THE UNIVERSITY OF CALGARY

Red/Far-Red light mediated stem elongation response and regulation of anthocyanin biosynthesis in alpine and prairie ecotypes of *Stellaria longipes*

by

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Abstract

In order to further our understanding of phenotypic plasticity, I studied some of the molecular aspects of differences between alpine and prairie ecotypes of *Stellaria longipes* in stem elongation and accumulation of anthocyanin under varied R/FR ratios. Both the ecotypes responded to the quality of light. Prairie plants were more responsive, low R/FR produced a significant increase in stem elongation, leaf area and decrease in anthocyanin accumulation as compared to alpine ecotype. Photoregulation of Phenylalanine ammonia-lyase (PAL) and Chalcone synthase (CHS) in the accumulation of anthocyanin was also compared. The results show that light is required for the induction of PAL and CHS and accumulation of anthocyanin. The subtle variations in the duration and magnitude of mRNA expression and significant differences in PAL and CHS activity between the alpine and prairie ecotype may account partially for variation in the degree of anthocyanin accumulation between these two ecotypes.

Acknowledgements

I would like to take this opportunity to thank all of those people who helped me in making my research work possible and very exciting. I express my indebtedness to Dr. C. C. Chinnappa for taking me in as a graduate student and forever gratefulness for his continuous encouragement and support throughout my MSc. program. I would like to thank Dr. David Reid for his excellent co-supervision of my research project and ideas (though I hated the idea of assignments in the beginning, I must say that it helped me immensly in gaining background knowledge). A very special thanks goes to Dr. Peter Facchini for his great advice, valuable suggestions and kind gift of coumaroyl-CoA ligase clone. I thank Dr. Jim Mukherjee for his well wishes and lending me his text books whenever I needed them.

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With greatest gratitude and love I dedicate this thesis to:

my parents for their support and encouragement through out my academic pursuits

and

my husband, Giri for his continuous and unconditional love and emotional support

Table of Contents

List of Figures

7B	prairie ecotype of *S.longipes* collected from the Chain Lakes Kananaskis Country, AB
1D	alpine ecotype of *S.longipes* collected from the top of Plateau Mountain, Kananaskis Country, AB
CHS	chalcone synthase
dNTP	deoxyribonucleotide
EDTA	ethylene diamine tetra-acetic acid
EODFR	end of day far-red light treatment
LDW	long day warm
LMP	low melting point
MOPS	3-morpholinopropane sulfonic acid
PAL	phenylalanine ammonia lyase
PAR	photosynthetically active radiation
PCR	polymerase chain reaction
Pfr	far-red light absorbing form of phytochrome
Pfr / P (φ)	phytochromephotoequilibrium
Pr	red light absorbing form of phytochrome
PhyA	phytochromeA
PhyB	phytochromeB
R/FR	red / far-red light ratio
RT – PCR	reverse transcription-polymerase chain reaction

SDC	short day cold
SDS	sodium dodecyl sulfate
SPS	sucrose phosphate synthase
SSC	saline sodium citrate

Chapter one

General Introduction

Phenotypic plasticity:

Phenotypic plasticity is the ability of a single genotype to exist in different morphological and physiological states in response to the varying environmental conditions (West-Eberhard, 1989). This adaptive feature is particularly important in plants to transcend their sessile life style and be able to survive the variable ambient conditions in an opportunistic manner. Adaptive phenotypic plasticity may often lead to natural selection of a species in a given population, thereby contributing to an evolutionary change (Bradshaw, 1965; Schlichting, 1986; Smith, 1990; Schmid, 1992). However the degree of plasticity varies from one genotype to another and environmentally induced phenotypic variation is not always directly related to the fitness of an individual (Scheiner and Goodnight, 1984; Schlichting and Levin, 1986). The evolution of phenotypic plasticity relies on the ability to minimize any deleterious effects of the environment and maximize any advantageous effects (Bradshaw, 1965). Maximal fitness also involves interactions between physiological and morphological plasticities. Physiological plasticity may include subtle changes in gene transcription, mRNA translation or post-translational processing of proteins that often lead to obvious morphological changes. Due to the difficulties involved in measuring physiological changes, most of the information on phenotypic plasticity is obtained from morphological studies. Morphological and physiological plasticity together can be related to ecological strategies (Sultan, 1987). The most common examples of physiological and morphological plasticity include (i) variation in the length of the internodes depending

upon the densities, some plants have longer stems in the shade than in the sun (ii) allocation of more biomass is made to roots in nutrient poor than in nutrient rich soil (iii) variation of leaf shape in amphibic plants, for example, *Ploygonum amphibium* produces erect shoots out of water but floating shoots and leaves in water (Schmid, 1992).

Although phenotypic plasticity is a property of specific traits in response to environmental cues, the pattern and degree of plastic response can evolve independently. This property of phenotypic plasticity is very important in the selection of crop plants that are more responsive to withstand hostile environments, for better yields (Schlichting, 1986). For a complete analysis of phenotypic plasticity as a selective force in plant evolution, integration of various disciplines like ecology, physiology, developmental morphology, genetics, evolution and molecular biology is required. Although some aspects of the evolutionary and ecological significance of phenotypic plasticity are starting to be understood, the molecular mechanisms of this phenomenon remain relatively unclear. In this study an attempt has been made to understand the phenotypic plasticity in *Stellaria longipes* using physiological, molecular and biochemical approaches.

Phenotypic plasticity in *Stellaria longipes*:

Stellaria longipes is a herbaceous weed belonging to the family Caryophyllaceae, with circumpolar distribution (Chinnappa and Morton, 1976). It is an ideal system in which to study phenotypic plasticity, as it is a very successful species in diverse habitats, such as sand dunes, alpine and the prairie habitats (Macdonald and Chinnappa, 1989). The various phenotypic plastic traits that help *Stellaria longipes* to grow in different habitats include stem elongation, leaf shape and inflorescence development (Chinnappa

and Morton, 1984). Among the various populations that colonized divergent habitats, alpine (1D) and prairie (7B) ecotypes have been extensively used in comparative studies (Macdonald, 1988; Emery, 1994; Zhang and Chinnappa, 1994; Kathiresan, 1997; Chuong, 1998; Tatra, 1999). Even though both of these ecotypes are tetraploid (2n = 52) the degree of plasticity between them varied greatly. For instance when these ecotypes were grown under short day (8 h photoperiod) and cold (8° C day/5° C night) conditions (SDC), both alpine and prairie ecotypes showed short stems and ovate leaves. When transferred to long day (16 h photoperiod) and warm (22° C day/18° C night) conditions (LDW) both the ecotypes showed elongated stems and leaves. However, the phenotypic plasticity trait for stem and leaf elongation under LDW conditions is greater in prairie ecotype as compared to alpine ecotype. Also the amount of phenotypic plasticity varied among populations of *Stellaria* from different habitats (Macdonald et al. 1988). This variation in the morphological plasticity can be easily explained when the natural environmental conditions of these two habitats are taken into account. The alpine ecotypes are naturally dwarf to withstand the wind stress in their mountain habitat and the prairie ecotypes on the other hand grow in a habitat with intense competition for light from the neighbouring plants, where greater stem elongation plasticity provides them with an adaptive advantage.

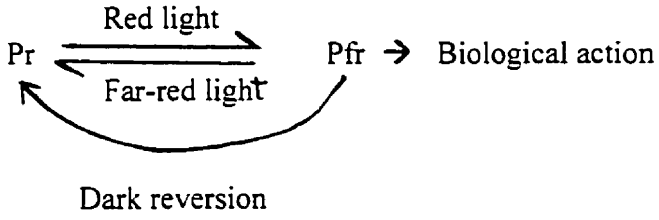
Studies on the physiological aspects of phenotypic plasticity showed that ethylene plays an important role in governing the variation in stem elongation plasticity between alpine and prairie ecotypes. Both the ecotypes exhibit diurnal rhythmicity in ethylene production and the amplitude of the rhythm is slightly smaller in alpine ecotype than in prairie ecotype (Emery et al., 1994; Kathiresan et al, 1996). Also the differential

regulation of 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) gene may contribute to the phenotypic plastic responses in the two ecotypes (Kathiresan et al., 1998). Anatomical studies showed that stem elongation occurs through both cell division and cell elongation, while the epidermal cells regulate the rate of stem elongation (Chuong, 1998). It has also been suggested that phenylalanine ammonia lyase (PAL) mediated deposition of phenolic compounds like lignin and suberin may have a possible role in the regulation of stem elongation plasticity in *Stellaria* under varied temperature and photoperiod treatments Chuong, (1998). Recent studies on the levels of DNA methylation showed that the levels of the genomic methylation in alpine and prairie ecotypes in response to shaded environment are different which may be responsible for the differential stem elongation in these ecotypes (Tatra et al., 2000). Despite the extensive physiological, biochemical, anatomical and molecular studies on stem elongation plasticity in *Stellaria longipes*, the cellular mechanisms for sensing the environmental signals and mediating the appropriate phenotypic response remain elusive.

Role of Phytochromes in sensing R/FR ratio:

Depending upon the type of environmental signal, the nature and degree of phenotypic responses may vary in plants. The various environmental cues that mediate phenotypic plasticity in plants include temperature, photoperiod, light, wind and humidity. Both the quality and quantity of light particularly play a significant role in stem elongation (Morgan and Smith, 1979). Plants perceive the quality of light and photoperiod through photoreceptors or photosensors known as phytochromes. Phytochrome is a chromic-bili protein that absorbs principally in the red/far-red spectral regions (600-800 nm) and exists in two forms, Pr (red light, R-absorbing) and Pfr (far-red

light, FR-absorbing). The Pr and Pfr forms are mutually inter-convertible by appropriate irradiation and Pfr is considered to be the physiologically active form of phytochrome.



Dark reversion

The spectral properties of Pr are distinct from those of Pfr, reflecting the structural differences in their respective chromophores (Furuya, 1994). The phytochrome apoproteins (Phy) are a family of 120 – 130 kD soluble proteins that result from the expression of diverse phytochrome genes (PHY). Based on their physiological and physiochemical properties the various Phy genes are broadly classified into Type I and Type II (Furuya, 1989). Type I or 'etiolated tissue' phytochromes (PhyA) are regarded as being light labile and Type II or 'green tissue' phytochromes (PhyB, PhyC) are light stable.

Phytochromes have an important function in regulating plant growth and development in response to signals perceived from the natural light environment. The ratio of red light (R) to far-red (FR), R/FR varies remarkably in different environments. The R/FR light ratio is considerably lower in habitats where the plants are shaded by neighbouring vegetation (Morgan and Smith, 1976). The adaptive responses of plants to shade by other vegetation are viewed in terms of two extreme strategies, shade intolerance (avoidance) and shade tolerance (Smith, 1995). The shade intolerant plants unlike the shade tolerant plants, were found to show dramatic shade avoidance responses

like stem elongation, suppressed branching and accelerated transition to flowering when grown in competing vegetation or reduced R/FR light ratio.

