reduction of 47 to 67% in bract area was observed in the different transgenic lines (Figure 7D). The reduced bract size may have impact on the ornamental value of poinsettia. About 41% higher total leaf area and 8% lower specific leaf area were observed in the transgenic line TL1 compared to control plants (Table 2). Leaf colour and alterations were visually observed where the transgenic lines did not show any differences from the control plants. In Figure 6C, both serrate lobed and non-serrate lobed leaves are present. We have observed both types of leaves in the same plants of three transgenic lines and the control plants. Thus, there was no difference in leaf shape of transgenic and control plants. Fridborg et al. [24] observed darker green leaves in *Arabidopsis*, whereas Lütken et al. [19] did not observe any differences in leaf colour in *Kalanchoë* but *AtSHI* was over-expressed in both plants. The overexpression of *AtSHI* caused pleiotropic changes during the developmental stages of *Arabidopsis*. The reason for differences of phenotypic characteristics might be due to the different habits of growth and flowering stage of *Arabidopsis* and *Kalanchoë* as well as poinsettia. Life span and vegetative stage are very short in *Arabidopsis* compared to *Kalanchoë* or poinsettia. No significant difference in relative chlorophyll content was observed among the transgenic lines and control plants. This might suggest that the *AtSHI* overexpression does not reduce photosynthesis in plants. Rather, the relative chlorophyll mean value was higher in the transgenic lines compared to the WT control plants. However, 56% total dry weight in TL1 was significantly lower compared to the WT controls. About 23% reduction in shoot diameter in TL1 was observed compared to the control plants. This result is similar to *SHI* overexpressing *Kalanchoë*, but in contrast to *Arabidopsis* [19,24]. There was no significant difference of branching among the transgenic *AtSHI* poinsettias and control plants (data not shown). This is in contrast to the *shi* mutant in *Arabidopsis*, where more branches were observed in *SHI* overexpressing plants [24].

Reduced Elongation Growth Correlates with Reduced IAA and Higher Expression Levels of *AtSHI*

The reduced stem extension of the plants expressing the *AtSHI* gene was correlated with reduced endogenous levels of IAA levels (Tables 2, 3), with the lowest levels in the shortest lines (TL1). SHI/STY family members regulate plant development. In *Arabidopsis* the *STY1* interacts with the promoter of the auxin biosynthesis gene *YUC4* and induces its transcription [36,46]. YUC family proteins act as rate limiting enzymes of the tryptophan-dependent auxin biosynthesis pathways [47,48]. In *Arabidopsis* it was shown that the SHI/STY family controls the developmental process through regulation of auxin biosynthesis [27]. Our results with reduced height and reduced IAA levels in poinsettia overexpressing *AtSHI* are similar to those obtained in a previous study of the moss *Physcomitrella patens* [27]. Two genes of *PpSHI* reduced elongation and reduced endogenous auxin levels in this moss [45]. The *Arabidopsis* auxin mutant (*ettin-1*) is affected by *SHI/STY* family mutants [27,49]. It is also reported that elongation involves cell division and cell elongation due to cell wall modification activated by auxin among others [50]. Elongation is also well known to be related to GA levels or sensitivity to GA [20,51,52]. However, it has been reported that IAA promotes the biosynthesis of active gibberellin (GA) and that auxin transport inhibitors reduce the active GA content in pea and *Arabidopsis* [37,38,53,54]. This reduction of shoot length and internode length is also consistent with results obtained in the moss *P. patens* [45]. The transgenic moss lines showed reduced levels of auxin presumably related to the overexpression of *PpSHI*.

Figure 6. Transgenic *AtSHI* overexpressing poinsettia plants (A), petioles (B) and leaves (C). In each figure: from the left different transgenic lines; TL1, TL2, TL3 and non-transformed control plants are shown. The plants were grown at 21±2°C, a 10 h photoperiod at an irradiance of 100±20 µmol m^{-2} s^{-1}.
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In *AtSHI* overexpressing lines of *Populus*, the internode number and the concentration of cytokinin were increased. The height and number of shoot apical meristems (SAM) was reduced due to reduced content of cytokinins [55]. The auxin level was also lower in transgenic plant tissue. This supports the *Arabidopsis* data where auxin levels and biosynthesis were reduced by the introduction of the *SHI* family member gene *sty1* [27,36]. In contrast, *AtSHI* expressing *Populus* did not show any change in auxin concentration, but the cytokinin levels were decreased [18]. No significant difference was observed in the petiole length in poinsettia and a similar result was obtained in *Arabidopsis*.

The *AtSHI* transcript levels correlated well with the observed phenotype. Plants that contained relatively high levels of the *AtSHI* transcript were severely dwarfed, whereas less dwarfed plants contained lower transcript levels (Figures. 4, 5A).

Flowering Time and Keeping Quality are Not Affected in the *AtSHI* Overexpressing Transgenic Poinsettia Plants

In the ornamental industry, the most important feature is flowering. The attractive part of poinsettia is the bract colour (formation of red anthocyanin pigment in the transition leaves) formation, which was observed at nearly the same time in both control and transgenic plants. The completion of bract colour formation was a little bit faster in control plants compared to transgenic plants. This is quite similar to previous studies, where no difference in flowering time was found in *sty1–2* and wild type

Table 2. Comparison of growth parameters among transgenic (T) lines of poinsettia overexpressing the *AtSHI* gene, TL1, TL2, TL3 and control plants under short day conditions of a 10 h photoperiod in a controlled environment.

	Line TL1	Line TL2	Line TL3	Control
Height (mm)	71.5±7.7 b	75.3±8.8 b	104.5±8.2 b	148.8±16.3 a
Relative chlorophyll content	27.7±1.0 a	28.0±2.1 a	25.5±2.1 a	24.6±2.2 a
Shoot diameter (mm)	10.2±0.6 b	12.0±1.1 a	13.9±0.3 a	13.2±0.3 a
Total leaf area (cm^2)	199.8±17.3 c	247.6±32.3 bc	385.1±29.0 a	341.0±27.0 ab
Specific leaf area (cm^2 g^{-1})	283.3±3.7 a	271.9±6.6 ab	265.1±5.8 b	262.7±5.0 b
Total dry weight (g)	1.2±0.1b	1.8±0.3 ab	2.7±0.3 a	2.7±0.3 a

Different letters within a parameter indicate significant differences (Tukey's test at $p<0.05$). n = 11–12. Mean value ± SE (standard error) are given.
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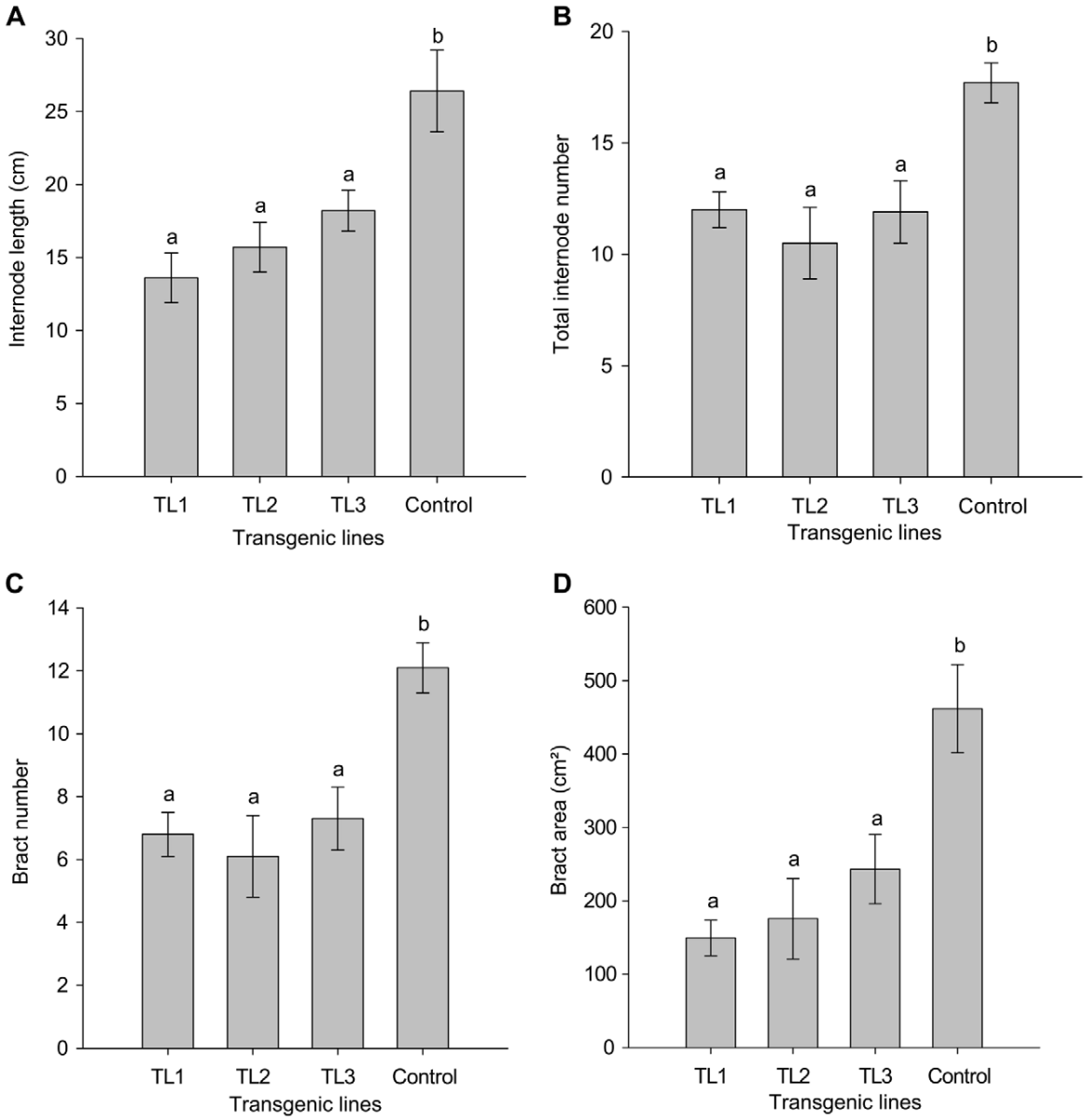

Figure 7. Effects of *AtSHI* overexpression on internode length (A), total internode number (sum of bracts and leaves) (B), bract number (C) and bract area (D) of different transgenic lines and control plants of poinsettia. Plants were grown under short day conditions of a 10 h photoperiod, n = 11–12. Mean values with different letters are significantly different based on ANOVA followed by a Tukey's test at $p \leq 0.05$. Vertical bars represent the $\pm$ SE (standard error).
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Arabidopsis plants [46]. Multiple inputs like photoperiod, light quality and GA converge to regulate flowering [56,57]. GA promotes flowering in some LD plants like *Arabidopsis* and *Lolium* and inhibits flowering in SD plants such as rice [58,59]. However, GA's involvement in floral initiation is complex and varies from species to species [60,61]. The bract formation was visible after four weeks when plants were kept under SD conditions. Cyathia abscission was observed in the postharvest room under standard conditions. The cyathia abscission started after three weeks and at the end of 4 weeks about 90% bracts were abscised in both transgenic and WT control plants (data not shown). Furthermore, no negative effects were seen on cyathia abscission or bract necrosis or the postharvest quality due to *AtSHI* overexpression in poinsettia. In *Arabidopsis* a negative effect of *SHI* overexpression is late flowering, but this was not observed in either *Kalanchoë* or in our study of poinsettia [19].

Conclusions

The economic importance of poinsettia provides a driving force to improve this important ornamental plant by using genetic engineering. With respect to control of plant morphology, this method is time saving, convenient and environmentally friendly compared to conventional breeding and application of hazardous chemical growth retardants. We have here demonstrated that compact poinsettia plants without delay in flowering or change in keeping quality can be obtained by using ectopic *AtSHI* expression

Table 3. Endogenous levels of auxin and their metabolites (pmol g^{-1} DW) in transgenic lines and control plants of poinsettia grown under a short photoperiod of 10 h.

Line	IAA	IAA-Asp	IAA-Glu	Total auxin
Transgenic line TL1	2004.8±14.2 a	169.9±23 a	99.6±7.5 a	2274.3±35.3 a
Transgenic line TL2	2575±104 a	240.3±29.7 a	89.4±4.0 ab	2905±127 a
Transgenic line TL3	2290±371 a	171.8±6.3 a	62.63±4.6 b	2524±375 a
WT control	2892±328 a	204.3±10 a	98.19±9.59 a	3194±340 a

Different letters within a parameter indicate significant differences (Tukey's test at $p \leq 0.05$). Mean value ± SE (standard error) are given; Three shoots of one plant were treated as one replicate and three separate replicates were analyzed.
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as a tool. For poinsettia, dwarf characteristics with good keeping quality are required for high ornamental and market value. Dwarf plants are also convenient for handling and transportation compared to more elongated plants, and need less space in expensive production facilities. Although there is generally, especially in Europe, a negative attitude towards genetic engineering of food crops which are consumed by humans, the attitude might be less negative for plants grown for other purposes such as non-food, non-feed ornamental plants. Hence, the use of *Agrobacterium*-mediated transformation to introduce *AtSHI* into commercially grown poinsettia cultivars can be very promising in the poinsettia industry, being environmentally friendly, beneficial to the economy and to human health by avoiding hazardous effects of plant growth retardant application. Due to the vegetative propagation nature of poinsettia, the acquired dwarfing effect and other desirable characteristics will be stably inherited in vegetative cuttings used in propagation.

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Author Contributions

Conceived and designed the experiments: MAI HL DRB ST SKR JEO JLC. Performed the experiments: MAI HL SH JR. Analyzed the data: MAI HL DRB ST JR SKR JEO JLC. Contributed reagents/materials/analysis tools: MAI HL JR. Wrote the paper: MAI HL DRB ST SKR JEO JLC.