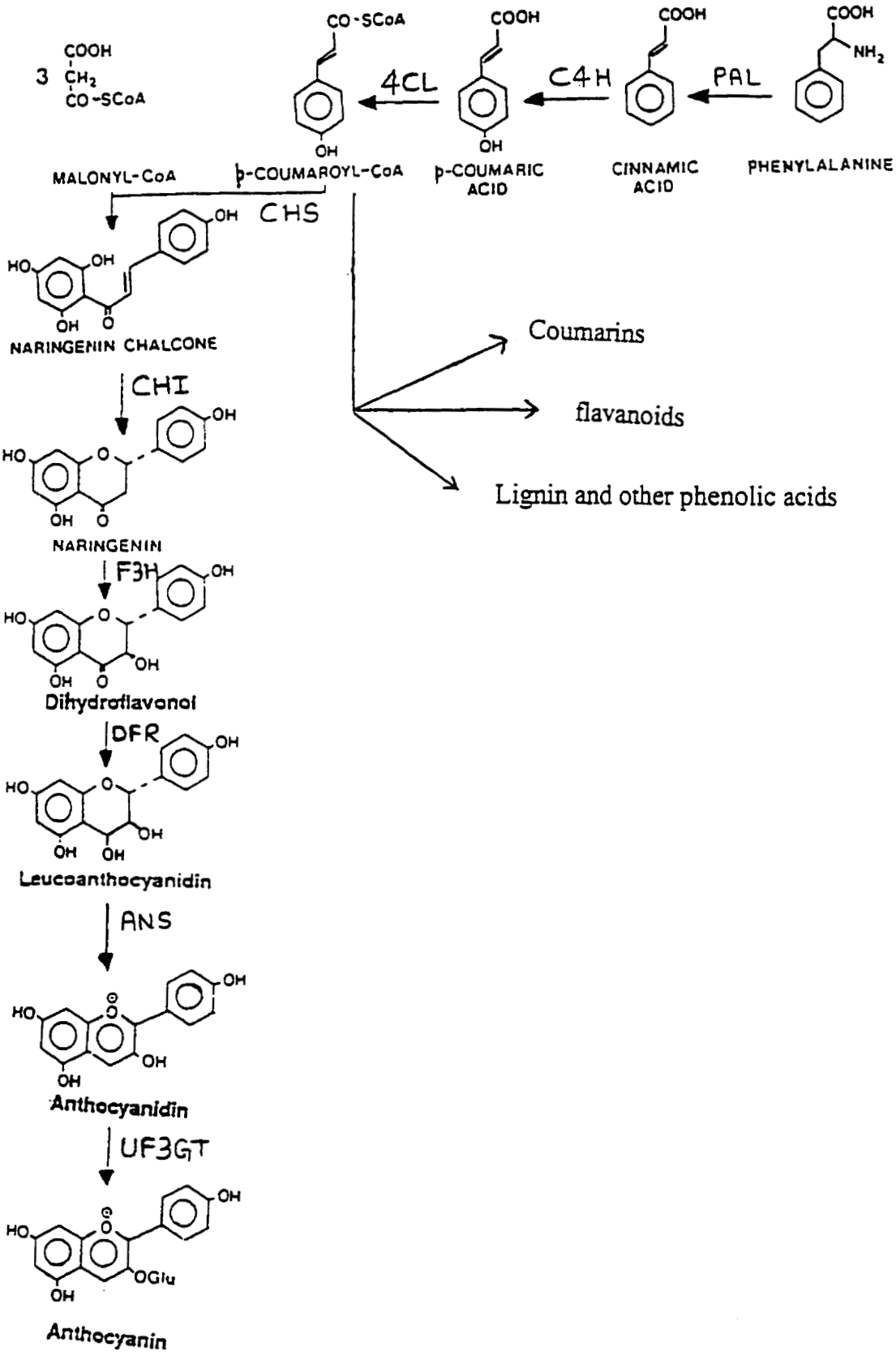
Anthocyanin biosynthesis and photoregulation:

In addition to the stem elongation response under varied R/FR light, variation in the accumulation of anthocyanin levels was studied in some plant species and mutants (Kerckhoffs et al., 1992; Yanovsky et al., 1998). Anthocyanins belong to the general class of phenolic compounds known as flavonoids. Flavonoids have many important functions in plants. For examples, they may have a significant role in structural support, as filtering agents against UV light (Hahlbrock and Scheel, 1989), as physiological inhibitors of auxin transport (Ozeki et al., 1990), as precursors for the pigments in flowers (Jacobs and Rubery, 1988) and as signal molecules for the activation of nodulation genes of nitrogen-fixing *Rhizobium* (Peters et al., 1986). Besides the developmental control by growth regulators like auxin and cytokinins (Ozeki et al., 1990), flavanoid biosynthesis is also controlled by several environmental parameters such as light (Hrazdina and Creasy 1979), temperature (Rabino and Mancinelli, 1986), fungal elicitors (Dixon, 1983), microbial pathogens and wounding (Tanaka et al., 1989). Most plant systems need light as an essential prerequisite for flavonoid synthesis in both vegetative and reproductive tissues (Duell-Pfaff and Wellman, 1982; Mancinelli, 1984), with few exceptions such as oat primary leaves which can produce flavonoids in the dark (Margna and Laanest, 1984). Studies on photoregulation of anthocyanin biosynthesis have shown the interrelationship between photoreceptor activation and accumulation of anthocyanin (Mancinelli, 1985; 1990; Mancinelli et al., 1991). Although earlier studies were focussed mostly on the involvement of phytochrome (Siegelman and Hendricks,

1957; Downs and Seigelman, 1963; Drumm and Mohr, 1974), recent studies have shown evidence for the involvement of both phytochrome and UV-A/blue-light-photoreceptor, often referred to as cryptochrome (Duell-Pfaff and Wellmann, 1982; Oelmuller and Mohr., 1985; Sponga, et al., 1986; Hashimoto et al., 1991). Various photomorphogenic mutants of tomato *au, hp, auhp* along with the wild type have been used to show the role of phytochromes in the photoregulation of anthocyanin synthesis (Adamse et al 1989). The *au* mutant seedlings which are deficient in phytochrome showed rapid cessation of anthocyanin synthesis unlike the *hp* seedlings which have same level of phytochrome as in WT, showed 8 - 10 fold accumulation of anthocyanin (Peter et al 1989). The HY4 mutant of *Arabidopsis thaliana* which are defective to encode CRY1 protein with characteristics of a blue-light photoreceptor show a possible correlation between blue-light-mediated inhibition of hypocotyl elongation and increased levels of anthocyanin accumulaton. (Ahmad et al., 1995).

The anthocyanin biosynthetic pathway in plants is complex. This is summarized in Fig. 1.1. The early steps of the pathway involve deamination of phenylalanine by the enzyme phenylalanine ammonia lyase (PAL, EC 4.3.1.5) to cinnamic acid, which upon a series of reactions produces 4-coumaroyl-CoA. The first reaction specific to flavonoid biosynthesis is the condensation of three molecules of malonyl-CoA and one molecule of p-coumaroyl-CoA by the enzyme chalcone synthase (CHS, EC 2.3.1.74) to produce naringenin chalcone. This step is frequently considered to be the rate-limiting step for this pathway (Lewis et al, 1998). Naringenin chalcone is finally converted to the coloured anthocyanin through a series of steps involved in the biosynthetic pathway. Of all the enzymes involved in this pathway, the photoregulation of PAL and CHS activity and the

Figure 1.1: A simplified pathway for anthocyanin biosynthesis. PAL = Phenyl alanine ammonia lyase, 4CL = 4-Coumarate CoA ligase, C4H = Cinnamic aid 4-hydroxylase, CHS = Chalcone synthase, CHI = Chalcone isomerase, F3H = Flavonone 3-hydroxylase, DFR = Dihydroflavanol 4-reductase, ANS = Anthocyanin synthase and UF3GT = UDP glucose: flavanoid 3-O-glucosyl transferase. (Adapted from Dixon and Steele, 1999).


3
COOH
CH2
CO-SCoA
CO-SCoA
COOH
COOH
COOH
NH2
4CL
C4H
PAL
OH
OH
MALONYL-CoA
p-COUMAROYL-CoA
p-COUMARIC ACID
CINNAMIC ACID
PHENYLALANINE
CHS
NARINGENIN CHALCONE
Coumarins
flavanoids
Lignin and other phenolic acids
CHI
NARINGENIN
F3H
Dihydroflavonol
DFR
Leucoanthocyanidin
ANS
Anthocyanidin
UF3GT
OGlu
Anthocyanin

expression of their respective genes have been extensively studied (Beggs et al 1987; Hahlbrock and Scheel 1989; Ehmann et al 1991). The accumulation of CHS mRNA is shown to be under the control of all three photoreceptors, UV-B receptors, blue-light receptors and phytochrome (Bruns et al., 1986; Ohl et al., 1989, Frohnmeyer et al., 1992). It has also been reported that there is a correlation between anthocyanin synthesis and the transcript levels and activity of PAL and CHS in response to UV-light and/or red light in maize (Taylor and Briggs, 1990), *Sinapsis alba* (Brodenfeldt and Mohr, 1988) and tomato (Kerckhoffs and Kendrick, 1997).

Specific objectives of the project:

The goal of the present project was to study R/FR induced differential photomorphogenic responses like shade avoidance stem elongation and anthocyanin accumulation in the alpine and prairie ecotypes of *Stellaria longipes*. Also an attempt was made to gain some insights into the phytochrome mediated regulation of anthocyanin biosynthesis. In order to achieve this goal specific objectives were designed:

to study the physiological responses like stem elongation, leaf size and anthocyanin accumulation in alpine and prairie ecotypes under varied R/FR (chapter 2);

to clone a cDNA encoding phytochromeB and study the mRNA expression of PhyB in alpine and prairie ecotypes under varied R/FR (chapter 3);

to study the enzyme activity and mRNA expression of PAL in alpine and prairie ecotypes under varied R/FR (chapter 4); and

to clone a cDNA encoding chalcone synthase, study the enzyme activity and mRNA expression of CHS in alpine and prairie ecotypes under varied R/FR (chapter 5).