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Paper II

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Artificial light from light emitting diodes (LEDs) with a high portion of blue light results in shorter poinsettias compared to high pressure sodium (HPS) lamps

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ABSTRACT

Strict control of morphogenesis is essential in production of potted poinsettia. Commonly, this is obtained by the use of plant growth retardants (PGRs), often in combination with early morning temperature drops. Due to negative effects on human health and the environment, the use of PGRs is becoming restricted. Also, energy-saving growth regimes and periods of high temperatures limit effective use of temperature drops. In the present study the use of a high proportion of blue (B) light provided by light emitting diodes [LEDs, 20% blue (B), 80% red (R)] was compared with traditional high pressure sodium (HPS) lamps (5% B) providing similar phytochrome photostationary state to produce compact poinsettia plants. Both in the greenhouse and growth chamber, all cultivars were 20–34% shorter for LED compared to HPS grown plants. Also, leaf and bract area as well as chlorophyll content and total dry matter accumulation were lower under LED. The LED did not delay bract color formation, visible cyathia and flowering compared to HPS, and no difference in post production performance (cyathia/bract abscission or necrosis) between the two light treatments was found. The effect of end of day-red (EOD-R) lighting combination with LED and HPS supplemental lamps during the photoperiod in the greenhouse was also investigated. Reduced stem extension (13%) was observed under HPS only and for one of the two cultivars tested, whereas under the LED regime, there was no effect of EOD-R lighting.

1. Introduction

Poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) is a very popular and economically highly significant potted plant in North America, Europe, Asia and Australia (Ecke et al., 2004). In northern Europe, poinsettias are commonly produced during autumn in greenhouses with the use of supplementary photosynthetic lighting due to low natural solar radiation. The most common light source is high pressure sodium (HPS) lamps which has a high emission of photosynthetic active radiation (PAR) and a high electrical efficiency but contain only 5% blue (B) light (400–500 nm) which is low compared to natural sun light (18% B). Different plant growth regulators (PGRs) such as chlormequat, daminozide or paclobutrazol are commonly used to inhibit the elongation growth in order to grow compact plants. However, PGRs have some negative impacts on human health and the environment, and are expensive and time-consuming to apply (De Castro et al., 2004; Sørensen and Danielsen, 2006). Thus, to control elongation growth of poinsettias different alternative strategies like manipulation of temperature, light quality and light duration have previously been tested (Clifford et al., 2004; Cockshull et al., 1995; Myster and Moe, 1995; Ueber and Hendriks, 1992). Until now a combination of PGRs and early morning temperature drop treatment have been the most common methods in commercial production in northern areas (Myster and Moe, 1995). Depending on the temperature regime, temperature drop in the morning can reduce the elongation in poinsettia by up to 25% compared to constant temperatures (Moe et al., 1992a). However, the implementation of a temperature drop treatment may be difficult early in the autumn when the temperature and solar radiation are high.

Moreover, energy saving in greenhouse production has received much attention lately (Körner and Van Straten, 2008). Climate strategies where ventilation is avoided and the growing temperature follows more the natural variation in day (DT) and night temperatures (NT) to reduce energy consumption are of current interest (Blanchard et al., 2011; Fitz-Rodríguez et al., 2010; Lund et al., 2006; Markvart et al., 2007). However, energy-saving growth regimes with reduced ventilation limit the use of temperature

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drops. Thus, there is interest in alternative height control strategies and especially focus on light quality.

Physiological knowledge on the action of photoreceptors involved in plant morphogenesis is available in many plant species (Smith, 2000). The main light receptors known to have prominent effects on plant morphology are the B light absorbing cryptochromes and the red (R) and far-red (FR) light absorbing phytochrome light receptors. Phytochromes are inter converted between a biologically inactive (P_R) and an active form (P_{FR}) depending on the light quality (Smith, 2000). However, the phytochrome responses vary with plant species, cultivar, age, irradiance, spectral quality and temperature. Low levels of FR light in the spectrum or a high ratio between R and FR commonly result in short, compact plants (Mata and Botto, 2009). Plants are usually more sensitive to R and FR light at the end of the day (EOD), and ten to sixty min of EOD-R light may be as effective as a high R/FR ratio during the whole lighting period (Hisamatsu et al., 2005; Ilias and Rajapakse, 2005; Symons and Reid, 2003). Also, cryptochromes are known to affect stem extension, and a variety of plants respond to B light by suppressing the shoot elongation (Hoenecke et al., 1992). However, the opposite effect with increased shoot elongation under pure B light compared to R light has also been reported in a number of species such as *Salvia* and marigold (Heo et al., 2002).

Another reason for the interest in utilizing light quality to modulate plant growth and morphology is the recent development of light emitting diodes (LEDs) as a lighting source in greenhouse production (Barta et al., 1992; Bula et al., 1991). Such small diodes can easily be placed close to the canopy and can be used to apply narrow-band light spectrum to the plants. Specific requirements for light spectral distribution for specific processes like morphogenesis, photosynthesis, chlorophyll and anthocyanin synthesis have been determined in different species (Robin et al., 1994a, 1994b; Stutte, 2009; Tripathy and Brown, 1995). However, little information is available on the effects of such light regimes on the growth of poinsettia, and no photoreceptors are until now identified in this species. Experiments with selective plastic films absorbing FR light or manipulation of the R/FR ratio have indicated that the morphology of poinsettia is responsive to the R/FR ratio (Clifford et al., 2004). On the other hand, experiments with a B deficient environment under spectral filters have suggested that B light does not play a significant role in controlling stem extension in this species (Clifford et al., 2004; Mortensen and Strømme, 1987). However, to the best of our knowledge, there are no studies where poinsettia is grown with increased B light.

The objective of this study was to examine the use of LED light as a tool to control elongation of poinsettias as well as on flowering time and postharvest quality. Specifically, the aim was to test if LED light with a higher portion of B light (20% B) than the commonly used HPS lamp (5% B) with similar phytochrome photostationary states (PSS), can suppress elongation. Further, to evaluate the effect of manipulating the phytochrome apparatus under these light conditions, poinsettias were exposed to low fluence rate R light in the end of the day (EOD-R).

2. Materials and methods

2.1. Plant material and pre-cultivation

Cuttings of *Euphorbia pulcherrima* Willd. ex Klotzsch (poinsettia) cultivars 'Christmas Spirit', 'Christmas Eve' and 'Advent Red' with 6–7 leaves rooted in Jiffy-7 (G3 Ljones Gartneri AS, Tørvikbygd, Norway), were potted in 13 cm plastic pots filled with *Sphagnum* peat (Veksttorv, Ullensaker Almenning, Nordkisa, Norway). The plants were kept in a growth chamber at 20 °C with an average relative air humidity of 70 ± 5%, corresponding to an average of 0.7 kPa

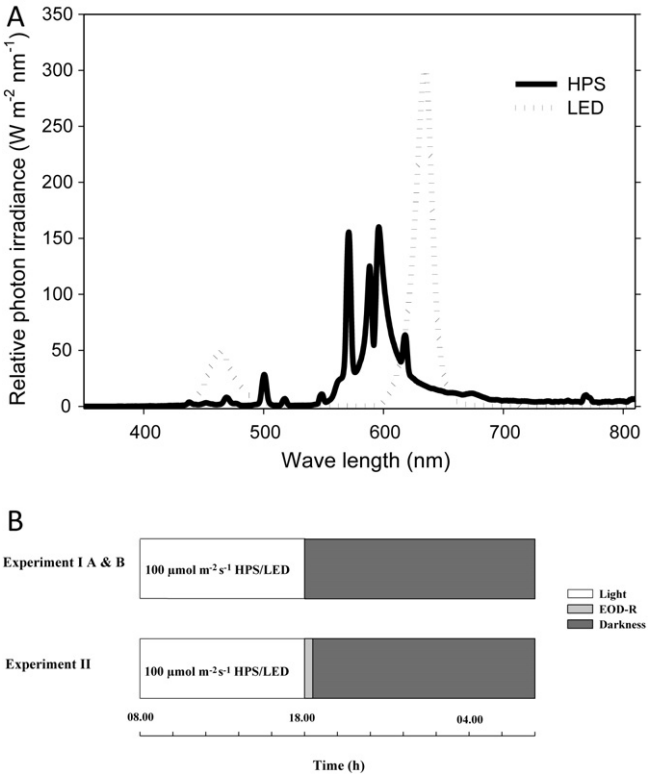

Fig. 1. (A) Light spectra of HPS (LU400/XO/T40) and LED lamps (SoLa-co round high power 162 W LED-light), used in the experiments. (B) Schematic illustration of the light conditions in experiment I (A and B) and II. In experiment II, 5 μmol m^{-2} s^{-1} of R-light was given at the end of the day for 30 min in greenhouse.

water vapor deficit (VPD), under 18 h photoperiod provided by cool white fluorescent (OSRAM L 58 W/640, Munich, Germany) at a photosynthetic photo flux density (PPFD) of 80–90 μmol m^{-2} s^{-1} for 6 weeks. The plants were pinched up to 3–4 leaves, and when the new shoots were 0.5–1 cm long, plants were transferred to different light quality treatments in growth chambers or greenhouse compartments. Three shoots were allowed to grow per plant. The plants were watered daily during the whole experimental period with a commercial nutrient solution [red superba and calcinit (Yara, Oslo, Norway)] with an electrical conductivity (EC) of 1.5 mS cm^{-1} and a pH of 5.6–5.8.

2.2. Experimental set-up and light quality treatments

Two experiments (experiments I and II) were performed at Centre for Plant Research in Controlled Climate, Norwegian University of Life Sciences, Ås, Norway (59°39′47″N, 10°47′38″E) from September to December in 2009 and 2010. In experiment I, we compared HPS and LED (Fig. 1A and B) as supplementary lighting in the greenhouse (experiment I A) and in closed growth chambers (experiment I B). Also, an experiment was performed to test the effect of EOD red light (EOD-R) in combination with HPS and LED as supplemental lighting (experiment II). In experiment I A 'Advent Red' (2009) and 'Christmas Eve' and 'Christmas Spirit' (2010) were supplemented with a PPFD of 100 ± 20 μmol m^{-2} s^{-1} for 10 h daily (14 h darkness) with HPS lamps containing 5% B (LU400/XO/T/40; General Electric Company, NelaPark, Cleveland, Hungary) or light emitting diodes (LEDs) containing 20% B (wavelength 450–500 nm, peak at 465 nm) and 80% R (wavelength 600–700 nm, peak at 630 nm) (Round LED-light 162W, VA–24150T, SoLa-co, Grimstad, Norway) as supplemental lighting. The spectra for the lamps were measured with a spectrophotometer (OceanOptics, model-SD2000,

USA) (Fig. 1A). The phytochrome photostationary states (PSS), was calculated according to Sager et al. (1988) by multiplying the irradiance at each wavelength against the relative absorption for each form of phytochrome and were 0.89 and 0.85 for LEDs and HPS, respectively. The lamps were turned on in the morning (08.00 am) and turned off right after the black cloth was pulled on in the evening (18.00 pm). The average natural solar radiation during the experimental periods (September–December) were 9.3 and 9.5 mol m^{-2} d^{-1} for 2009 and 2010, respectively (Meteorological data from Ås, Norway). In experiment I B, 'Christmas Eve' and 'Christmas Spirit' were grown in growth chambers with similar HPS and LED lamps as described above (Fig. 1A) at a PPFD of 100 ± 20 μmol m^{-2} s^{-1}.

In experiment II (greenhouse 2009), the cultivars 'Advent Red' and 'Christmas Eve' were grown in a greenhouse compartment with LED or HPS (as described above, Fig. 1A) as supplemental lighting with and without 30 min of low irradiance (5 μmol m^{-2} s^{-1}) R light at the end of day (EOD-R) provided by small LED strings placed close to the plant canopy (LED power drivers, peak 630 nm, 177358/4908, 24 VDC/max 10W, Philips, Eindhoven, The Netherlands). The EOD-R treatment from 18.00 to 18.30 h was given right after the black cloth was pulled (Fig. 1B).

The temperature in the greenhouse and the growth chambers was 21 ± 2 °C and the relative air humidity (RH) was 70 ± 5%. In the growth chambers the CO_2 concentration was at ambient level (400 ppm) whereas in the greenhouse compartments the air was enriched to 800 ppm pure CO_2 (Yara, Oslo, Norway). The climate data were recorded in 5 min intervals by a computer control system (Integro 3, Priva, Ontario, Canada). The irradiance was measured at the plant canopy level using a LI-COR Quantum/Radiometer/Photometer (Model LI-250, Li-COR, Lincoln, Nebraska, USA).

2.3. Growth analysis and postharvest quality testing

The length from the base of the stem to the shoot apical meristem was measured on each shoot once a week or every two weeks. At flowering (cyathia formation), growth analysis was performed on all three shoots of 10 greenhouse-grown plants, for each cultivar in each experiment. The leaf length of four mature leaves per stem and the stem diameter at the middle of each shoot was measured. The number of leaves and bracts were counted and the average internode lengths were calculated by dividing stem length by the number of leaves. Bracts were defined as transition leaves which had formed red color and were counted if the length exceeded 3 cm (petiole + bract). Leaf and bract area was measured by a leaf area meter (Model 3100, LI-COR). Fresh and dry weight of stem, leaves and bracts were recorded after drying at 65 °C until a constant mass was reached. Total dry matter (DM) was calculated from the total sum of dry weight of stem, leaves and bracts. Specific leaf and bract area (SLA and SBA, respectively) were determined from area/dry weight for each of them. Total chlorophyll content was measured on the middle leaf of the three shoots on each plant by a chlorophyll content meter (Model CL-01, Hansatech Instruments, Norfolk, England). In experiment I A, post harvest quality was tested on 5 plants of the 'Christmas Spirit' and 'Christmas Eve' for 5 weeks. The temperature in the test room was kept at 21 °C and RH at 30–40%. An irradiance of 10 μmol m^{-2} s^{-1} for 12 h per day was provided by fluorescent tubes (Philips Master TL-D 58W/830, Holland).

2.4. Statistical analysis

Effects of treatments on growth, morphology and length of shoots were analyzed by analysis of variance (General Linear Model procedure) and Tukey's pair wise comparison test ($p < 0.05$) using Minitab Version 16 (Minitab Inc., State College, PA, USA).

3. Results

A difference in growth pattern between the light treatments appeared after about 5–6 weeks for all cultivars both in the greenhouse and in growth chambers (Figs. 2 and 3). At the marketing stage after about 12 weeks of treatment, the total shoot length of all the cultivars tested was significantly reduced under LED. 'Christmas Spirit' showed the strongest response (Figs. 2–4) with a reduction in plant height by 34% both in the greenhouse and growth chambers under LED compared to HPS, whereas 'Christmas Eve' showed 27% and 21% height reduction, respectively. 'Advent Red', which was tested only in the greenhouse, showed a 20% height reduction.

In addition to a reduced shoot length, plants grown under LED had significantly shorter petioles, reduced leaf and bract area, resulting in reduced plant diameter and more compact plants, compared to HPS (Table 1 and Fig. 4). On average, petiole lengths were reduced by 36 and 37%, leaf area by 40 and 46%, bract area by 61 and 49% and stem diameter by 15 and 14% for 'Christmas Spirit' and 'Christmas Eve', respectively. The LED-grown plants also had significantly shorter and fewer internodes than the HPS grown ones (Table 1 and Fig. 5). The internode length was reduced by 24 and 17% and the number of internodes by 26 and 17% for 'Christmas Spirit' and 'Christmas Eve', respectively. 'Christmas Spirit' and 'Christmas Eve' showed a 17% and 30% decrease in chlorophyll content but a 43% and 35% decrease in total dry matter content respectively (Table 1). However, no significant difference was observed in the distribution of DM (%) in leaves, bracts and shoot between the two light treatments (data not shown). In 'Christmas Eve' the SLA and SBA was reduced 12 and 16% respectively in LED light compared to HPS (Fig. 6).

No significant difference in time to visible cyathia was observed between plants grown under LED and HPS either in the greenhouse or in growth chambers (data not shown). Visible cyathia were observed after about 9 weeks in both cases. Similarly, there was no significant difference in the keeping quality in the 'Christmas Eve' or 'Christmas Spirit' grown under LED and HPS. Only a small number of bracts abscised in 'Christmas Eve' under both light qualities but no bract necrosis or cyathia drop appeared during 5 weeks of testing (data not shown). Overall, no significant differences in post harvest quality parameters were observed between the plants grown under the two light qualities.

The effectiveness of an EOD-R treatment in reducing shoot elongation further was investigated in 'Christmas Eve' and 'Advent Red' grown under HPS and LED light under short day conditions. EOD-R light significantly reduced the height of 'Advent Red' by 13% when HPS was used as a supplemental lighting but had no effect on height under LED (Fig. 7). EOD-R had no significant effect of height on 'Christmas Eve' (Fig. 7).

4. Discussion

In the present study, a height reduction by 20–34% was achieved when poinsettia plants were grown under LED with a higher portion of B light (20%) than provided by the traditional HPS lamps (5% B). The internodes (17–24% reduction) and petioles (36–37% reduction) were shorter and the expansion of the leaves and bracts was reduced under LED compared to HPS, resulting in very compact plants (Table 1 and Figs. 2–4).

The phytochrome photostationary states (PPS) were 0.85 and 0.89 for the HPS and LEDs, respectively, indicating that mainly the blue light and not the PPS, was critical in regulating the stem extension growth (Sager et al., 1988; Stutte, 2009). This suggests that the

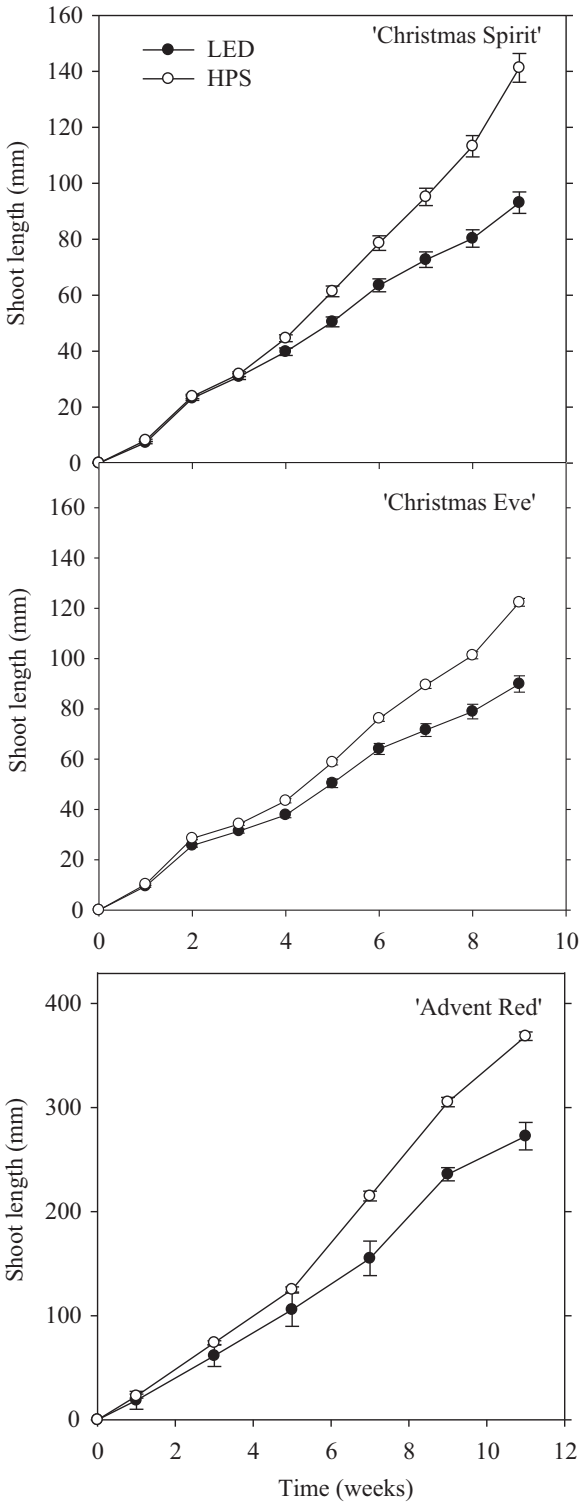

Fig. 2. Effect of LED light (20% B and 80% R) compared with HPS as supplementary lighting on shoot length of poinsettia 'Christmas Spirit' ($n = 45$), 'Christmas Eve' ($n = 48$) and 'Advent Red' ($n = 12$). HPS/LED was provided 10 h daily in a greenhouse compartment and black cloths were pulled in the evening to prevent natural light from the outside (14 h darkness). The average natural solar radiation during the experimental periods (September–December) was 9.4 mol m^{-2} d^{-1}. Vertical bars represent the ±SE (standard errors).

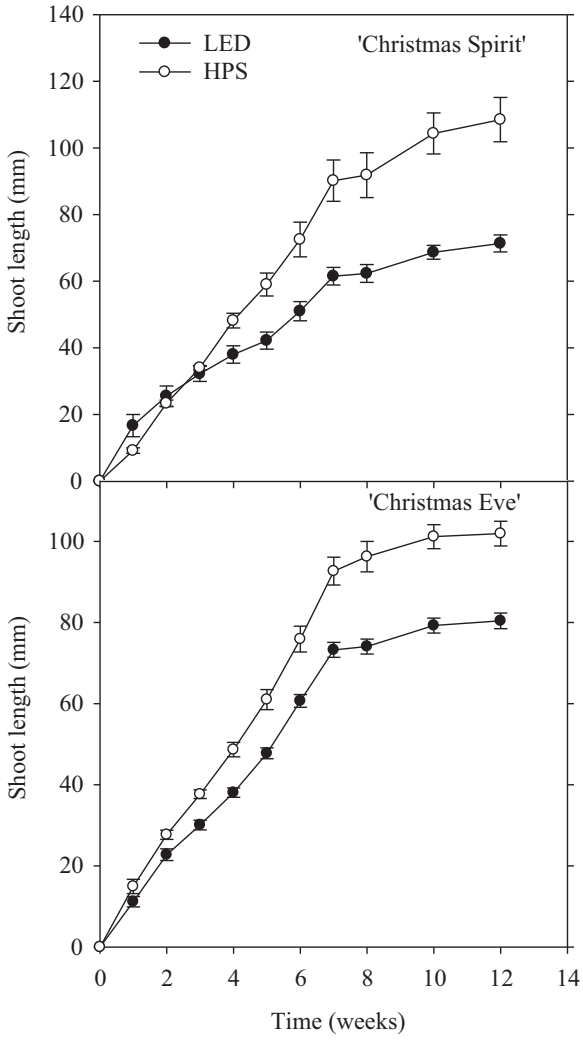

Fig. 3. Effects of LED light (20% B and 80% R) compared with HPS on shoot length of poinsettia 'Christmas Spirit' ($n = 6$) and 'Christmas Eve' ($n = 12$) under short day conditions (10 h) at an irradiance of 100 μmol m^{-2} s^{-1} in growth chambers. Vertical bars represent the ±SE (standard errors).

cryptochrome photoreceptors are important in the control of stem elongation of poinsettia. In earlier studies with the use of spectral films to manipulate light quality, it was claimed that B light did not play an important role in mediating stem extension of poinsettia (Clifford et al., 2004; Mortensen and Strømme, 1987). In those studies, no response or only a small promotion of stem extension was found under a B light deficient environment. However, the current study clearly shows that B light is effective in the control of stem extension of poinsettia when more B light is added to the environment.

B light suppression of petiole and stem extension as well as hypocotyl elongation have been demonstrated in a variety of horticultural plants species including soybean, pepper and lettuce (Brown et al., 1995; Hoenecke et al., 1992; Holmes and Schäfer, 1981; Schuerger et al., 1997; Wheeler et al., 1991). Typically, increasing B light decreases stem length down to a maximum threshold level (Wheeler et al., 1991). The amount of B light in the spectrum for maximum inhibition of stem extension of poinsettia is not known. An increase in B light from 5% to 20% in the current study reduced the stem extension extensively but it also reduced the relative chlorophyll content and the total DM accumulation (Table 1). It is probably not practical to increase the amount of blue light >20%.

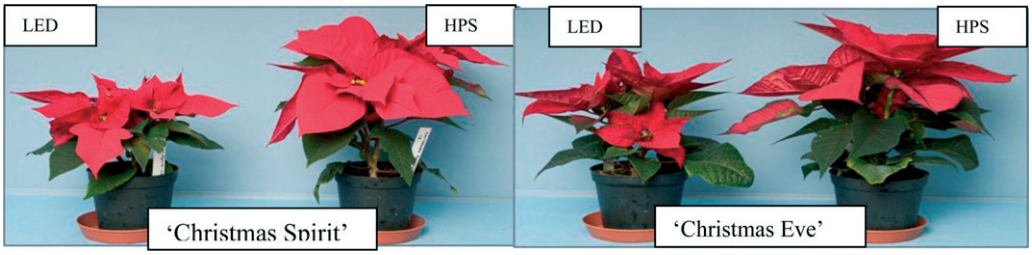

Fig. 4. Morphology of poinsettia grown under LED light (20% B and 80% R) and HPS as supplementary lighting (100 μmol m^{-2} s^{-1} for 10 h daily) in greenhouse compartments. Plants were grown at 21 °C, 70% relative air humidity and 800 ppm CO_2.

Table 1
Growth and morphology of poinsettia plants grown under LEDs (20% B and 80% R) or HPS as supplementary lighting (100 μmol m^{-2} s^{-1} for 10 h daily in 12 weeks) in a greenhouse compartments.

Growth parameter	'Christmas Spirit'		'Christmas Eve'	
	LED	HPS	LED	HPS
Shoot length (mm)	93.1 ± 3.8 a	141.3 ± 5.2 c	89.9 ± 3.3 a	122.4 ± 1.6 b
Relative chlorophyll content	16.6 ± 0.7 a	20.0 ± 0.8 b	14.6 ± 0.8 a	20.8 ± 0.7 b
Petiole length (cm)	2.8 ± 0.1 b	4.4 ± 0.1 d	2.2 ± 0.1 a	3.5 ± 0.1 c
Shoot diameter (mm)	5.3 ± 0.1 a	6.2 ± 0.1 b	5.5 ± 0.1 a	6.4 ± 0.2 b
Internode length (mm)	20.6 ± 0.8 b	27.2 ± 0.8 c	15.4 ± 0.6 a	18.5 ± 0.4 b
Total leaf area (cm^2)	148.1 ± 8.4 a	247.1 ± 12.1 c	112.9 ± 8.4 a	208.4 ± 11.1 b
Total bract area (cm^2)	79.7 ± 5.6 a	205.6 ± 9.4 b	183.4 ± 15.1 b	359.7 ± 17.1 c
Total dry matter (g)	1.6 ± 0.1 a	2.8 ± 0.2 b	1.8 ± 0.1 a	2.8 ± 0.1 b

Mean ± SE followed by non-similar letters within a parameter are significantly different at $p < 0.05$ according to Tukey's test. $n = 20–30$.

The total bract area was reduced by 50% or more under LED compared to HPS, and the expansion of the bracts was more reduced than the expansion of the leaves. The reduced bract size may lower the ornamental value and the marketability of some cultivars of poinsettia. Especially, the 'Christmas Spirit' developed small bracts under LED. To avoid reduction in bract expansion the B light proportion may be reduced in the later stage of the production during the active bract expansion phase. The use of LED technologies may thus be an effective method to optimize the different phases in the production of poinsettia.

No difference in time to open flowers was found between LED and HPS, showing that poinsettia tolerates LEDs with a high proportion of B light in the spectrum without negative effects on the time to the marketing stage. However, the plants grown under LED had developed fewer leaves and bracts at flowering than plants grown under HPS (Fig. 5), indicating a faster floral induction on a leaf number basis under increased B light. A similar response has been reported in the long day plant (LDP) *Arabidopsis thaliana* where either FR or B light is known to hasten the transition to flowering, while R delays it (Kenneth, 1992). In this species both phytochrome and cryptochrome light receptors are involved in the floral control (Simpson and Dean, 2002). However, less is known on the effects of B light on flower transition of short day plants (SDPs). Both the B light photoreceptor-and phytochrome-mediated pathways are probably involved in the flowering of poinsettia. A delay in time to the stage with visible cyathia was reported in poinsettia under selective films lacking FR light but no effects was found when the plants were grown in a B light deficient environment (Clifford et al., 2004). Although the HPS grown plants in the current study flowered at a higher leaf number, the time to visible cyathia or time to open cyathia was not significantly different between the different light treatments. Thus, the growth and development of the inflorescence was probably faster under HPS than the LED. The larger leaves with longer petioles probably resulted in a more effective light capturing and a higher potential for fast growth and development compared to the smaller leaves of compact LED-grown plants. Further, about 1.5 °C higher leaf temperature was measured under HPS compared to LED because of higher infra-red radiation exposed directly to the plants (results not shown) which may also have compensated for the extra leaves.