Chapter two

Physiological responses of alpine and prairie ecotypes of *Stellaria longipes* under varied R/FR light

Introduction

Light is one of the most complex and variable environmental factors to which a growing plant is exposed. It is not only a source of energy for the plant but also an information medium to synchronize development with seasonal changes, to allow for appropriate responses that have adaptive advantages over the environmental perturbances (Smith and Whitelam, 1990). Plant form is greatly controlled by the quality, quantity, direction and periodicity of light within the environment, which is generally termed photomorphogenesis (Kendrick and Kronenberg, 1994). The perception of light quantity, photosynthetically active radiation (PAR) involves photon counting while the perception of light quality involves the estimation of ratio of photons in two or more photosynthetically active wavelength bands, such as Red to Far Red photon ratio (R/FR) (Smith, 1994). Higher plants effectively utilize only visible light (400-700 nm) for photosynthesis and most of the FR (700-800 nm) is either transmitted or reflected. Thus the spectral quality (R/FR) and the quantity of light (PAR) varies greatly depending on the location. For example in a canopy, there is a greater depletion in the R due to vegetational shade. Phytochromes as photoreceptors can detect the presence of neighbouring vegetation through the perception of the FR reflection signals and regulate morphological and physiological changes in the plants, in response to the signals perceived (Smith, 1982). The ratio of red to far red light (R/FR) is a parameter of the light environment, which is directly related to the spectral properties of phytochrome.

Morgan and Smith (1976) studied the effect of variation of R/FR on the physiology of plant growth, in particular stem elongation. The results of their experiments showed that when simulated natural radiation was used to vary the FR content, the higher the FR content, the higher the stem extension rate for shade avoiders. This type of shade avoidance stem elongation response provides the plant an adaptive advantage in competing for light resources by overtopping its neighbours.

In addition to the shade avoidance stem elongation, phytochromes were shown to be correlated with anthocyanin synthesis under differing R/FR treatments (Drumm and Mohr, 1974) and also by using various photomorphogenic mutants (Kerckhoffs et al., 1992). Chinnappa and Morton (1984) have demonstrated the plastic responses of *S.longipes* for stem, leaf shape and inflorescence development under different environmental conditions. When the plants are grown under long day (16 hours photoperiod) and warm (18^0C - 24^0C), LDW conditions, all the plants from prairie habitat elongate their leaves and stems to a significantly greater degree than the plants from alpine region do. Also under the same conditions, all prairie ecotypes produce multiple flowers unlike their alpine counterparts, which produce only single flowers. These observations are similar to shade avoidance responses listed by Smith (1982) and hence the plants from the prairie region with competing vegetation are more plastic and considered to be shade avoiders while the plants from the alpine region with no competing vegetation are dwarf. Thus, *Stellaria* provides an ideal model system for comparative studies on the role of phytochromes in shade avoidance stem elongation and anthocyanin production in plants belonging to two different habitats.

Materials and methods

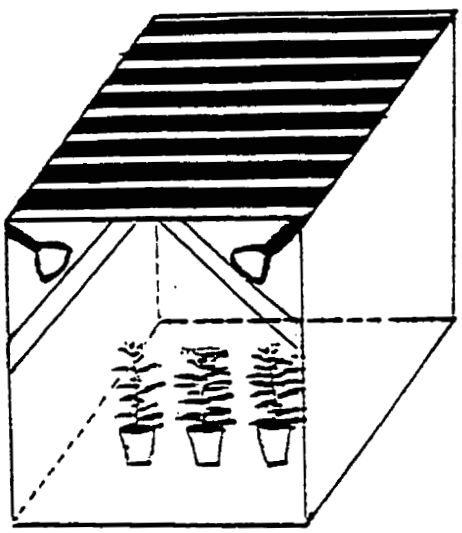
Plant material:

Plants were originally collected from the Chain Lakes area of Southern Alberta (Prairie ecotype) and Plateau Mountain of Southern Alberta (alpine ecotype). Genotypes from alpine habitat at higher elevation (2,453m) are referred to as 1D and from prairie habitat at lower elevation (1,310m) are referred to as 7B. All the plants were potted in 4cm pots containing peat moss, sand and terra green (2:1:1) and were maintained in growth chambers under short day and cold conditions (SDC, with 8 h photoperiod and 8^0C day / 5^0C night) for a minimum of 90 days to simulate winter cycle (Macdonald etal, 1984). Then the plants were transferred directly to appropriate chambers with either high or reduced R/FR ratio to study the shade avoidance stem elongation responses. For some of the experiments involving anthocyanin studies, the plants from SDC were first etiolated for 6 days in a chamber with dark warm conditions (temperature of 22^0C), prior to their transfer to varied R/FR light chambers.

Growth chambers with varied R/FR light:

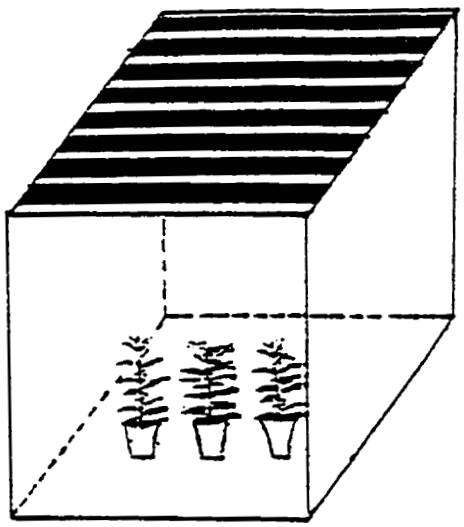
The experimental lay out, to study the response of prairie and alpine ecotypes to varied R/FR light required, two growth chambers, one with a low R/FR of 0.7 and the other with high a R/FR of 1.9 (Fig. 2.1). All other environmental conditions within the chamber were identical, with photosynthetically active radiation (PAR) of 120 µmol m^{-2} s^{-1}, 16h photoperiod, 22^0C day and 18^0C night temperature. Lighting in the chambers with high R/FR was provided by eight white fluorescent tubes and four 60 W bulbs, and in the chamber with low R/FR the white light was supplemented with additional FR light sources. To obtain FR, additional light from the lamps was filtered through 4cm of cooled flowing water and one layer (3 mm) of FRF 700 filter (West Lake plastics, PA). Fluence

Figure 2.1: Experimental layout of the growth chambers to study the effect of varied R/FR light on alpine and prairie ecotypes of *Stellaria longipes*. Growth chamber I has low R/FR, 0.7 and growth chamber II has high R/FR, 1.9 while all other environmental conditions within the chambers are identical with PAR 115 $\pm$ 5 µmol m^{-2} s^{-1}, 16 h photoperiod, 22^{0}C day and 18^{0}C night temperature.



Growth chamber I

Reduced R/FR, 0.7



Growth chamber II

High R/FR, 1.9

rates and the spectral distribution of the light sources were recorded by the cosine corrected remote probe of calibrated LI-1800 Spectroradiometer (LI-COR, Lincoln, NE, USA), probe held horizontally at the level of the shoot apices. The PAR and photon irradiance were measured in the range of 400-800nm and R/FR was calculated as the ratio of fluence rates over 630-670nm and 715-745nm wavelength intervals.

Growth measurements:

Twenty ramets for each type, from two of 1D and two pots of 7B were selected randomly, marked at day zero and transferred into each of the high R/FR light and low R/FR light chamber. The total length of the ramets was measured every two days for 30 days with a ruler to the nearest millimetre from the soil (from an ink mark made where the thread was tied) to the shoot apex. Other parameters such as size of leaf, fresh weight and dry weight of the ramets were also measured. To obtain the size of the leaf, the length (l) and breadth (b) of 10 samples of leaves at the third internode of 1D and 7B plants were measured on day zero and day 30 after their transfer to the respective chambers. The average area and l/b for 10 replicates of the fully developed leaf on day 30 were measured to represent the size and shape of the leaf respectively. The length and breadth were measured with a ruler and the breadth was measured at the centre of the midrib region. The area of the leaf was measured using an area meter. The experiments were repeated at least twice until quantitatively similar results were obtained and the data from one experiments obtained was analysed by appropriate statistical analysis, standard error (SE).

Anthocyanin measurements:

Anthocyanin was extracted for every two or three days from 200mg of elongating

ramets in 1.5ml of acedified methanol (1% HCl v/v), for 24hrs in darkness at 4 ^{0}C with occasional shaking. A partitioning was performed by the addition of 1ml H_2O and 2.4ml chloroform to the extracts and centrifugation for 30 min at 5000 rpm. The absorption of the top phase was determined at 530 nm (λmax for anthocyanin) and 657 nm (peak of absorption for chlorophyll). The values for anthocyanin yield, $A_{530} - 0.25A_{657}$ were expressed as anthocyanin / g fr wt (Mancinelli et al, 1991). Each value for anthocyanin reported in figures is a mean of 4 replicates.

Results

Absorption spectra of light under natural and simulated environments:

To study the physiological and morphological responses of prairie and alpine ecotype under varied R/FR, one chamber with low R/FR (0.7) and another one with high R/FR (1.9) were assembled (Fig. 2.1). The spectral irradiance of light in these two chambers, along with that seen in the natural alpine and prairie habitats is shown in figures 2.2, 2.3, 2.4, 2.5 and 2.6. Absorption spectra in the chambers with low R/FR and high R/FR showed that except for the red (600 – 670 nm) and far-red (715 – 780 nm) light regions, there are no differences in the distribution of light in the other spectral regions, especially in the blue light region. In the chamber with low R/FR the absorption is slightly lower in the red region and much higher in the far-red region as compared to the chamber with high R/FR (Figs. 2.2 & 2.3). The spectral irradiance in the natural alpine and prairie habitats did not show any significant differences through out the visible region (Figs. 2.4 & 2.5). However there is a significant difference between the absorption spectra of unfiltered light and partially filtered light by neighbouring plants in the natural prairie habitat (Figs. 2.5 & 2.6). In the regions where the light is partially

Figure 2.2: Spectral distribution of light in the chamber with reduced R/FR, 0.7, PAR 115 ($\pm$ 5) μmol m^{-2} s^{-1}, 16 h photoperiod, 22^{0}C day and 18^{0}C night temperature.