It should be noted that no significant difference was observed in the post production performance of poinsettia grown under LED and HPS. The plants were transferred directly from the greenhouse into the post harvest test room without any simulated transport or storage. The keeping quality of the plants was very good irrespective of the light treatment without cyathia drop or bract necrosis during the 5 weeks of testing. In earlier experiments where diurnal temperature manipulation was used to reduce stem extension a negative effect on the postharvest quality was found. After four weeks of testing under similar conditions as in the current study, all the cyathia abscised in the cultivars 'Lilo' and 'Starlight' (Moe et al., 1992a).

All cultivars tested were more compact under LED and the response to B light was similar in greenhouse compartments and growth chambers indicating that the natural light during the experimental periods (autumn) did not influence the response qualitatively. However, the shoot length showed a linear increase over time in the greenhouse, but a saturating response in the growth chamber experiment. The extra energy the plants get from the natural light in combination with the high CO_2 concentration in the greenhouse (800 ppm) probably contributes to the increased extension in the greenhouse experiment compared to the growth chamber. The magnitude of this effect of LED treatment is similar to or stronger than other height control strategies commonly used in the greenhouse industry. About 20–25% height reduction was found in poinsettia using FR-removing plastic filters, and up to 25% reduction has been demonstrated in temperature drop experiments (Clifford et al., 2004; Moe et al., 1992a, 1992b). In the current experiment, more B light did not delay flowering time like it has been observed when photoselective films or temperature manipulations were used as tools to control height (Mata and Botto, 2009; Moe et al., 1992a). Thus, supplementary LED lighting with

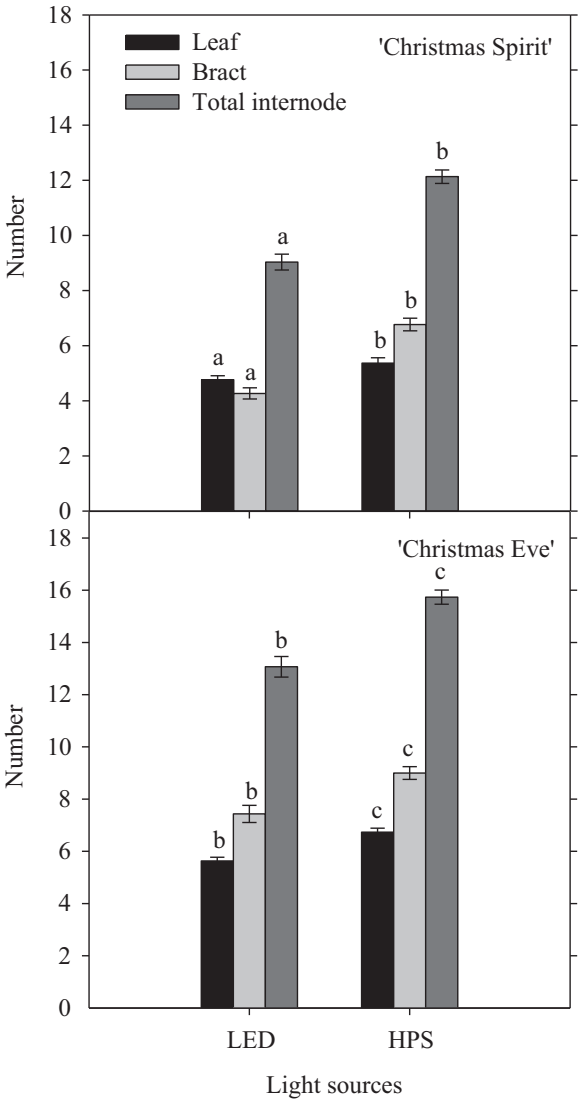

Fig. 5. Number of leaves, bracts and the total internodes (sum of leaves and bracts numbers) of poinsettia grown in greenhouse compartments with LED light (20% B and 80% R) or HPS as supplementary lighting at an irradiance of 100 μmol m^{-2} s^{-1} during a 10 h photoperiod. Vertical bars represent the ±SE (standard errors) (n = 30). Mean values with the same letter are not significantly different based on ANOVA followed by a Tukey's test at p < 0.05.

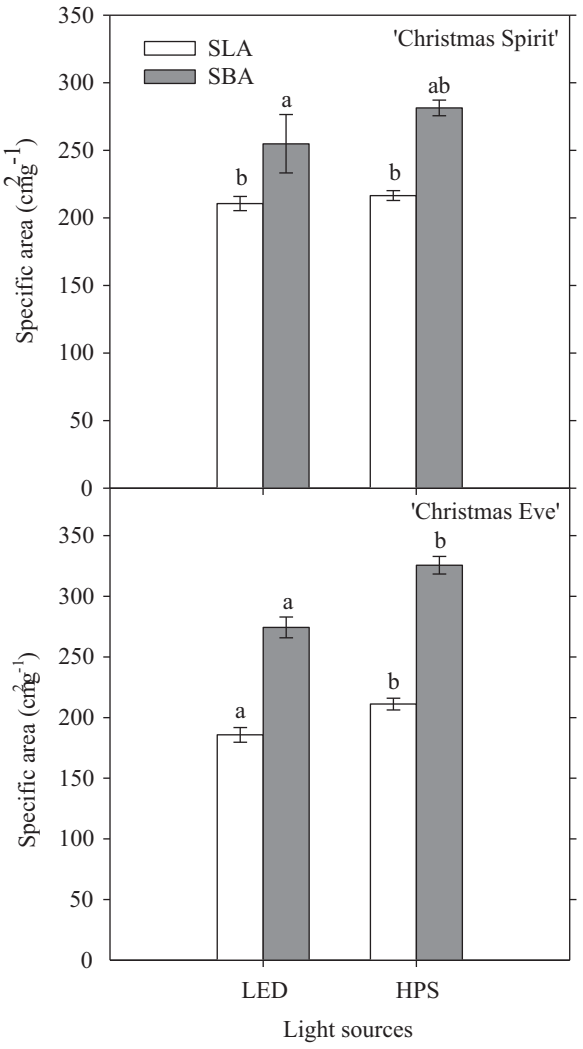

Fig. 6. Specific leaf area (SLA) and specific bract area (SBA) of poinsettia grown in greenhouse compartments under LED light (20% B and 80% R) or HPS as supplementary lighting at an irradiance of 100 μmol m^{-2} s^{-1} during a 10 h photoperiod. SBA and SLA were calculated from leaf or bract area divided by their respective dry weights. Vertical bars represent the ±SE (standard errors) (n = 30). Mean values with the same letter are not significantly different based on ANOVA followed by a Tukey's test at p < 0.05.

increased B light has a potential to be an effective method to control stem extension in commercial culture of poinsettia without quality reduction.

The use of EOD treatment with low intensity light (5 μmol m^{-2} s^{-1}) to control stem extension requires little energy. In the present study, EOD-R was found equally effective in one of the cultivars tested as the traditional temperature drop treatment when the supplemental light was provided by HPS (Fig. 7). Thus, EOD-R in combination with HPS supplemental light might possibly substitute the traditionally used temperature drop treatment. Others also reported on suppressive effects of EOD-R for species like *Petunia*, *Chrysanthemum*, tobacco and soybean (Ilias and Rajapakse, 2005; Kasperbauer, 1971, 1987; Rajapakse et al., 1993). The effect of EOD-R on poinsettia was somewhat weaker than the response to increased B light, and one of the cultivars tested ('Christmas Eve') did not respond to EOD-R either in the greenhouse (Fig. 7) or in chamber experiments (Islam, unpublished results). Others also report on variable results or no significant effect of EOD-R light treatment on poinsettia (Mata and Botto, 2009). This might be due to different sensitivity to light qualities in different species or cultivars as a consequence of differences in the action of photoreceptors. The differential light quality response might also be due to differential hormonal regulation of stem extension growth of poinsettia, but so far knowledge on this is limited. It is well known however, that both the phytochrome and the cryptochrome are involved in regulating the content of the plant hormone gibberellins (Hisamatsu et al., 2005; Zhao et al., 2007).

Since no effect of EOD-R was observed when LED was used as supplemental light, it is possible that the inhibition of the extension growth of poinsettia is saturated under a light spectrum with high B portions (Fig. 7). In contrast to the LED light source, the HPS contain low B light. The LEDs appear already to be saturated in inhibition of shoot elongation, and accordingly EOD-R does not result in further inhibition as it did when HPS was used as supplementary light (Fig. 7).

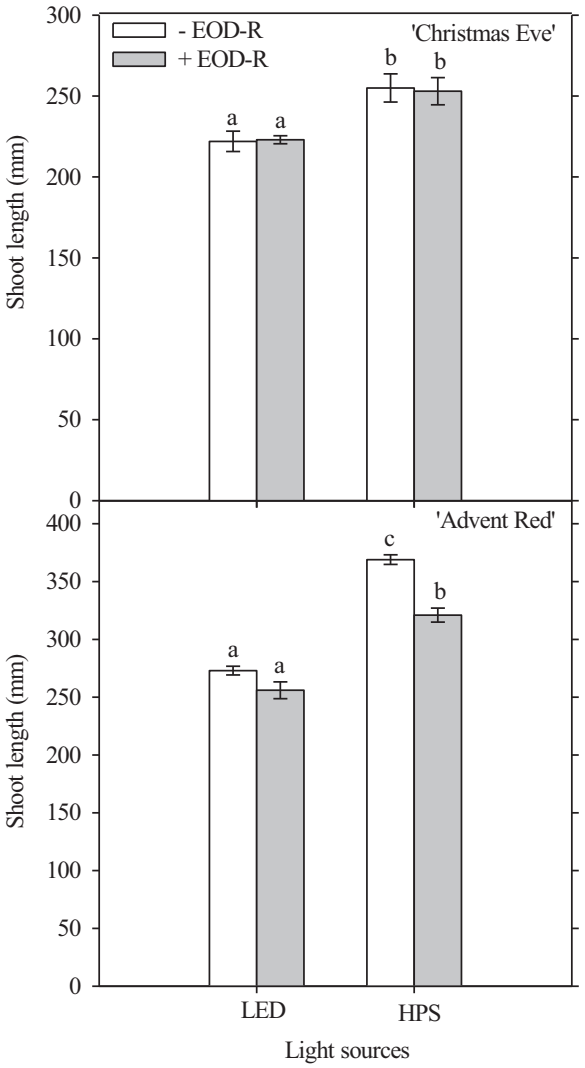

Fig. 7. Effect of 30 min end-of-day red light (EOD-R) at an irradiance of 5 μmol m^{-2} s^{-1} on poinsettia plant height grown in a greenhouse compartment under supplementary lighting with LED lamps (80% R and 20% B) or HPS lamps at 100 μmol m^{-2} s^{-1}. Vertical bars represent the ±SE (standard errors) ($n = 12$). Mean values with the same letter are not significantly different based on ANOVA followed by a Tukey's test at $p < 0.05$.

In conclusion, LED with a high proportion of B light was effective in reducing the stem extension growth of all the poinsettia cultivars tested compared to HPS. The plants were compact with 20–34% height reduction without any effect on the flowering response or post harvest quality. EOD-R light reduced shoot length by up to 13% in one of the cultivars tested when the supplemental light was provided with HPS (5% B) but had no effect under LED top light (20% B). This in generally shows that adding more B light to the spectrum by using LED light is more effective than manipulation of the phytochrome status at the end of the day.

Acknowledgements

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Paper III

Impact of end-of-day red and far-red light on plant morphology and hormone physiology of poinsettia

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Abstract

Control of morphology is essential in greenhouse production of poinsettia, which is among the largest and economically most important ornamental pot plant cultures worldwide. Plant growth regulators are used to reduce elongation but due to their negative impacts on human health and the environment, there is an urgent need to replace these. Exploiting responses to light quality as an alternative has been investigated to a limited extent only in poinsettia. Also, information about elongation-controlling hormones in poinsettia is scarce. In this study the phytochrome status was manipulated by exposing plants to 30 min red (R) or far-red (FR) light at the end of the day (EOD) to study effects of elongation growth and hormone physiology. Depending on cultivar, shoot elongation was reduced 34-54% by exposure to EOD-R compared to EOD-FR treatment. This was apparently due to reduced internode lengths. Compared to EOD-FR, reduced elongation under EOD-R was correlated with 28% and 21% lower levels of the active gibberellin GA_1 and indole-3-acetic acid, respectively, as well as 19% decrease in abscisic acid content. Specific leaf and bract area were also significantly different in the two light treatments. Time to flowering (bract formation and visible cyathia) did not differ between the light treatments in any cultivar. In conclusion, manipulation of the phytochrome system by EOD-R in order to modulate the contents of the growth-controlling hormones can be a useful tool in control of shoot elongation in greenhouse-grown poinsettia.

Keywords: Abscisic acid, end of day (EOD) red (R) and far red (FR), indole-3-acetic acid, gibberellin, shoot elongation

Introduction

Poinsettia (*Euphorbia pulcherrima*) is among the economically most important ornamental potted plants worldwide (Ecke et al., 2004). Due to low natural solar radiation and low temperatures in northern areas during the autumn when poinsettia is produced in greenhouses, the use of supplementary light and heating is necessary. Control of plant height is then among the main challenges (Ecke et al., 2004; Lütken et al., 2012). In greenhouse culture of ornamental species such as poinsettia, lilies, geraniums and chrysanthemum graphical tracking is a commonly used tool for height management (Currey and Lopez, 2010; Heins and Carlson, 1990; Lopez and Currey, 2011; Moe et al., 1992). The graphical tracking method is based on a static sigmoid curve to determine the progress toward a desired plant height. The actual growth is then compared with the predicted curves. Plant growth regulators (PGR) such as chlormequat, daminozide or paclobutrazol are commonly used in this respect to control shoot elongation. However, due to the negative effects of PGRs on human health and the environment, their use is becoming restricted and desirable to phase out. In addition, application of PGRs is time-consuming. In northern areas temperature drop treatments in the morning is commonly used in combination with PGRs to reduce elongation growth, but use of such temperature drops is limited by energy-saving regimes in semi-closed or closed greenhouses as well as periods of high temperatures and high solar radiation in early autumn. As an alternative, exploiting the responses of the plants to different light qualities provides an interesting possibility in greenhouse production of poinsettia.

In plants light quality is sensed by different photoreceptors, which are involved in a wide range of developmental processes. Red (R) and far-red (FR) light are sensed by phytochromes, which exist in two inter-convertible forms, the R-light absorbing inactive form (P_r) and the FR-

absorbing active form (P_{fr}) (Smith, 1995). Although the effect vary with species, the R:FR-ratio during the entire light phase or at a short duration of (10-60 min) at the end of the day (EOD) has been shown to be very effective to control shoot elongation in a number of species with FR and R enhancing and reducing elongation, respectively (Blom et al., 1995; Gilbert et al., 1995; Kasperbauer and Peaslee, 1973; Smith, 1995). Thus, the amount of P_{fr} relative to the total amount of phytochrome at the beginning of the dark period may regulate plant morphology and photosynthetic partitioning (Rajapakse et al., 1993). Furthermore, the phytochrome responses vary with other environmental conditions such as irradiance, other aspects of spectral quality and temperature (Smith, 1995).

Although effects of R:FR ratios have been studied to a limited extent in poinsettia, experiments where poinsettia plants were covered by selective films absorbing FR or R light, indicated that morphology is affected by the R:FR ratio (Clifford et al., 2004). Also, when greenhouse-grown poinsettia was provided with a 30 min EOD-R treatment plant height was reduced compared to those where only high pressure sodium lamps were used as supplementary light (Islam et al., 2012). However, no systematic comparison of effects of EOD-R and FR on shoot elongation and the hormones involved in poinsettia has been reported. In the poinsettia growing season in greenhouses (Sept-Dec) in northern areas, the R:FR ratio of natural sun light is decreasing at the EOD (Nilsen, 1985). Manipulation of the light quality at the EOD might thus provide an interesting, energy-efficient option for controlling shoot elongation in poinsettia.

Light-dependent changes of plant growth and development are known to be regulated by plant hormones. In investigated, responsive species internode elongation is influenced by the R:FR-ratio through modulation of the levels of hormones, especially gibberellin (GA) and auxin. In cowpea (*Vigna sinensis*) enhanced elongation in response to EOD-FR is apparently due to

reduced inactivation of GA_1 as a consequence of removal of the active phytochrome form P_{fr} (Martínez-García et al., 2000). In hybrid aspen (*Populus tremula x tremuloides*) phytochrome A (*PHYA*) overexpression resulted in decreased shoot elongation due to reduced cell numbers and cell lengths, and this was correlated with reduced GA and IAA levels (Olsen et al., 1997). However, after exposure of *PHYA* overexpressing lines and wild type plants to EOD-FR light, the plant length, cell lengths and cell numbers as well as GA levels were similar in both genotypes, indicating that phyA is not involved in the response to EOD-FR (Olsen and Junttila, 2002). As shown in a variety of species responses to different R:FR and EOD treatments appear to depend on light stable phytochromes such as phyB, phyD and phyE (Devlin et al., 1999; Smith, 1995).

The biologically active GAs, such as the 13-hydroxylated GA_1, GA_3, GA_5 and GA_6 as well as the non-13-hydroxylated GA_4 and GA_7 are synthesised in the early 13-hydroxylation pathway and the non-13-hydroxylation pathway, respectively (Yamaguchi, 2008). In some species, such as the herbaceous pea (*Pisum sativum*), *Petunia,* cowpea and *Silene*, as well as the woody species of the *Populus* and *Salix* genera, GA_1 is dominating in vegetative tissues, whereas GA_4 is dominating e.g. in *Arabidopsis thaliana* and conifers like Norway spruce (*Picea abies*) (Graebe, 1987; Yamaguchi, 2008). In poinsettia, GA biosynthesis has not been characterised and information about hormone physiology in general is scarce.

Indole-3-acetic acid (IAA) is the predominant auxin in plants and an important determinant of phytochrome-mediated growth suppression during de-etiolation (Chory et al., 1996). Auxin is implicated in stem elongation as well as phototropism, gravitropism, vascular tissue differentiation and cell expansion. In pea endogenous IAA was increased under EOD-FR (Behringer and Davies, 1992). Upregulation of auxin-induced genes under low irradiance and

low R:FR ratio resulted in increased hypocotyl elongation in *A. thaliana* (Steindler et al., 1999; Vandenbussche et al., 2003).

The role of cytokinins (CKs) in mediating effects of light on stem elongation is unclear but somehow appears to interact with other hormones (Halliday and Fankhauser, 2003; Miller, 1956; Thomas et al., 1997). CKs can apparently promote stem elongation in light-grown *A. thaliana* either through blocking ethylene action or IAA transport (Smets et al., 2005). Furthermore, the effects of CKs may vary in light- and dark-grown plants. In light-grown *A. thaliana* there was no effect of CK application on hypocotyl elongation but in the dark CK inhibited hypocotyl elongation (Su and Howell, 1995). Taken together, the role of CKs in photomorphogenesis is still unclear.

The relationship between light and abscisic acid (ABA) levels is not clearly established (Kraepiel and Miginiac, 1997). The phyA-deficient mutant *pew 1* in tobacco (*Nicotiana tabacum*) has higher levels of ABA compared to wild type plants (Kraepiel et al., 1994). In sunflower (*Helianthus annuus*) the R:FR ratio and ABA levels in leaves were correlated with internode length (Kurepin et al., 2007b). Similarly, FR-treated *Lemna gibba* showed higher ABA content compared to R light alone, whereas ABA levels decreased with decreasing elongation under R light (Weatherwax et al., 1996). Recently, reduced height was reported in an ABA deficient mutant in tomato (*Solanum lycopersicum*) (Nitsch et al., 2012).

Under natural light conditions, the R:FR ratio is decreasing at the EOD in the poinsettia growing season (Sept-Dec) in greenhouses in northern areas (Nilsen, 1985). In this study, we aimed to manipulate the phytochrome status at the EOD in poinsettia by applying EOD-R or FR and to study hormone metabolism to get a general overview on light quality-regulation of elongation growth controlling hormones in this species. Furthermore, in addition to the effect on

shoot elongation, the role of the phytochrome status of poinsettia on other quality parameters like bract number, bract size, leaf morphology and time to flowering was studied.

Materials and methods

Plant materials

Two cultivars (cvs Christmas Spirit and Christmas Eve) of poinsettia (*Euphorbia pulcherrima* Willd. ex Klotzsch) were used to test the effect of red (R) and far-red (FR) end-of-day (EOD) treatments on plant morphology. Cuttings of poinsettia with 6-7 leaves were rooted in Jiffy-7 (G3 Ljones Gartneri AS, Tørvikbygd, Norway) and thereafter potted in *Sphagnum* peat (Veksttorv, Ullensaker Almenning, Nordkisa, Norway) in 13 cm plastic pots. The plants were kept for 6 weeks in a growth room at 20ºC with an average relative air humidity (RH) of 70% ± 5%, corresponding to a an average of 0.7 kPa water vapour deficit (VPD). Light at a photon flux density of 80-90 μmol m^{-2} s^{-1} (OSRAM L 58W/640 Cool White fluorescent tubes, Munich, Germany) was provided during an 18 h photoperiod. Plants were pinched above 3-4 leaves and three side shoots per plant were allowed to grow.

Growth conditions and light treatments

To induce flowering, all plants were then transferred to a growth chamber where plants were grown at 21^{o}C ± 2^{o}C under a 10 h photoperiod provided by high pressure sodium lamps (HPS, LU400/XO/T/40; General electric company, Fairfield, CT, USA) at an irradiance of 100 μmol m^{-2} s^{-1}. At the EOD, 30 min of either R (peak 630 nm, Philips green power LED module HF deep red, 10 W, Eindhoven, The Netherlands) or FR (peak 730 nm, Philips green power LED module HF far red, 10 W) light at 10 μmol m^{-2} s^{-1} was provided (measured by Skye instruments

660/730 sensor, Powys, Wales, UK) by light emitting diode (LED) bars. The RH was adjusted to 70% ± 5%, and the CO_2 concentration was at ambient level. The plants were watered daily during the experimental period with a nutrient solution of an electrical conductivity (EC) of 1.5 mS cm^{-1} (Red superba and Calcinit, Yara, Oslo, Norway) at pH 5.6-5.8. The irradiance was measured at the plant canopy level using a LI-COR Quantum/Radiometer/Photometer (Model LI-250, Li-COR, Lincoln, NE, USA). The spectra for the lamps were measured with a spectrometer (OceanOptics, model-SD2000, Dunedin, FL, USA). Temperature and RH data were recorded in five minute intervals by a PRIVA Integro 3 environmental computer (Priva, Vineland station, ON, Canada). Two cultivars with 10 replicate plants of each cultivar were used in each treatment and two replicate experiments were performed.

Growth analysis

Plants were grown until flowering (open cyathia) and growth analysis was performed. The length of the stem (shoot) from the base of each shoot to the shoot apical meristem was measured once a week. Petiole length of four mature leaves in each shoot and the stem diameter at the middle of each shoot were measured after flowering. The number of leaves and bracts was counted and the average internode lengths were calculated by dividing final height by the number of leaves. Bracts were defined as transition leaves which had formed red colour and were counted if the length exceeded 3 cm (petiole + bract). An area meter (Model 3100 area meter, LI-COR, Licor Biosciences, Lincoln, NE, USA) was used to measure the leaf and bract area. Fresh and dry weight (dry matter; DM) of stems, leaves and bracts were recorded after drying at 65°C until a constant mass was reached. Specific leaf and bract area (SLA and SBA, respectively) were determined from the area/dry weight for each of them. Total chlorophyll content was

measured in the middle leaf of the three shoots on each plant by a chlorophyll content meter (Model CL-01, Hansatech Instruments, Norfolk, England).

Samples for hormone analyses

After one month under each EOD treatment, the elongating part of the shoot tips (stem) (0.5 to 1 cm) from each of 6 plants of the cv Christmas Eve were harvested into liquid nitrogen. The samples were freeze-dried using a freeze dryer machine (Heto Holten A/S, Gydevang 17-19, DK-3450 Allerød, Denmark). For each light treatment six samples, each consisting of 3 shoot tips from one plant, were used for hormone analysis. Of these three samples were used for the analysis of auxin, abscisic acid (ABA), cytokinins (CKs) and their metabolites and the three other samples were used for gibberellin (GAs) analysis.

Analyses of auxin, cytokinins, abscisic acid and their metabolites

Fifty mg dry weight (DW) of homogenized plant tissue was used for extracting auxin, cytokinin and ABA using 3 ml of isopropanol:water:glacial acetic acid (80:19:1, v/v). The samples were agitated in the dark for 24 h at 4 ºC. Deuterated forms of the hormones and their metabolites (Abrams et al., 2003; Chiwocha et al., 2003) were added as internal standards. These included [$^2H_3$]-dihydrophaseic acid (DPA), [$^2H_5$]-ABA-β-glucose ester (ABA-GE), [$^2H_3$]-phaseic acid (PA), [$^2H_4$]-7'-OH-ABA, [$^2H_3$]-neoPA, [$^2H_4$]-ABA, [$^2H_4$]-*trans*-ABA, [$^2H_3$]-indole-3-acteyl-leucine (IAA-Leu), [$^2H_3$]-IAA-alanine (IAA-Ala), [$^2H_3$]-IAA-aspartate (IAA-Asp), [$^2H_3$]-IAA-glutamate (IAA-Glu) and [$^{13}C_4$]-indole-3-butyric acid (IBA) [all synthesised at Plant Biotechnology Institute-National Research Council, Canada, according to Abrams et al. (2003) and (Chiwocha et al., 2003)], [$^2H_5$]-indole-3-acetic acid (IAA) (Cambridge Isotope Laboratories,

Andover, MA, USA) and [$^2H_3$]-dihydrozeatin (DHZ), [$^2H_3$]-dihydrozeatin-riboside (DHZR), [$^2H_5$]-zeatin-O-glucoside (ZOG), [$^2H_6$]-isopentenyl adenosine (iPA) and [$^2H_6$]-isopentenyl adenine (iP) (OlChemIm Ltd., Olomouc, Czech Republic). The samples were centrifuged, the supernatant isolated and dried on a Büchi Syncore Polyvap (Büchi, Flawil, Switzerland). The samples were then purified according to Abrams et al. (2003) and Chiwocha et al. (2003). The samples were reconstituted in 100 µL acidified methanol, adjusted to 1 ml with acidified water and then partitioned against 2 ml hexane. After 30 min, the aqueous layer was isolated and dried as described. Dry samples were reconstituted in 800 µL acidified methanol and adjusted to 1 ml with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak C18 cartridges (Waters, Mississauga, ON, Canada), the eluate being dried on a LABCONCO centrivap concentrator (Labconco Corporation, Kansas City, MO, USA). An internal standard blank was prepared with 100 µL of the deuterated internal standards mixture. A quality control (QC) standard was prepared by adding 100 µL of a mixture containing all the analytes of interest, each at a concentration of 0.2 pg μL^{-1}, to100 µL of the internal standard mix. Finally, samples, blanks, and QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 pg μL^{-1} of each of the recovery standards.

Samples were injected onto a Genesis C18 HPLC column (2.1 mm x 100 mm, 4 µm, Chromatographic Specialties, Brockville, ON, Canada) and separated by a gradient elution of water against an increasing percentage of acetonitrile that contained 0.04% acetic acid. The analyses were performed on an HPLC system (2695 Waters HPLC, Waters) linked to a tandem mass spectrometer (Quattro Ultima, Micromass, Manchester, UK). Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters) control software was used for the analysis.

Quantification of auxins, CKs and ABA and their metabolites was performed according to Chiwocha et. al. (2003; 2005). The resulting chromatographic traces were quantified off-line by the QuanLynx v4.1 software (Waters) wherein each trace was integrated and the resulting ratio of signals (non-deuterated/internal standard) was compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed along each batch of tissue samples.