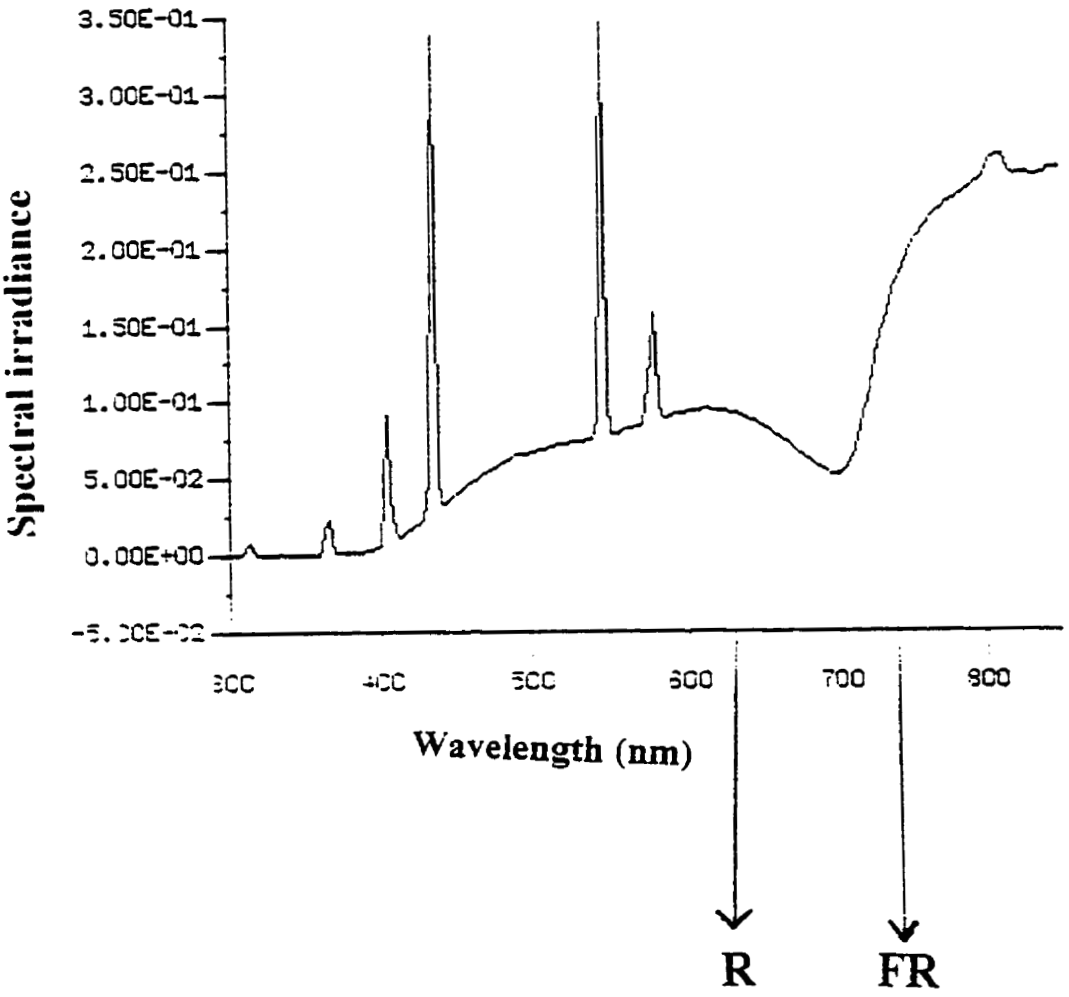
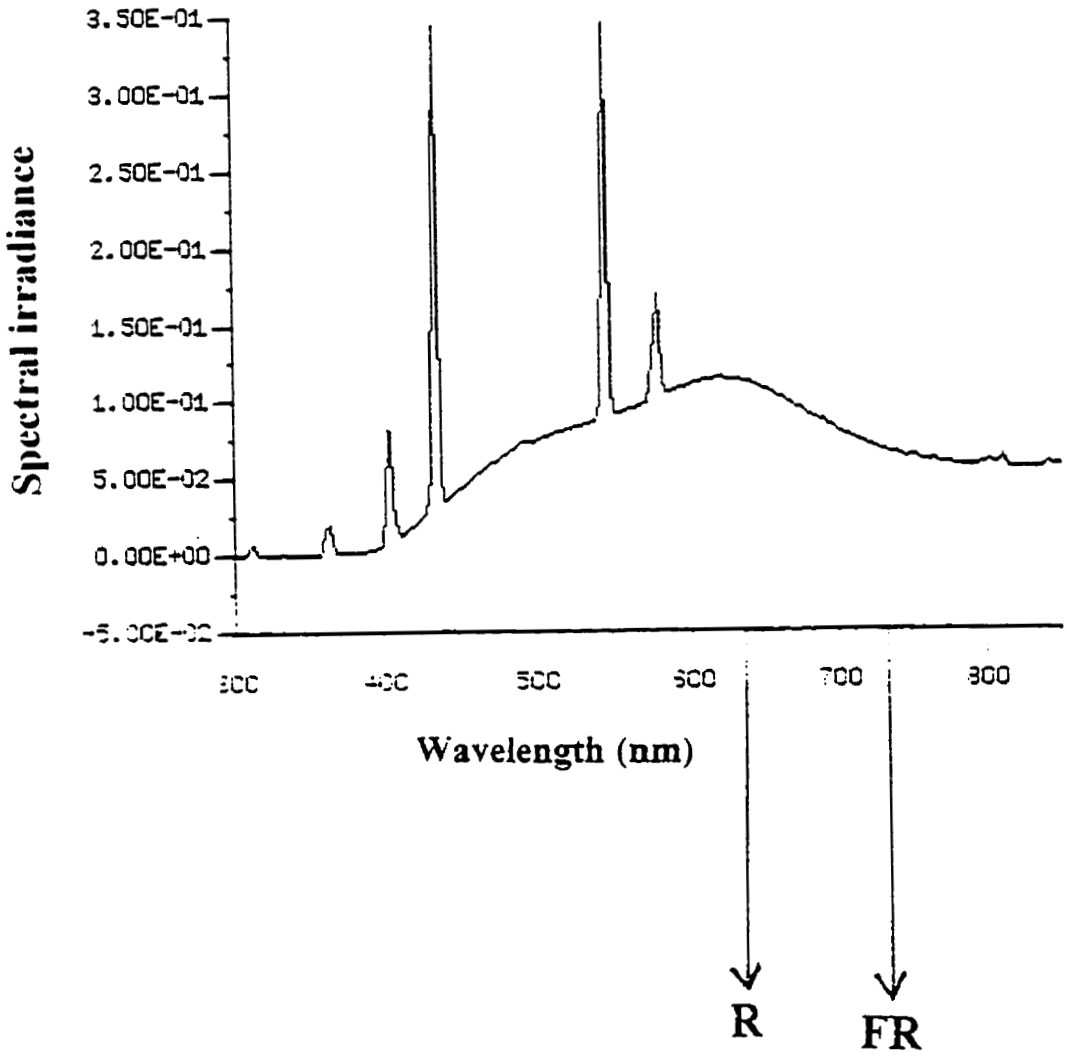

3.50E-01
3.00E-01
2.50E-01
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
Spectral irradiance
Wavelength (nm)
R
FR

Figure 2.3: Spectral distribution of light in the chamber with high R/FR, 1.9 and PAR 115 ($\pm$ 5) μmol $m^{-2} s^{-1}$, 16 h photoperiod, 22^{0}C day and 18^{0}C night temperature.


Spectral irradiance
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3.00E-01
2.50E-01
2.00E-01
1.50E-01
1.00E-01
5.00E-02
0.00E+00
-5.00E-02
300
400
500
600
700
800
Wavelength (nm)
R
FR

Figure 2.4: Spectral distribution of unfiltered light in the natural alpine habitat with R/FR 1.37 and PAR 1500 μmol m^{-2} s^{-1}.

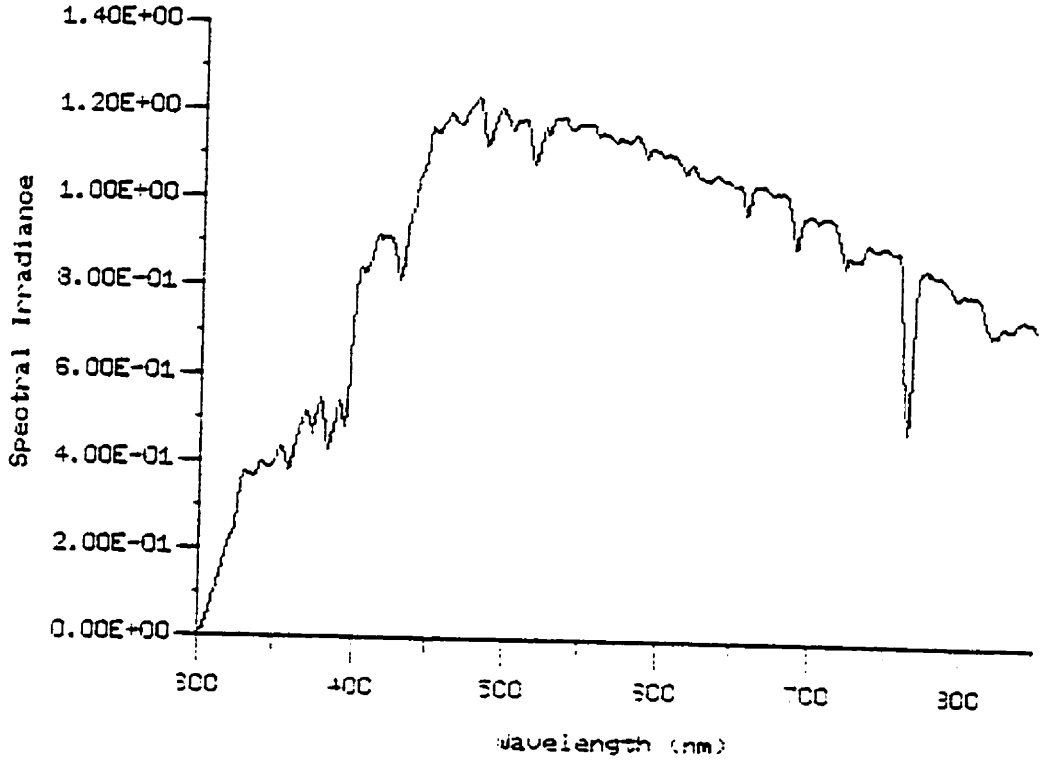

1.40E+00
1.20E+00
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
Spectral Irradiance
300
400
500
600
700
800
Wavelength (nm)

Figure 2.5: Spectral distribution of unfiltered light in the natural prairie habitat with R/FR 1.4 and PAR 141 μmol $m^{-2} s^{-1}$.

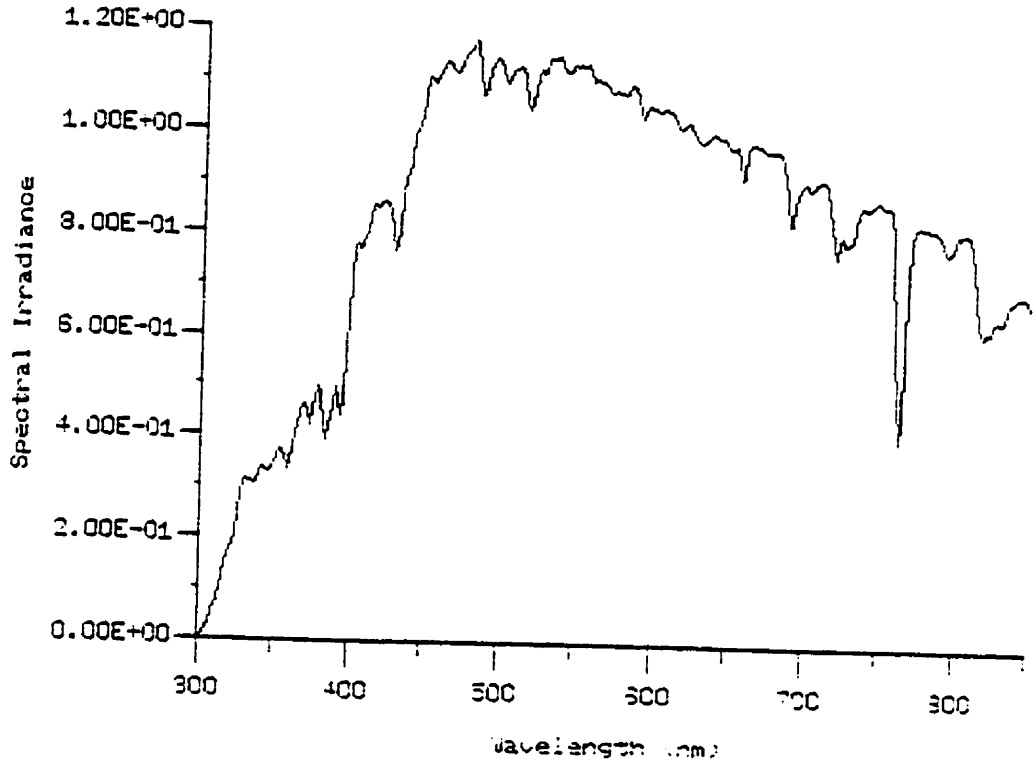
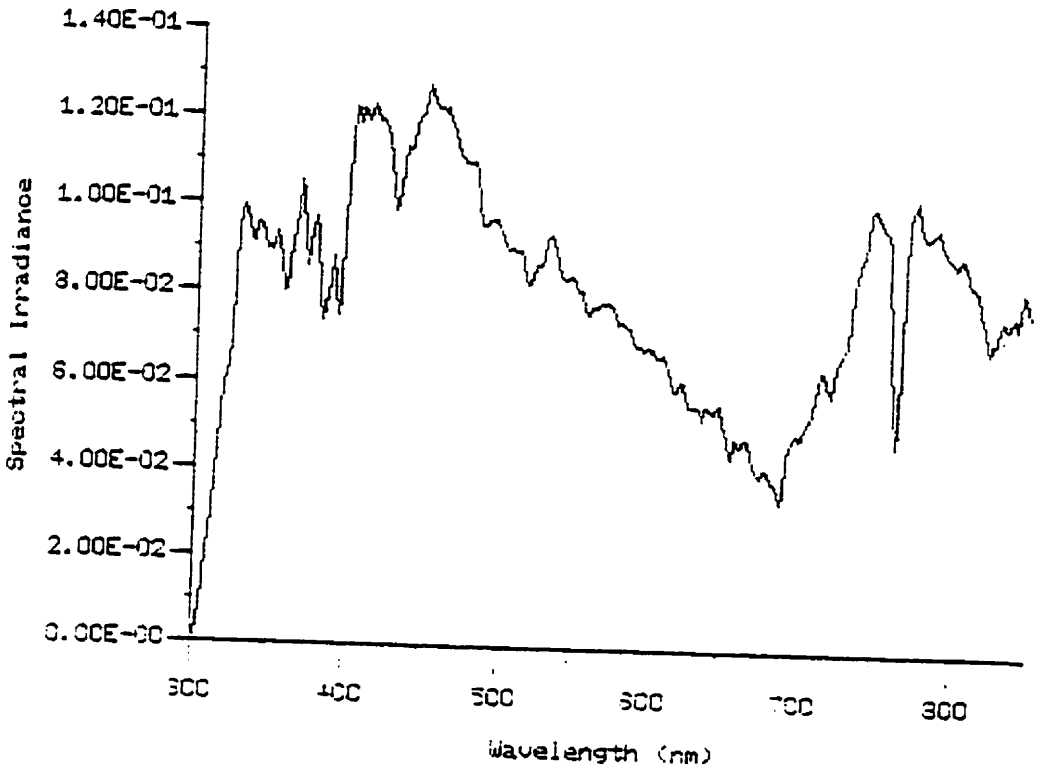

1.20E+00
1.00E+00
8.00E-01
6.00E-01
4.00E-01
2.00E-01
0.00E+00
Spectral Irradiance
300
400
500
600
700
800
Wavelength (nm)

Figure 2.6: Spectral distribution of partially filtered light by neighboring plants in the natural prairie habitat with R/FR 0.7 and PAR 107 μmol m^{-2} s^{-1}.


1.40E-01
1.20E-01
1.00E-01
8.00E-02
6.00E-02
4.00E-02
2.00E-02
0.00E-00
Spectral Irradiance
300
400
500
600
700
800
Wavelength (nm)

filtered by neighbouring plants there is a 3fold decrease in the UV-A region (350 – 390 nm), 10-fold decrease in the blue-light region (455 – 500 nm) and 16.66-fold decrease in the red light region as compared to the unfiltered light.

Stem elongation of alpine and prairie ecotypes under varied R/FR:

When SDC treated plants were transferred to LDW conditions with varied R/FR both alpine and prairie ecotype showed significant differences in the stem elongation. However prairie ecotype showed a contrasting degree of increased stem elongation in response to low R/FR (0.7) than under high R/FR (1.9), as compared to alpine ecotype which showed only slight increase in the stem elongation under the above conditions (Figs. 2.7 & 2.8). Difference in the total height of the ramets under varied R/FR was not distinct until day 10 in 1D unlike in 7B, which showed a marked difference from day 6 (Fig. 2.9). 7B showed stem elongation over an extended period, starting from day 6 until day 30, whereas in 1D the stem elongation was from day 10 until day 22. For both 1D and 7B significant difference in the elongation occurred between third to sixth internodes. The stem elongation response of prairie ecotype under low R/FR light is 22.72% greater than that of alpine ecotype.

Size and shape of the leaf under varied R/FR:

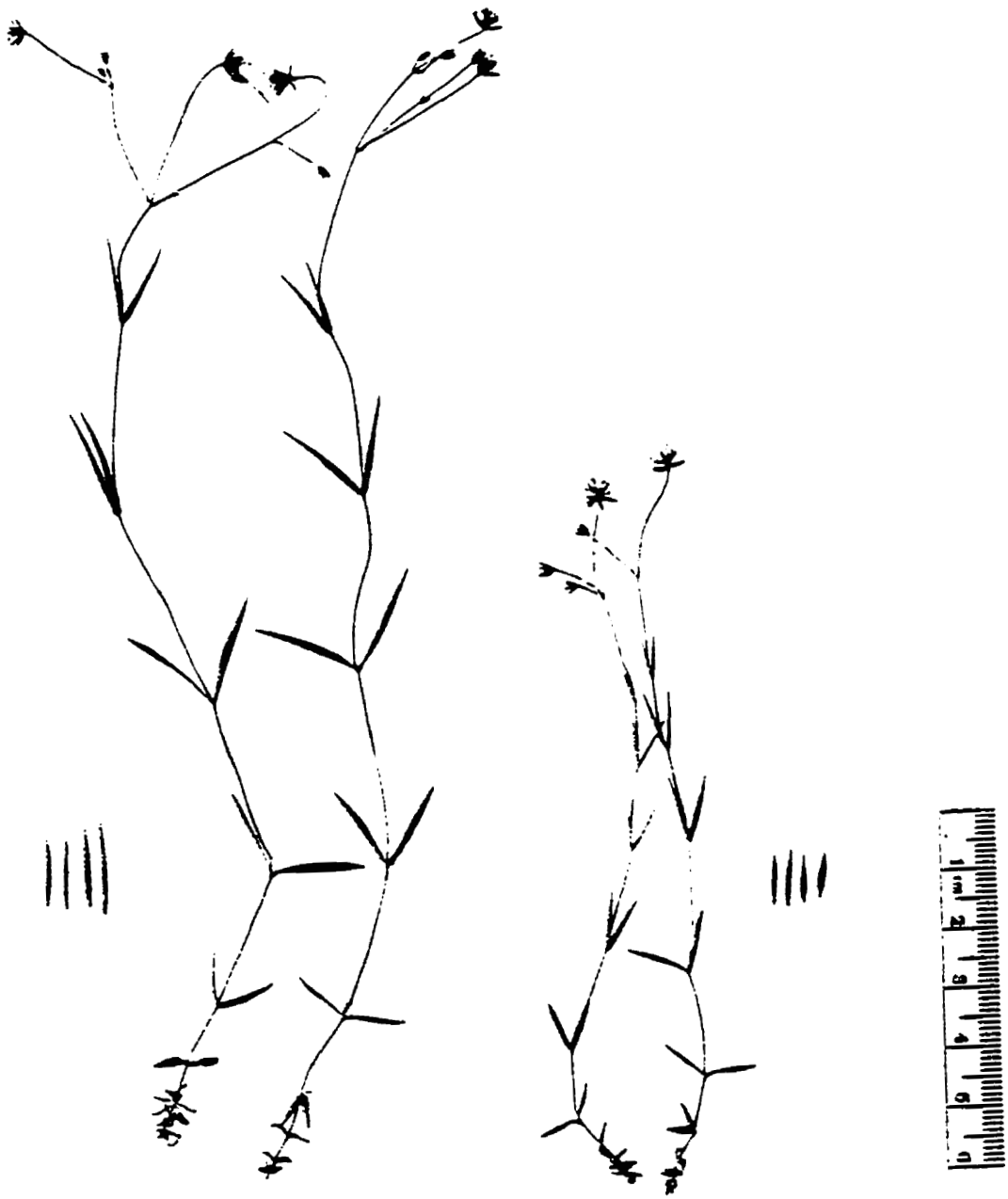
Thirty days after their transfer from SDC-conditions to the chambers with varied R/FR, area and l/b of fully developed leaves at the third node was measured to represent the size and shape of the leaf respectively (Fig. 2.10A and B). For both alpine and prairie ecotypes there was an increase in the length of the leaf under low R/FR compared to the plants under high R/FR. However the leaves of the alpine type were more ovate and those of prairie type were more lanceolate under both the light conditions (Figs. 2.7 & 2.8).

Figure 2.7: Ramets and leaves of alpine ecotype (1D) of *Stellaria longipes* grown under varied R/FR ratio for 22 days.