Analyses of gibberellins

Since the hormone profiling method described above was not able to detect GAs in the poinsettia shoot tip tissues, quantification of GAs was performed according to Urbanova et al. (2013). Briefly, samples of 20 mg fresh weight were homogenized in liquid nitrogen, extracted in ice-cold 80% acetonitrile containing 5% formic acid with 50 pmol of $[^2H_2]GA_1$, $[^2H_2]GA_3$, $[^2H_2]GA_4$, $[^2H_2]GA_5$, $[^2H_2]GA_6$, $[^2H_2]GA_7$, $[^2H_2]GA_8$, $[^2H_2]GA_9$, $[^2H_2]GA_{12}$, $[^2H2]GA_{12ald}$, $[^2H_2]GA_{15}$, $[^2H_2]GA_{19}$, $[^2H_2]GA_{20}$, $[^2H_2]GA_{24}$, $[^2H_2]GA_{29}$, $[^2H_2]GA_{34}$, $[^2H_2]GA_{44}$, $[^2H_2]GA_{51}$ and $[^2H_2]GA_{53}$ as internal standards, and purified on cation exchange extraction cartridges coupled to reversed-phase (C18) cartridges followed by anion exchange cartridges (all Waters, Milford, MA, USA). Dried elutes were re-solubilized in the mobile phase and analyzed by a UPLC-MS/MS system. The data were then analyzed using Masslynx 4.1 software (Waters) and quantified by the standard isotope-dilution method.

Statistical analysis

The effects of the different light treatments on the different growth parameters as well as endogenous level of hormones and their metabolites were analyzed using analysis of variance (ANOVA General Linear Model procedure) and Tukey's pair wise comparison test ($p < 0.05$) using Minitab Version 16 (Minitab Inc., State College, PA, USA).

Results

Effects of EOD-R and FR light

Poinsettia plants grown under controlled conditions in growth chambers showed significant reduction in shoot elongation under a daily 30 min treatment with EOD-R compared to EOD-FR (Fig. 1). At the marketing stage after 12 weeks of EOD-treatment under SD of 10 h photoperiod 'Christmas Spirit' showed 54% reduction in height under EOD-R compared to EOD-FR (Fig. 1-2). 'Christmas Eve' responded somewhat less, but still showed 34% height reduction under EOD-R. Similarly, internode lengths were decreased under EOD-R with 53% and 35% reduction in 'Christmas Spirit' and 'Christmas Eve', respectively, compared to FR light (Fig. 3). Leaf and bract numbers were not significantly affected by the light treatments in any of the cultivars (Table 1). Compared to EOD-FR 'Christmas Spirit' showed significantly reduced petiole length by 22% under EOD-R, whereas petiole length was not affected by the light treatments in 'Christmas Eve'.

Dry matter (DM) of the stem was decreased by 50% and 29% in 'Christmas Spirit' and 'Christmas Eve', respectively, under EOD-R treatment compared to EOD-FR (Table 1). There was no significant difference in DM of leaves and bracts under the different treatments. However, in both cultivars the specific leaf area (SLA) and bract area (SBA) were significantly

different between the two different EOD treatments. SLA was reduced by 15% and 10% in 'Christmas Spirit' and 'Christmas Eve', respectively, and SBA was reduced by 18% and 12% in 'Christmas Spirit' and 'Christmas Eve', respectively, under R light compared to FR light (Fig. 4). We did not find any difference in time to visible cyathia and bract formation by R and FR light (data not shown). Shoot diameter and the relative chlorophyll content did not differ in any cultivar under the different light treatments (Table 1).

Endogenous levels of auxin, GAs, ABA CKs and their metabolites

To assess the effect of the EOD treatments on hormone contents, elongating shoot tips of 'Christmas Eve' were analysed for their levels of IAA, GAs, ABA, CKs and their metabolites. The pathways of biosynthesis and inactivation of the different hormones are shown in Fig. 5 and the hormone metabolites found in poinsettia tissue are indicated (in bold). Endogenous IAA levels were significantly lower under EOD-R compared to EOD-FR light (Fig. 6). There was no significant difference in the contents of conjugated IAA (IAA-Asp and IAA-Glu) or the ratio of conjugated IAA and IAA, suggesting an effect of the EOD treatments on IAA biosynthesis and not conjugation. The endogenous GA levels showed a similar trend as that of IAA. The total content of GAs was significantly lower (29%) under EOD-R compared to EOD-FR (Table 2). The 13-hydroxylated pathway of GA biosynthesis was found to be the dominating one in the shoot tips of poinsettia. Among non-13 hydroxylated GAs, only GA_7 and the inactivation product GA_{34} of the active GA_4 were present in measureable amounts. However, their levels were far lower than those of the 13-hydroxylated GAs detected (Fig. 5, Table 2). There was a trend of lower levels (about 28%) of the 13-hydroxylated GA_1 in shoot tips under EOD-R compared to EOD-FR. The levels of GA_8, the inactive catabolite of GA_1, and GA_{29}, the C2-

hydroxylated product of the GA_1 precursor GA_{20}, were also significantly reduced by 46% and 29%, respectively, under the EOD-R light compared to EOD-FR. Furthermore, the ratio of GA_1/GA_{20} and GA_6/GA_5 were significantly lower (44% and 16%, respectively) under EOD-R compared to EOD-FR. Poinsettia contained GA_3 in levels similar to GA_1, and there was a trend of lower GA_3 contents in EOD-R compared to FR.

The endogenous content of ABA was significantly lower (19%) in poinsettia shoot tips under EOD-R compared to EOD-FR (Fig. 7). ABA is inactivated through oxidation to phaseic acid (PA) and dihydrophaseic acid (DPA) in sequence as well as by conjugation to ABA glucose ester (ABA-GE) (Kraepiel and Miginiac, 1997). There were no significant effects of the light treatments on any of the inactivation products.

The active free bases of cytokinins *trans*-zeatin (*t*Z) and isopentyladenine (iP) as well as the inactive and storage forms (nucleosides and nucleotides) *cis*-zeatin O-glucoside (*c*ZOG), *cis*-zeatin riboside (*c*ZR), *trans*-zeatin riboside (*t*ZR) and isopentenyladenosine (iPA) were detected in the poinsettia shoot tip tissues (Table 3). Only the nucleoside *t*ZR and iPA showed a statistically insignificant trend of reduced levels under EOD-R compared to EOD-FR.

Discussion

Control of shoot elongation is essential in poinsettia which is among the largest and economically most important ornamental pot plant cultures worldwide. In this species hormone physiology associated with shoot elongation has previously been characterised to a very limited extent. In this study, we have shown that the phytochrome status is controlling the levels of IAA, GAs and ABA in shoot tips of poinsettia. The EOD-R light treatment, which is known to convert most of the phytochrome to its active form P_{fr}, accordingly inhibits stem extension growth in

poinsettia through affecting the metabolism of these hormones. Shoot elongation of the two poinsettia cultivars tested was significantly reduced by 34-54% under EOD-R compared to EOD-FR light (Fig. 1-2). Similarly, the internode length was reduced (35-53%) significantly under the R light treatment relative to FR (Fig. 3). The height reduction can thus be explained by reduced internode length and not by different numbers of internodes (Table 1). Stem elongation or cell elongation basically depends on cell proliferation and cell expansion (Depuydt and Hardtke, 2011; Sánchez-Rodríguez et al., 2010). In hybrid aspen clear effects of EOD-R and EOD-FR on cell lengths and cell numbers have been demonstrated (Olsen and Junttila, 2002). Suppressive effects on shoot elongation by EOD-R light have also been shown in different crops like cowpea, *Petunia*, *Chrysanthemum*, tobacco and soybean (*Glycine max*) (Ilias and Rajapakse, 2005; Kasperbauer, 1971; Kasperbauer, 1987; Martínez-García et al., 2000; Mata and Botto, 2009; Rajapakse et al., 1993).

The two different poinsettia cultivars studied showed different sensitivity to the different light treatments. Whereas the cultivar Christmas Spirit showed 54% and 53% reduction in shoot elongation and internode length in response to EOD-R, the Christmas Eve cultivar showed 34% and 35% reduction in the same parameters, respectively (Fig. 1-3). The difference between cultivars might be due to differences in the action of the involved phytochrome light receptors. SLA and SBA were lower in both cultivars under EOD-R light compared to FR light (Fig. 4). The petiole lengths and DM of the stem were significantly higher only in ‘Christmas Spirit’ under FR although Christmas Eve showed similar statistically insignificant trends (Table 1). However, the DM accumulation in leaves and bracts did not show any significant differences under the R and FR treatments. Mata and Botto (2009) and Clifford et al. (2004) showed no significant differences in dry weight of any part of poinsettia plants including stems under

different R:FR ratios under photoselective films absorbing or transmitting FR. The different effect of light quality on stem dry weight in that study and the present might be due to use of different cultivars or other environmental conditions affecting light quality responses.

In our study, no difference in time to visible cyathia and bract formation was observed under EOD-R and FR light. On the contrary, flowering time was slightly delayed in the Freedom Red poinsettia cultivar grown under a high R:FR ratio under a photoselective film absorbing FR (Mata and Botto, 2009). The differences between the cultivars in elongation growth and other characteristics such as flowering might be due to different sensitivity to light quality as a consequence of the action of photoreceptors (Hisamatsu et al., 2005; Kurepin et al., 2007a, b; Mata and Botto, 2009; Olsen and Junttila, 2002; Zhao et al., 2007).

Reduction of internode length and plant height under the EOD-R light treatment was associated with reduced levels of IAA and GA (Fig. 6 and Table 2). The endogenous levels of IAA were 21% lower in EOD-R light than FR light. This is similar to in *A. thaliana*, where enhanced elongation under a low R:FR-ratio was shown to be IAA-dependent (Morelli and Ruberti, 2000; Steindler et al., 1999). Furthermore, in cowpea and sunflower the endogenous level of IAA was increased under FR light treatment and reduced by a high R:FR-ratio (Behringer and Davies, 1992; Kurepin et al., 2007a, b). Recently, it was observed that reduced shoot elongation of *AtSHI* overexpressing transgenic poinsettia plants was associated with lower IAA levels compared to the higher non-transgenic plants (Islam et al., 2013). That study and the present one demonstrate that IAA is an important determinant of shoot elongation in poinsettia like shown for a variety of species.

The detection of GA_{19}, GA_{20}, GA_{29}, GA_1, GA_8, GA_5, GA_6 and GA_3 of the 13-hydroxylation pathway but low levels of GA_{34} and GA_7 only of the non-13-hydroxylation

pathway (Urbanova et al., 2013), strongly indicate that the 13-hydroxylation pathway is the dominating one in poinsettia. The total GA levels were significantly lower (29%) under EOD-R compared to FR (Table 2). There was also a trend with 28% lower content of active GA_1 under EOD-R compared to EOD-FR treatment. The inactive metabolites GA_{29} and GA_8 which are formed by GA 2-oxidase (GA2ox; 2-β hydroxylase) activity from GA_{20} and GA_1, respectively, were significantly higher under the FR light treatment compared to R. On the other hand, there was no significant difference ($p \leq 0.05$) in the ratio of inactive GA_8 to active GA_1 and the ratio of GA_{29} to GA_{20} between the light treatments, only insignificant trends of higher ratios in EOD-FR. Thus, lower GA content under EOD-R compared to EOD-FR can apparently not be explained by a higher inactivation rates. Furthermore, although feed-forward effects resulting in increased *GA2ox* activity under conditions with increased levels of GA_1 is well known e.g. in *A. thaliana* (Thomas et al., 1999), such a relationship was not clear under EOD-FR in our study of poinsettia. Like a number of species, poinsettia has been shown to respond to a temperature reduction in light by reduced shoot elongation (Moe et al., 1992). In pea increased transcript levels of *PsGA2ox2* resulting in decreased GA_1 levels and thus reduced shoot elongation, have been demonstrated upon a temperature reduction during the light phase (Stavang et al., 2007; 2005). Furthermore, in *A. thaliana* increased temperature during continuous light resulted in down-regulation of *GA2ox1* (Stavang et al., 2009). However, it appears that regulation of GA metabolism by EOD-R and FR light in poinsettia differs from the effect of a temperature alteration in light. The significantly reduced GA_1/GA_{20}-ratio under EOD-R compared to FR indicates reduced biosynthesis of GA_1. This might be due to reduced GA3ox or GA20ox activities, catalysing the conversion of GA_{20} to GA_1 and among others GA_{19} to GA_{20}, respectively. In *A. thaliana* enhanced petiole elongation under a low R:FR-ratio in LD or a brief

EOD-FR exposure in SD was ascribed to increased *AtGA20ox2* expression (Hisamatsu et al., 2005). Taken together, the significantly lower levels of total GA as well as GA_{29}, GA_8 and the ratio of GA_1/GA_{20} and GA_6/GA_5 under EOD-R light compared to FR light correlated with the reduced stem elongation and internode length in poinsettia (Table 2). The reduction in GA content might also have affected leaf and bract area which were lower under the R compared to the FR light treatment. In sunflower exposed to a low R:FR ratio, Kurepin et al. (2007b) demonstrated higher endogenous levels of GAs; with significantly increased levels of the inactive GA_8 and GA_{20} (the precursor of GA_1), in leaf tissue. The situation in poinsettia is very similar to this. The detection of high levels of GA_3 in poinsettia, which were similar to the GA_1 levels, suggests that the side chain leading to GA_3 is highly active in poinsettia (Table 2). This phenomenon has been demonstrated also in some other crops such as *Marah marcocarpus*, apple (*Malus domestica*) and maize (*Zea mays*) (Albone et al., 1990; Spray et al., 1996).

When poinsettia plants were treated with EOD-R light, the ABA levels were significantly (19%) decreased compared to EOD-FR (Fig. 7). This is similar to the situation in sunflower, where ABA levels were lower in R light compared to FR, even in elongated leaves (Kurepin et al., 2007b). Also, in dark-grown *L. gibba*, ABA levels were lower after 4 h in R light treatment (Weatherwax et al., 1996). These studies suggest that phytochrome action somehow regulates the endogenous ABA level to a certain extent. In general, ABA is well known to increase under different stress conditions such as water logging, drought, cold or wounding (Arve et al., 2011). For example, drought stress is decreasing the turgor pressure in the cells, which in turn leads to reduced cell expansion, which again affects the growth of the whole plant. ABA inactivation occurs by either oxidation to PA or DPA or by covalent conjugation to another molecule such as a monosaccharide. While there were no significant difference in the levels of ABA inactivation

products (PA, DPA or ABA-GE), the ratio of inactive ABA metabolites to ABA was slightly, but significantly higher under EOD-R than FR (Fig. 7). Thus, it seems that the rate of inactivation is higher under the R light treatment compared to the FR light.