Reduced R/FR, 0.7

High R/FR, 1.9

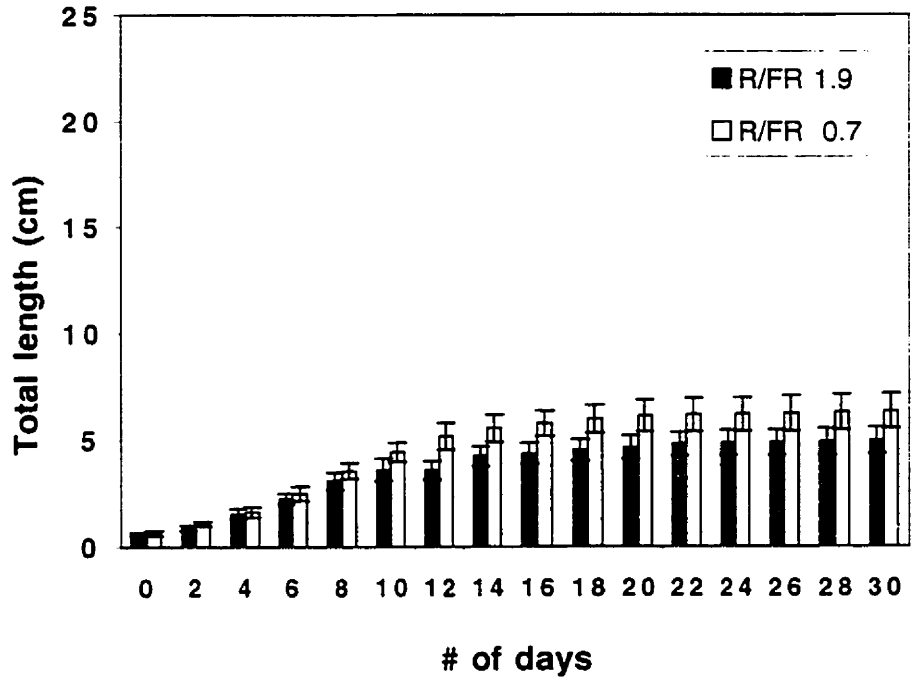
Figure 2.8: Ramets and leaves of prairie ecotype (7B) of *Stellaria longipes* grown under varied R/FR ratio for 22 days.



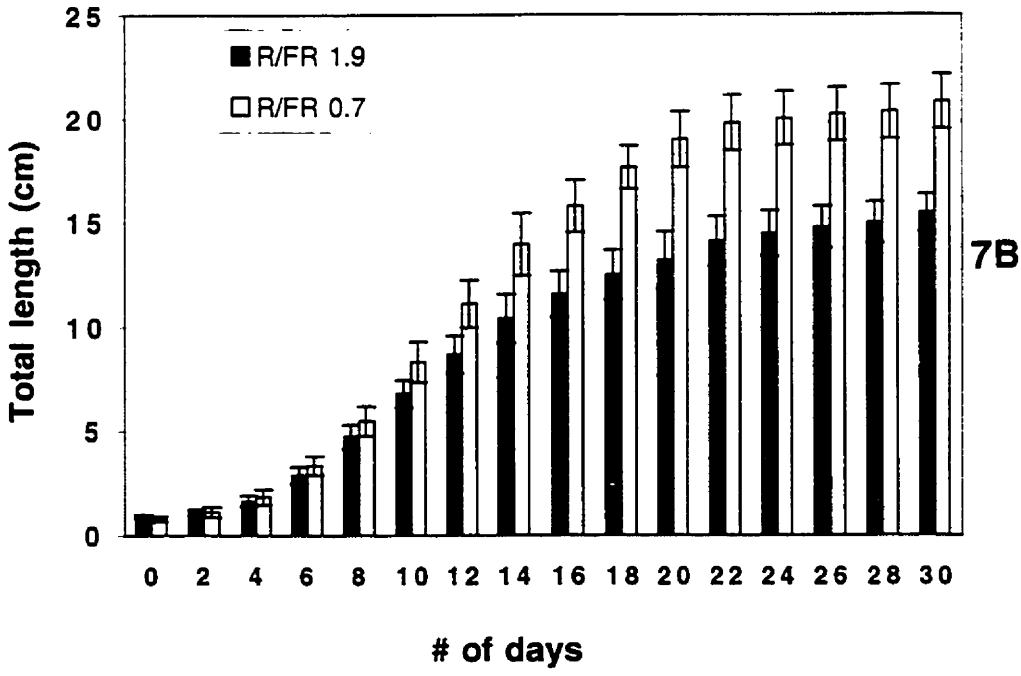
Reduced R/FR, 0.7

High R/FR, 1.9

Figure 2.9: Stem elongation in the alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* upon transfer from SDC to LDW chambers with varied R/FR light. Data are means of at least 20 observed values and the vertical bars indicate ± standard error (SE).


25
20
15
10
5
0
Total length (cm)
R/FR 1.9
R/FR 0.7
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
# of days

1D

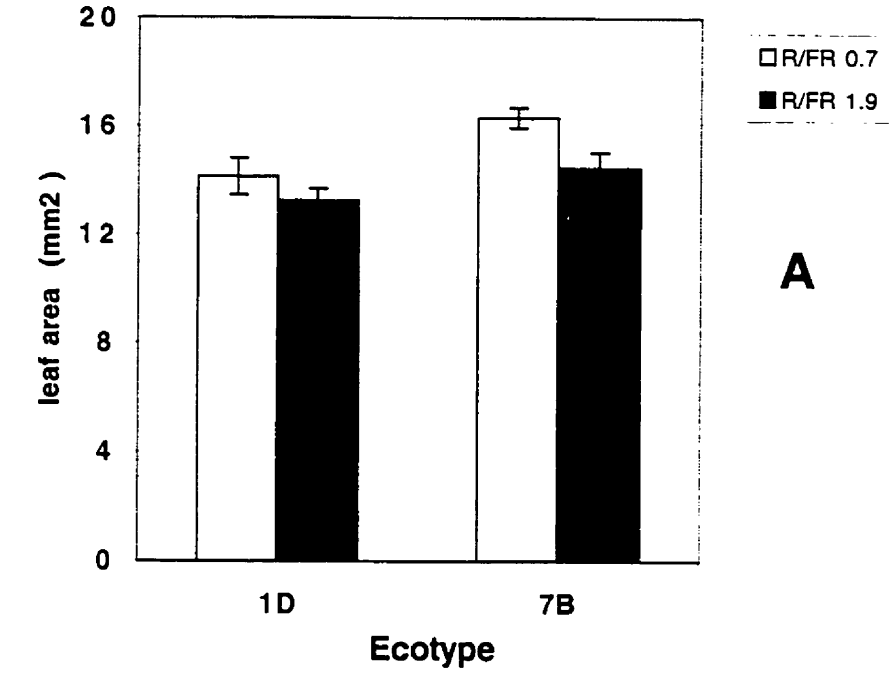

25
20
15
10
5
0
Total length (cm)
R/FR 1.9
R/FR 0.7
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
# of days

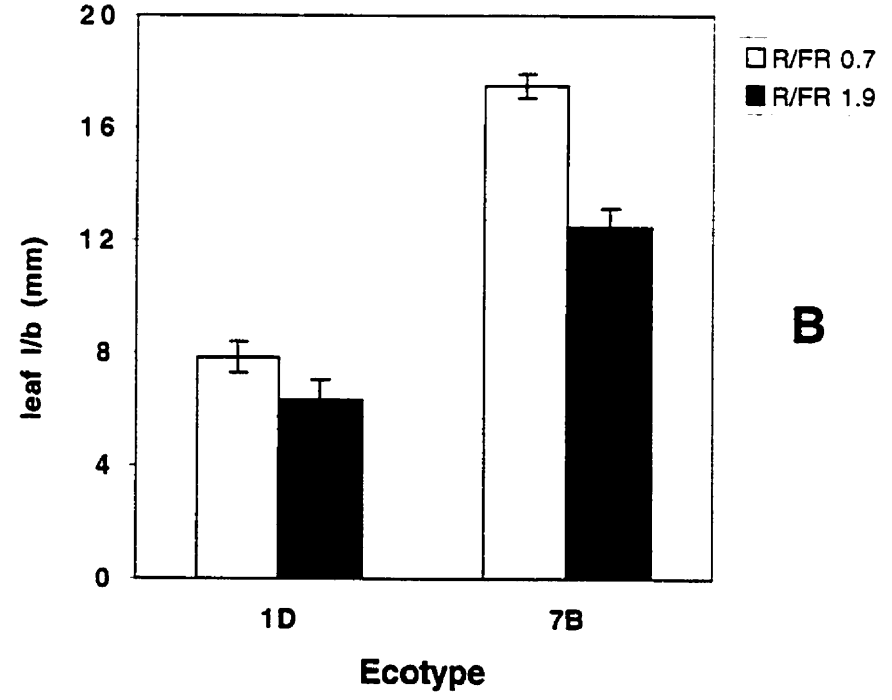
7B

Figure 2.10: Differences in the size and shape of the leaves of alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* grown for 30 days under varied R/FR light.

A: The size of the leaf, represented by leaf area in both the ecotypes.

B: The shape of the leaf, represented by ratio of leaf length to breadth (l/b).


20
16
12
8
4
0
leaf area (mm2)
R/FR 0.7
R/FR 1.9
A
1D
7B
Ecotype


20
16
12
8
4
0
leaf l/b (mm)
R/FR 0.7
R/FR 1.9
B
1D
7B
Ecotype

Under low R/FR conditions the prairie ecotype showed greater increase in the length of their leaves compared to the alpine ecotype. A concomitant variation in the angle of the leaves (from stem axis) was also observed for fully developed 7B leaves under varied R/FR. The 7B leaves opened up more widely when grown in the chamber with low R/FR than under high R/FR. This was not observed for 1D leaves (Figs. 2.7 & 2.8).

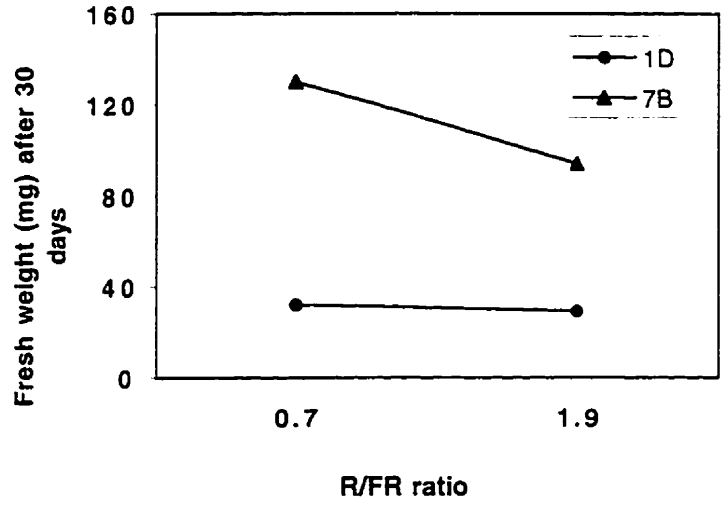
Variation in the biomass of ramets:

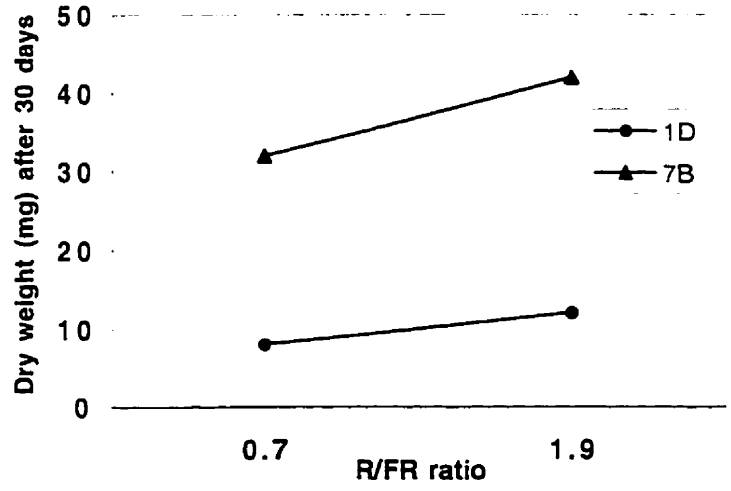
To study the effect of varied R/FR on the biomass of alpine and prairie ecotypes, fresh weight and dry weight of the ramets used for stem elongation measurements were obtained after 30 days. The alpine ecotype did not show much variation in the fresh weight under varied R/FR, unlike the prairie ecotype, which showed greater fresh weight under low R/FR than under high R/FR (Fig. 2.11). However, the dry weight was more under high R/FR than under low R/FR for both alpine and prairie ecotypes.

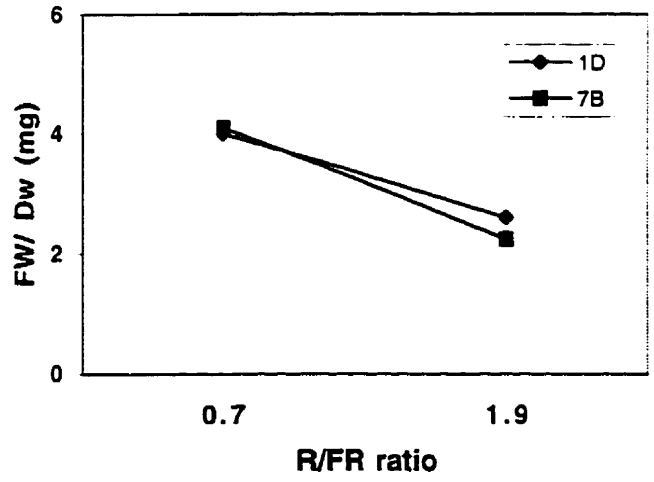
Accumulation pattern of anthocyanin:

When SDC plants were transferred to the chambers with varied R/FR, both alpine and prairie ecotype showed differences in the accumulation of anthocyanin. The anthocyanin that has already accumulated during SDC conditions accounted for the high values in first 6 days in alpine ecotype and first 4 days for prairie ecotype (Fig. 2.12). The prairie ecotype showed a more rapid decrease in the residual SDC anthocyanin under low R/FR as compared to their counterparts under high R/FR unlike the alpine ecotype, which did not show any significant variation under the same conditions. Also the prairie ecotype showed earlier accumulation of anthocyanin under high R/FR from day12 than under low R/FR where the anthocyanin accumulation started from day16. The alpine ecotype showed accumulation of anthocyanin from day16 under both the light conditions. The

Figure 2.11: The effect of varied R/FR on the growth parameters of alpine and prairie ecotypes of *Stellaria longipes*. The fresh weight and dry weight of the ramets grown under varied R/FR for 30 days was measured. Data plotted are means of at least 20 observed values. SE is smaller than the symbol used.


160
120
80
40
0
Fresh weight (mg) after 30 days
1D
7B
0.7
1.9
R/FR ratio


50
40
30
20
10
0
Dry weight (mg) after 30 days
1D
7B
0.7
1.9
R/FR ratio

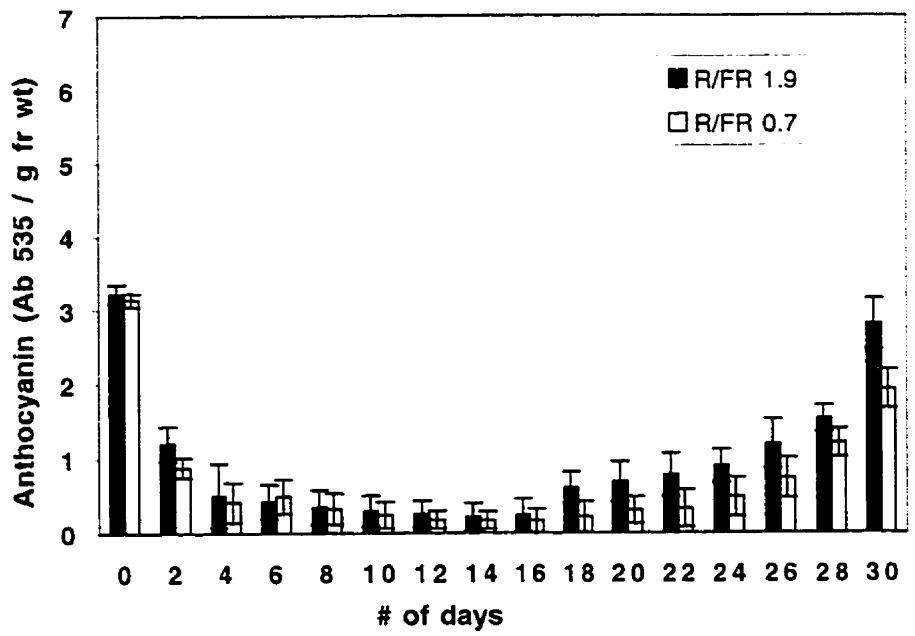

6
4
2
0
FW/ Dw (mg)
1D
7B
0.7
1.9
R/FR ratio

levels of anthocyanin in the alpine ecotype as compared to prairie ecotype were significantly higher under high R/FR and under low R/FR both the ecotypes showed almost the same levels of anthocyanin. High R/FR induced 1.5 and 2.5 fold increase in the levels of anthocyanin for alpine and prairie plants respectively by the end of 30days.

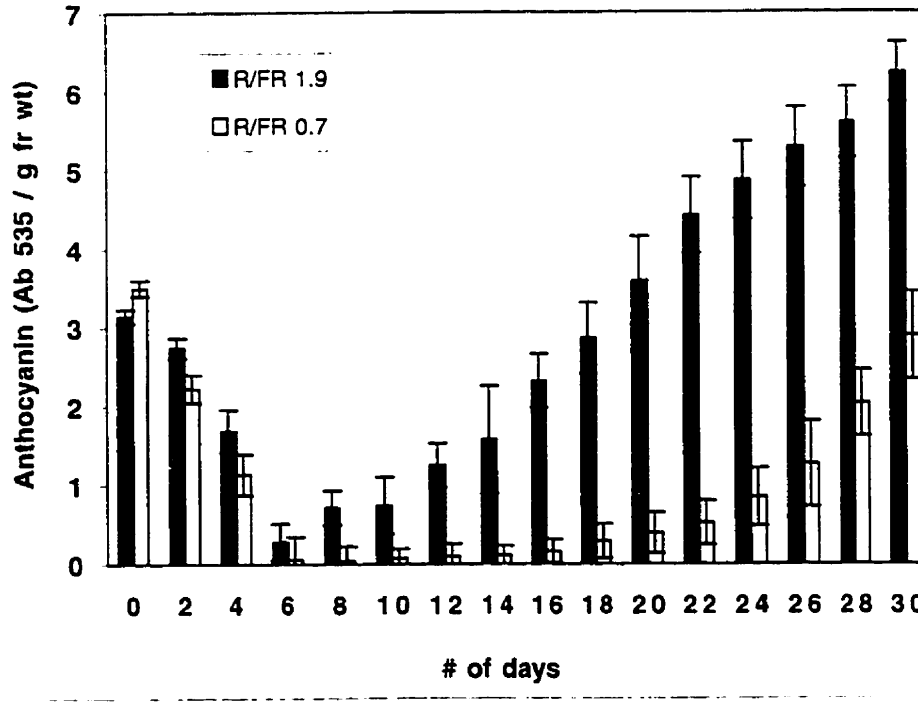
An interesting observation of accumulation of anthocyanin first in the stems and then in the leaves for both the ecotypes was made. The lower internodes showed higher levels of anthocyanin as compared to the upper internodes. The levels of anthocyanin in the leaves and stems of both the ecotypes after grown under varied R/FR conditions for 30 days were compared (Fig. 2.13). Both alpine and prairie ecotypes showed greater levels of anthocyanin in the stems as compared to leaves under low or high R/FR. Also the levels of anthocyanin were minimum in the leaves of both the ecotypes under low R/FR.

The effect of varied R/FR light on the accumulation of anthocyanin in etiolated alpine and prairie ecotypes was also studied. SDC plants from both the ecotypes were kept in dark for 7 days and the etiolated plants with no residual anthocyanin (Fig. 2.14) were transferred to varied R/FR conditions and the anthocyanin levels were measured for every 3days over a period of 30days. Etiolated plants of both the ecotypes showed higher levels of anthocyanin under low as well as high R/FR, as compared to the plants transferred directly from SDC (Fig. 2.15). Etiolated 1D and 7B plants under both low and high R/FR started to accumulate anthocyanin from day 9 and attained high levels from day 21 to day 30. From day 12 the etiolated 7B plants showed 3.5fold increase in the levels of anthocyanin than the etiolated 7B plants under low R/FR unlike the etiolated 1D plants, which showed only 1.5fold increase under the same conditions.

Figure 2.12: Amount of anthocyanin produced in the elongating stems of alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* upon transfer from SDC (0) to LDW chambers with varied R/FR. Data are means of at least three individual experiments and the vertical bars indicate ± standard error (SE).