Very little is known about the effects of light quality on CKs levels. There were no significant differences in any of CKs or their metabolites detected in the present study. This is similar to results found in sunflower exposed to a low R:FR ratio. Furthermore, application of CKs did not show any effect on elongation of sunflower, mustard (*Sinapis alba*) and light-grown *A. thaliana* (Kurepin et al., 2007a; Smets et al., 2005; Su and Howell, 1995; Tong et al., 1983).

Our study shows that poinsettia is very sensitive to the phytochrome status at the EOD, and that in periods of undesirably high growth rates, EOD-R may be used to inhibit growth. In northern areas much FR light is present at the EOD in the season when poinsettia is produced (Sept-Dec). Thus, removal of FR-light or light quality manipulation resulting in increased R:FR ratio at the EOD can be used to avoid unwanted extension growth. We show here that increased contents of elongation growth-controlling hormones in EOD-FR can be counteracted by providing an EOD-R treatment. The levels of IAA and GAs were significantly reduced under EOD-R compared to EOD-FR levels. Since manipulation of the phytochrome status by providing an EOD-R treatment has very small effects on other growth parameters than shoot elongation, including flowering time in poinsettia, such a treatment constitutes an interesting tool for manipulation of morphology. This is supported by the previous observation that 30 min EOD-R can reduce shoot elongation in some green-house-grown poinsettia cultivars compared to use of high pressure sodium lamps only as supplementary light (Islam et al., 2012). However, since morphological responses to a certain extent depend on cultivar, the effects on relevant cultivars must be investigated specifically.

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Table 1 Effects of 30 min end-of-day (EOD) treatment with red (R) or far-red (FR) light on morphology of two cultivars of poinsettia grown under a 10 h photoperiod

Parameters	Christmas Spirit		Christmas Eve	
	EOD-R	EOD-FR	EOD-R	EOD-FR
Petiole length (cm)	5.1 ± 0.3 b	6.5 ± 0.4 a	4.4 ± 0.2 b	5.1 ± 0.1 b
Leaf numbers per shoot	6.0 ± 0.2 a	6.0 ± 0.2 a	6.4 ± 0.1 a	5.8 ± 0.1 a
Bract numbers per shoot	10.0 ± 0.5 a	10.1 ± 0.5 a	12.9 ± 0.3 a	12.4 ± 0.3 a
Shoot diameter (mm)	5.3 ± 0.2 a	5.6 ± 0.3 a	5.4 ± 0.1 a	5.5 ± 0.1 a
Dry matter of stem (g)	0.5 ± 0.1 b	1.0 ± 0.1 a	0.5 ± 0.1 b	0.7 ± 0.1 b
Dry matter of leaves (g)	1.4 ± 0.2 a	1.4 ± 0.1 ab	1.1 ± 0.1 ab	1.0 ± 0.1 b
Dry matter of bracts (g)	1.3 ± 0.1 a	1.2 ± 0.1 a	1.3 ± 0.1 a	1.3 ± 0.1 a
Relative chlorophyll content	27.6 ±1.5 a	30.2 ± 1.5 a	29.2 ± 1.2 a	26.7 ± 1.4 a
Total leaf area (cm^2)	334.3 ± 34.8 a	372.3 ± 33.4 a	277.7 ± 15.1 a	288.6 ± 16.2 a
Total bract area (cm^2)	445.8 ± 39.4 a	526.6 ± 44.9 a	496.0 ± 23.0 a	551.6 ± 23.2 a

Mean values ± SE are given. n= 18-21. Mean values with different letters within a growth parameter are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

Table 2 Effects of 30 min end-of-day (EOD) treatment with red (R) or far-red (FR) on endogenous levels of gibberellins (GAs) (ng g^{-1} DW) in shoot tips of poinsettia (cv. Christmas Eve)

Gibberellins	EOD-R	EOD-FR
GA_{20}	40.9 ± 5.5 a	32.4 ± 6.9 a
GA_{29}	279.8 ± 9.2 b	395.8 ± 39.8 a
GA_1	169.0 ± 10.6 a	234.9 ± 33.7 a
GA_8	281.2 ± 6.1 b	521.6 ± 59.9 a
GA_5	35.5 ± 2.8 a	31.6 ± 7.5 a
GA_6	73.1 ± 3.3 a	77.8 ± 17.7 a
GA_3	200.0 ± 5.4 a	246.2 ± 30.6 a
GA_7	6.9 ± 1.7 a	3.5 ± 1.1 a
GA_{34}	13.7 ± 1.4 a	11.6 ± 3.3 a
Total GA	1099.9 ± 38.6 b	1555.0 ± 159.0 a
GA_8/GA_1	1.7 ± 0.1 a	2.3 ± 0.4 a
GA_{29}/GA_{20}	7.1 ± 1.0 a	13.3 ± 2.9 a
GA_1/GA_{20}	4.2 ± 0.4 b	7.5 ± 0.8 a
GA_6/GA_5	2.1 ± 0.1 b	2.5 ± 0.1 a
GA_3/GA_5	5.7 ± 0.3 a	8.6 ± 1.8 a

Mean values ± SE are given. n=3 with 3 pooled shoot tips in each. Mean values with different letters within a parameter are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

Table 3 Effect of 30 min end-of-day (EOD) treatment with red (R) or far-red (FR) on endogenous levels of cytokinins (ng g^{-1} DW) in shoot tips of poinsettia (cv. Christmas Eve)

Hormone Compounds	Christmas Eve	
	EOD-R	EOD-FR
cis-Zeatin-O-glucoside (c-ZOG)	8.0 ± 0 a	8.0 ± 0 a
trans-zeatin (t-Z)	3.0	3.0
trans-zeatin riboside (t-ZR)	16.0 ±1 a	25.0 ± 8 a
cis-zeatin riboside (c-ZR)	2.0 ±0 a	3.0 ±1 a
Isopentenyladenine (iP)	2.0 ± 0 a	3.0 ± 0 a
Isopentenyladenine adenosine (iPA)	47.0 ± 3 a	60.0 ±14 a
Total cytokinin (c-ZOG+t-Z+t-ZR+c-ZR+2iP+iPA)	75.0 ± 6.1 a	95.7 ± 20.9 a
Active cytokinin (t-Z+t-ZR+2iP)	18.1 ± 1.9 a	28.1 ± 8.8 a
Inactive cytokinin (c-ZOG+c-ZR+iPA)	53.7 ± 4.5 a	67.6 ± 12.1 a

Mean value ± SE are given, n=3 with 3 pooled shoot tips in each (except, n= 1 in t-Z and n=2 in c-ZOG). Mean values with different letters within a parameter are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

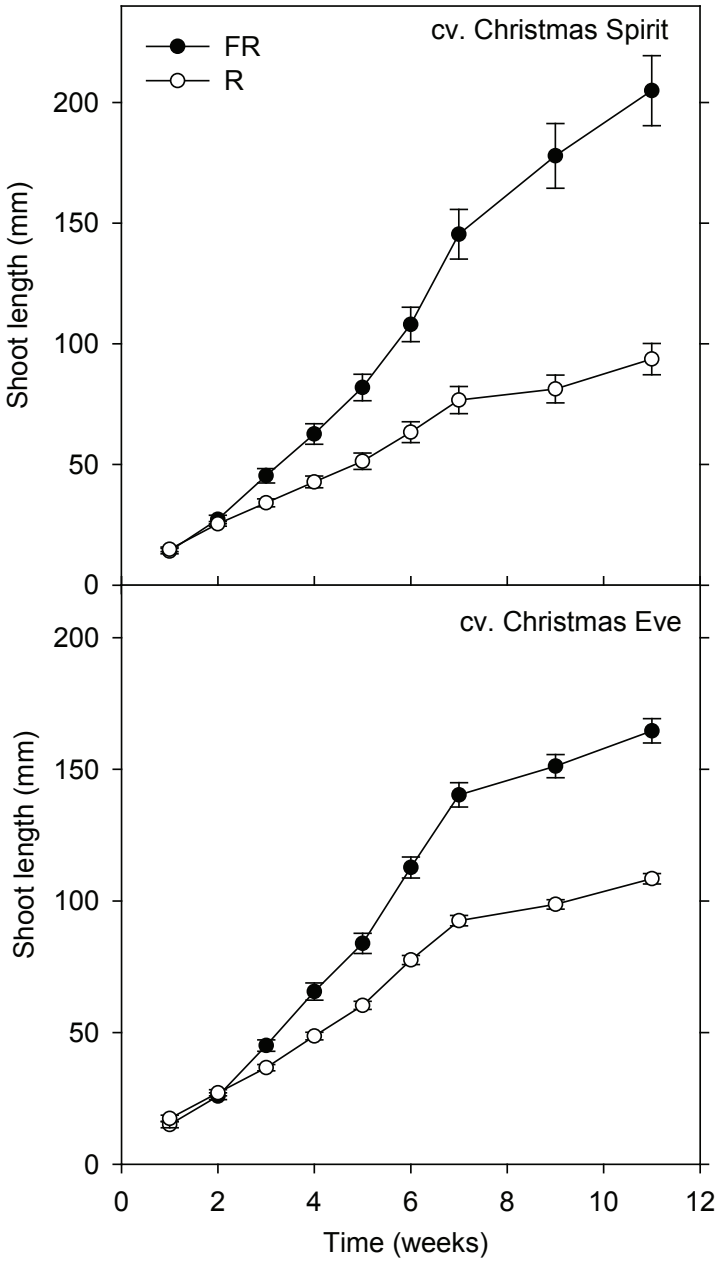

Fig. 1 Effects of 30 min end of day (EOD) treatment with red (R) or far-red (FR) light on shoot length of two poinsettia cultivars grown under a 10 h photoperiod. Mean values ± SE are given. n=18-21

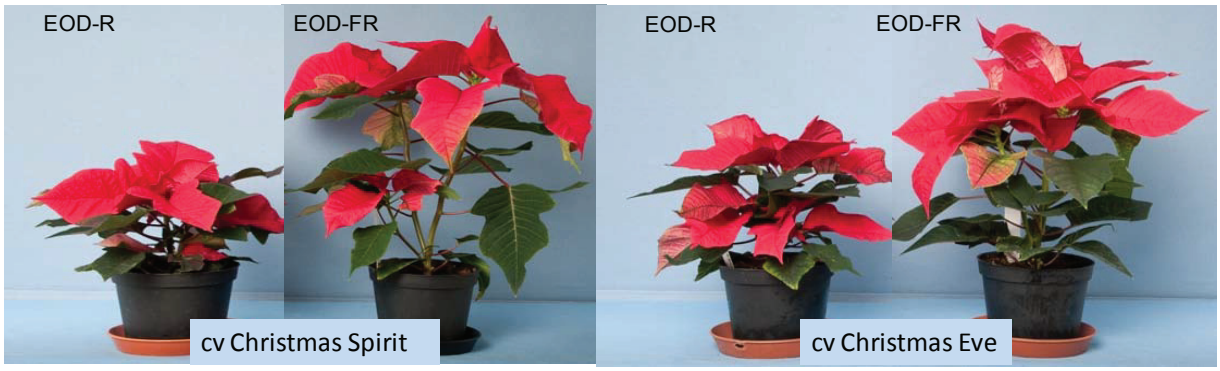

Fig. 2 Effects of 30 min end of day (EOD) treatment with red (R) or far red light (FR) light on morphology of two poinsettia cultivars grown under a 10 h photoperiod

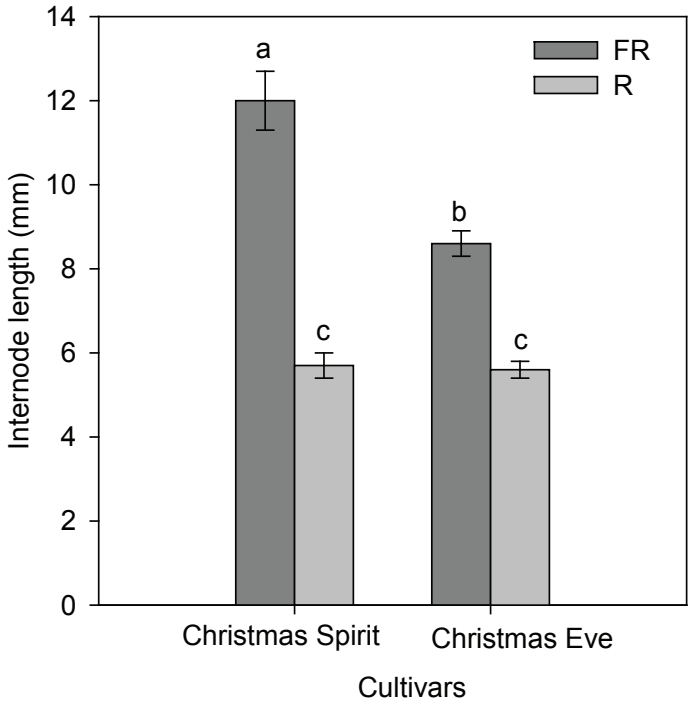

Fig. 3 Effects of a 30 min end of day (EOD) treatment with red (R) or far-red (FR) light on internode lengths of two poinsettia cultivars grown under a 10 h photoperiod. Mean values ± SE are given. n=18-21. Mean values with different letters are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

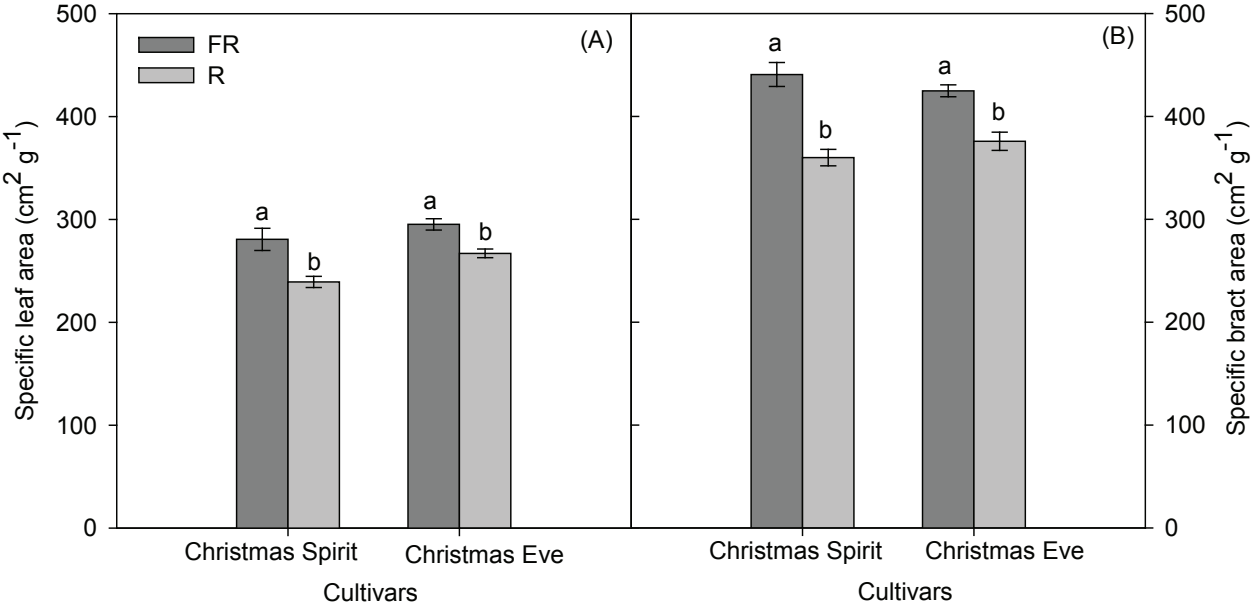

Fig. 4 (A) Specific leaf area (SLA) and (B) Specific bract area (SBA) of two poinsettia cultivars exposed to 30 min red (R) or far-red (FR) at the end of the day (EOD) (10 h main lighting period). SLA and SBA were calculated from area divided by the dry weight. Mean values ± SE are given. n=18-21. Mean values with different letters within subfigures are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

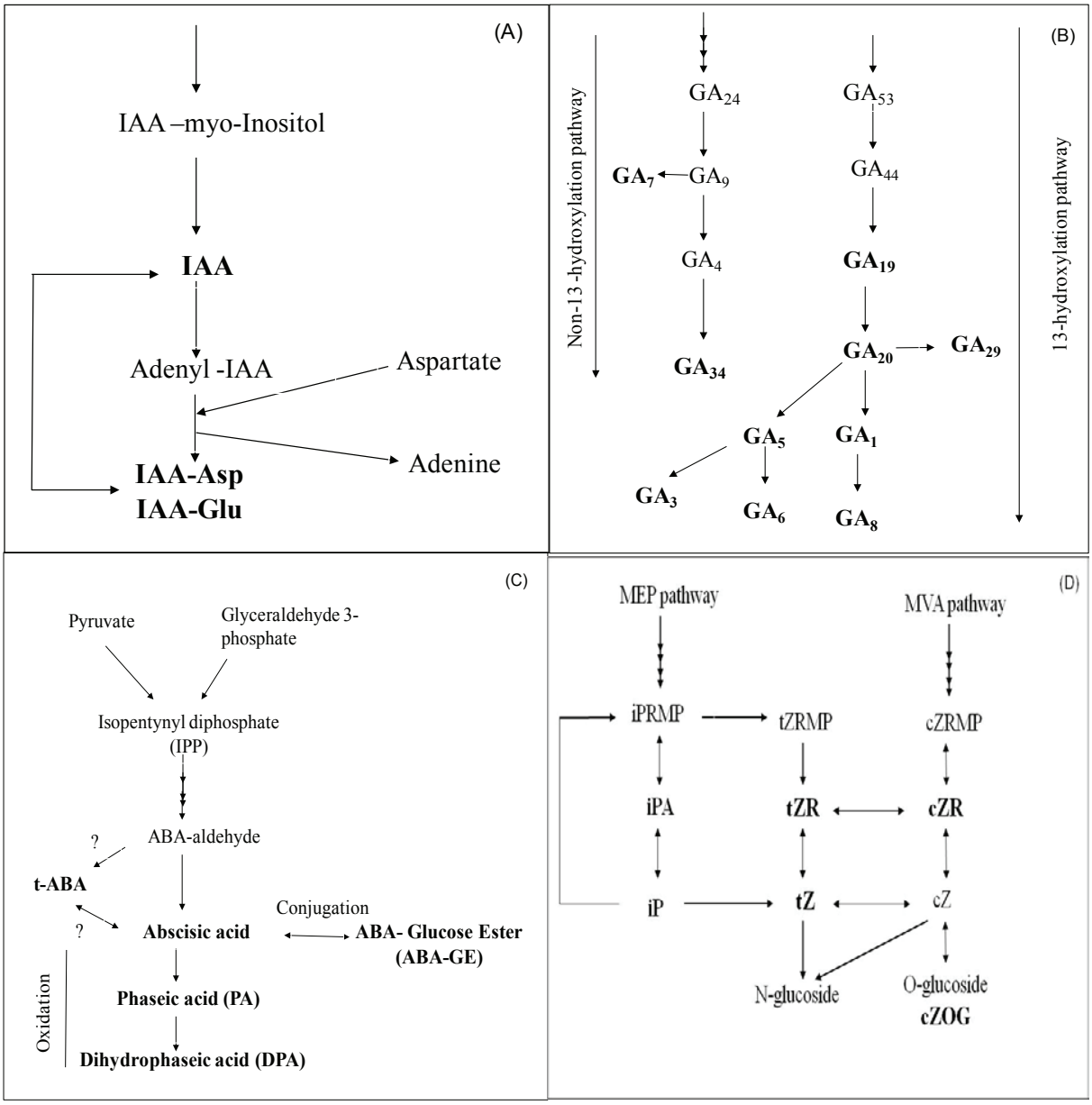

Fig. 5 Simplified biosynthesis and inactivation pathways of (A) the auxin indole-3-acetic acid (IAA), (B) gibberellin (GA), (C) abscisic acid (ABA) and (D) cytokinin (CKs). Bold letters indicate compounds detected in shoot tips of poinsettia (cv Christmas Eve). IAA-glu: IAA-glutamate, IAA-Asp: IAA-aspartate, MEP: methylerythritol phosphate, MVA: mevalonate, iPRMP: isopentenyladenine riboside 5`-monophosphate, tZRMP: *trans*-zeatin riboside 5`-monophosphate, cZRMP: *cis*-zeatin riboside 5`-monophosphate, c-ZOG: *cis*-Zeatin-O-glucoside, t-Z: *trans*-zeatin, t-ZR: *trans*-zeatin riboside, c-ZR: *cis*-zeatin riboside, iP: isopentenyladenine, iPA: isopentenyl adenosine

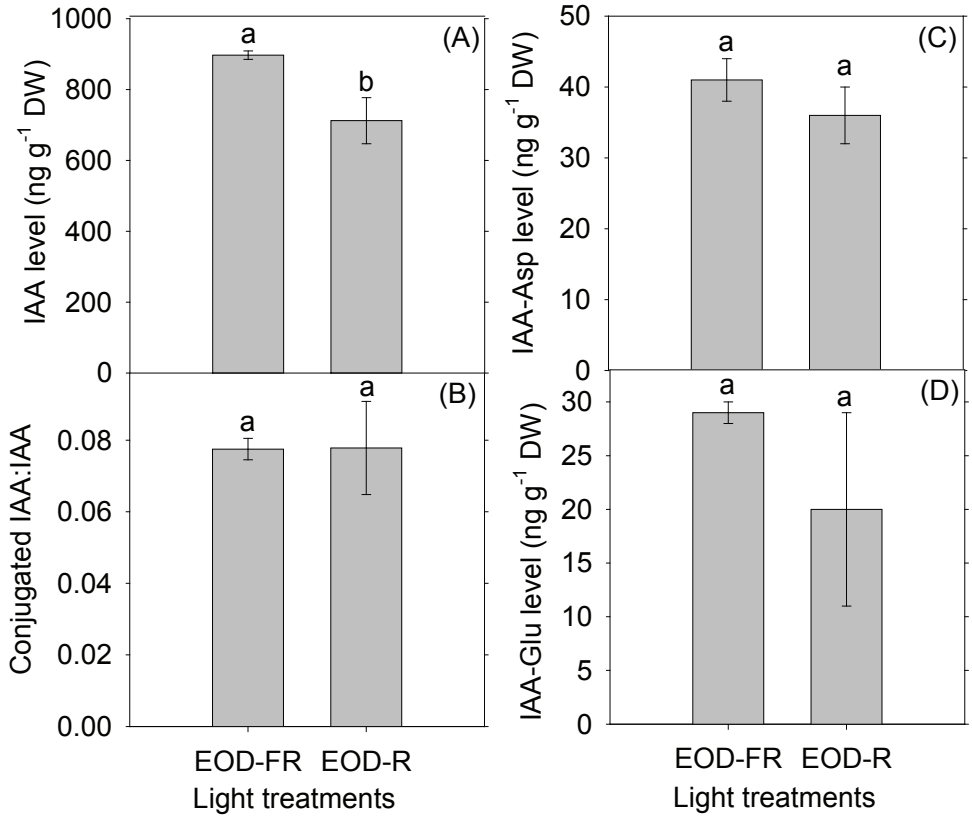

Fig. 6 (A) Endogenous level of IAA, (B) ratio of conjugated IAA [(IAA-Aspartate (IAA-Asp) + IAA-Glutamate (IAA-Glu)] and IAA, (C) IAA-Asp and (D) IAA-Glu in shoots tips of poinsettia (cv. Christmas Eve) exposed to 30 min red (R) or far-red (FR) at the end of the day (EOD) (10 h main lighting period). Mean values ± SE are given. n=3 with 3 pooled shoot tips in each. Mean values with different letters within subfigures are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$

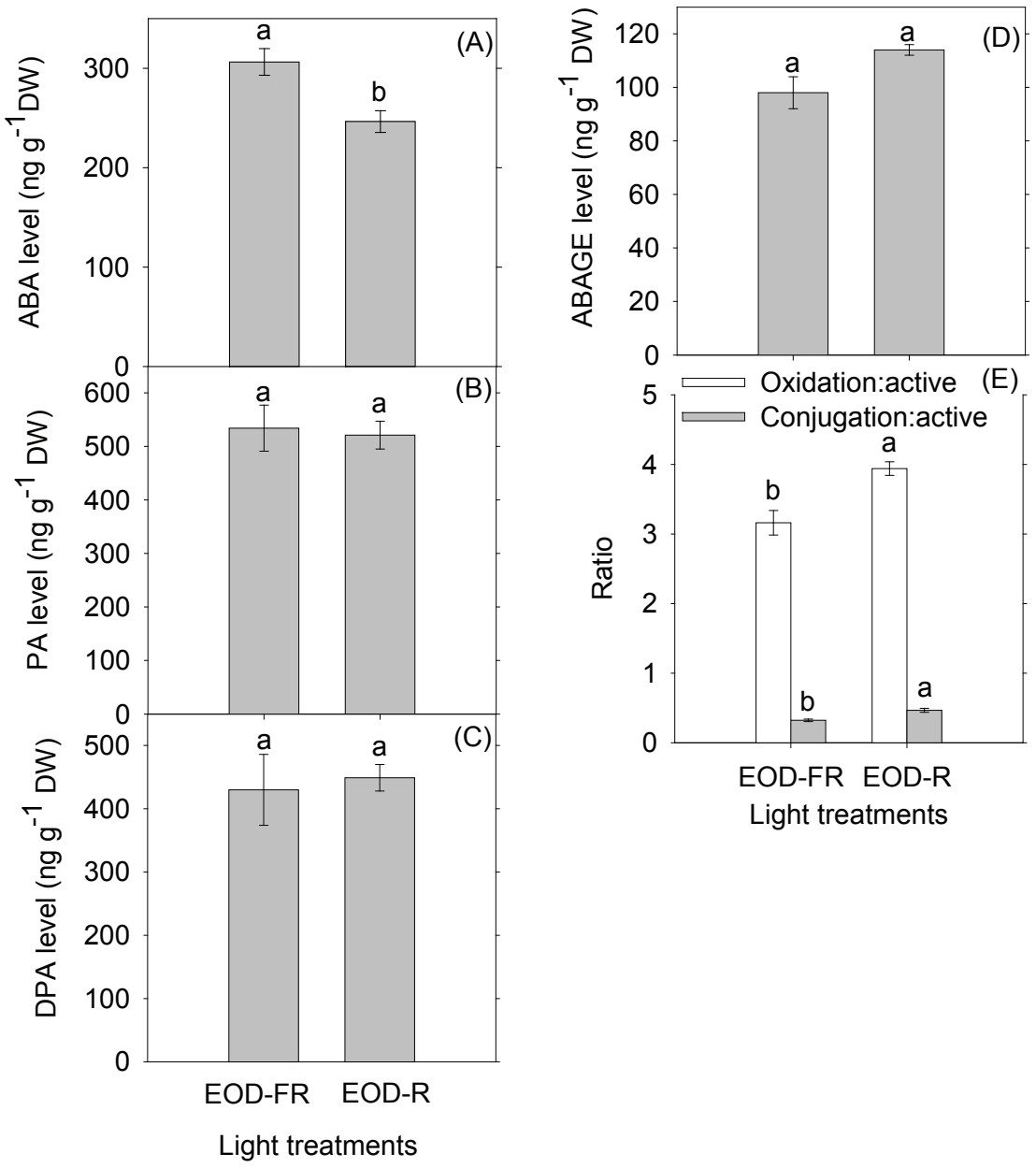

Fig. 7 (A) Endogenous levels of ABA (ABA + *trans*-ABA), (B) phaseic acid (PA), (C) dihydrophaseic acid (DPA), (D) ABA glucose ester (ABA-GE), and (E) ratio of oxidation products of ABA (PA+DPA) to ABA and ratio of conjugated ABA (ABA-GE) to ABA in shoots tips of poinsettia (cv. Christmas Eve) exposed to 30 min red (R) and far-red (FR) light at the end of the day (EOD) (10 h main lighting period). Mean values ± SE are given. n=3 with 3 pooled shoot tips in each. Mean values with different letters within subfigures are significantly different based on ANOVA followed by Tukey's test at $p \leq 0.05$