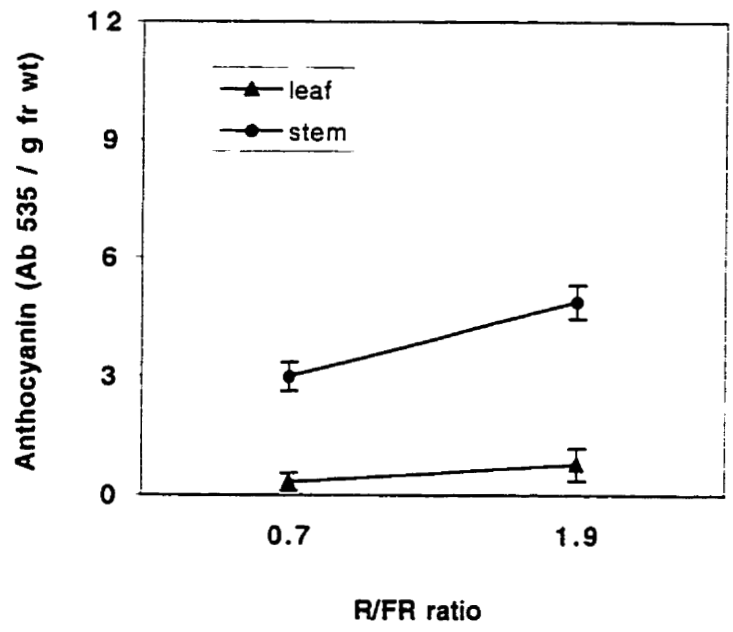

Anthocyanin (Ab 535 / g fr wt)
7
6
5
4
3
2
1
0
R/FR 1.9
R/FR 0.7
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
# of days

1D

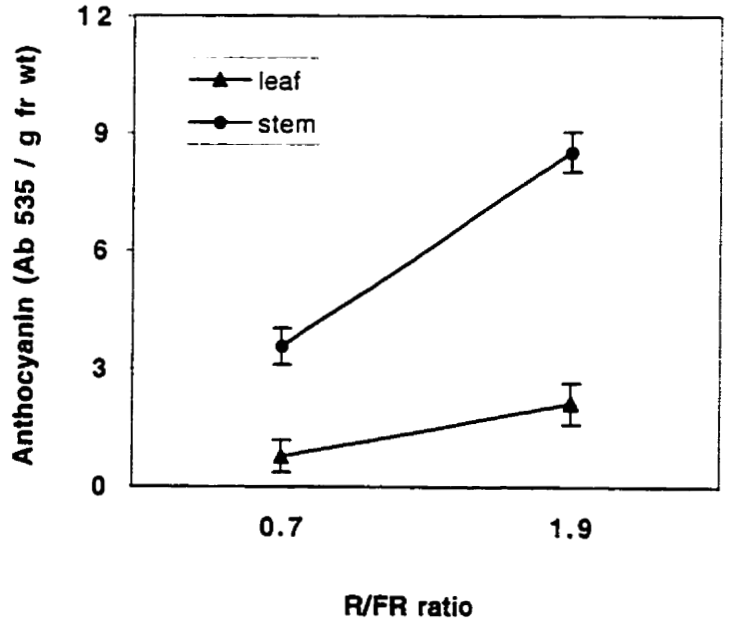

Anthocyanin (Ab 535 / g fr wt)
7
6
5
4
3
2
1
0
R/FR 1.9
R/FR 0.7
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
# of days

7B

Figure 2.13: Amount of anthocyanin in the stems and leaves of alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes*, after grown under varied R/FR for 30 days. Data are means of at least three individual experiments and the vertical bars indicate ± standard error (SE).

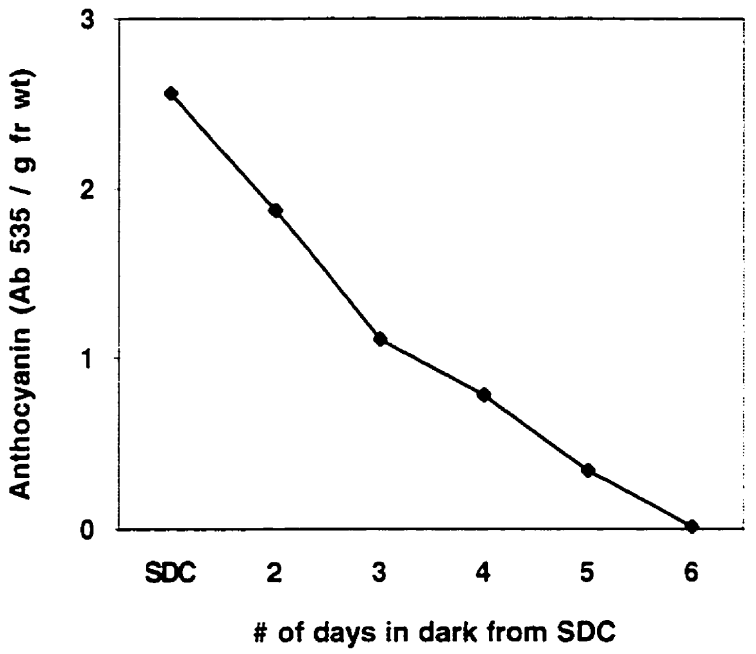

12
9
6
3
0
leaf
stem
Anthocyanin (Ab 535 / g fr wt)
0.7
1.9
R/FR ratio

1D

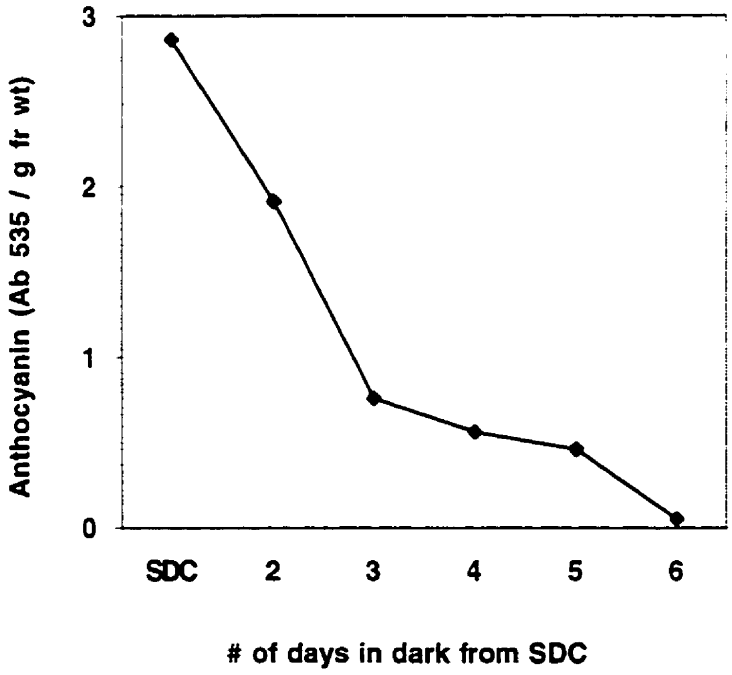

12
9
6
3
0
leaf
stem
Anthocyanin (Ab 535 / g fr wt)
0.7
1.9
R/FR ratio

7B

Figure 2.14: Decreasing levels of anthocyanin in alpine (1D) and prairie (7B) ecotypes during etiolation of the SDC plants for 7days before their transfer to the chambers with varied R/FR light.

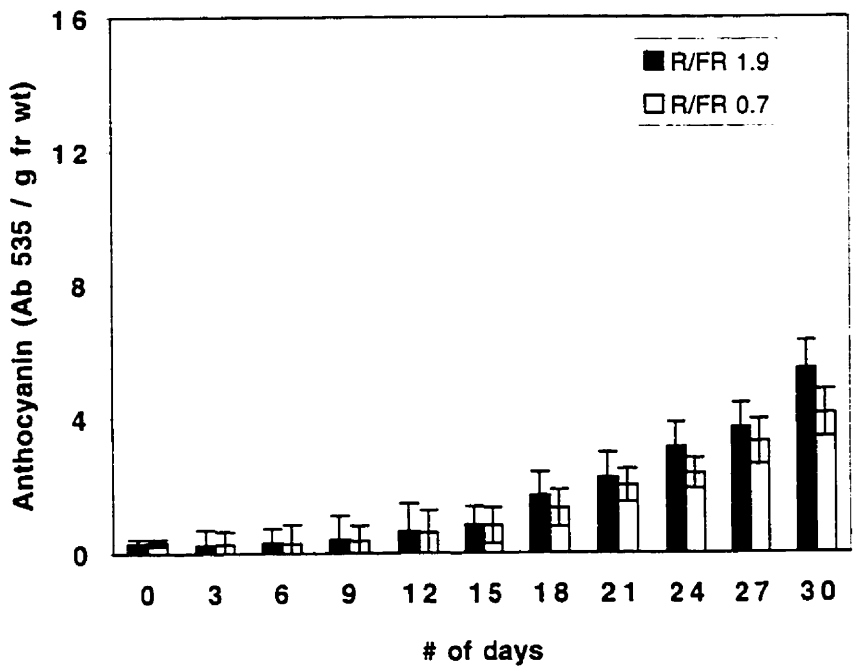

Anthocyanin (Ab 535 / g fr wt)
3
2
1
0
SDC
2
3
4
5
6
# of days in dark from SDC

1D

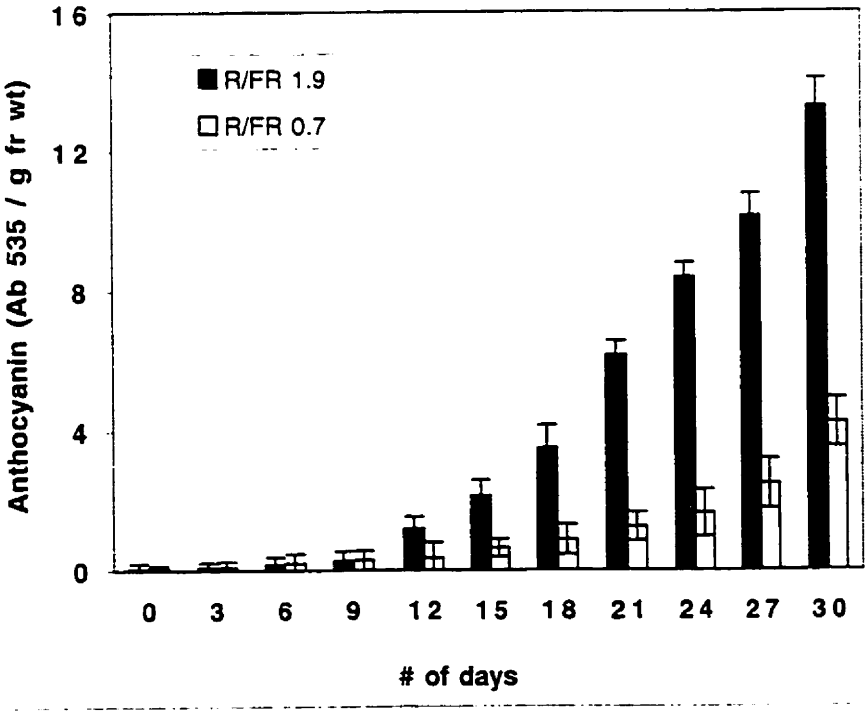

Anthocyanin (Ab 535 / g fr wt)
3
2
1
0
SDC
2
3
4
5
6
# of days in dark from SDC

7B

Figure 2.15: Amount of anthocyanin produced in the elongating stems of alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* upon transfer to varied R/FR light after etiolation of SDC plants for 7 days. Data are means of at least three individual experiments and the vertical bars indicate $\pm$ standard error (SE).


16
12
8
4
0
Anthocyanin (Ab 535 / g fr wt)
R/FR 1.9
R/FR 0.7
0 3 6 9 12 15 18 21 24 27 30
# of days

1D

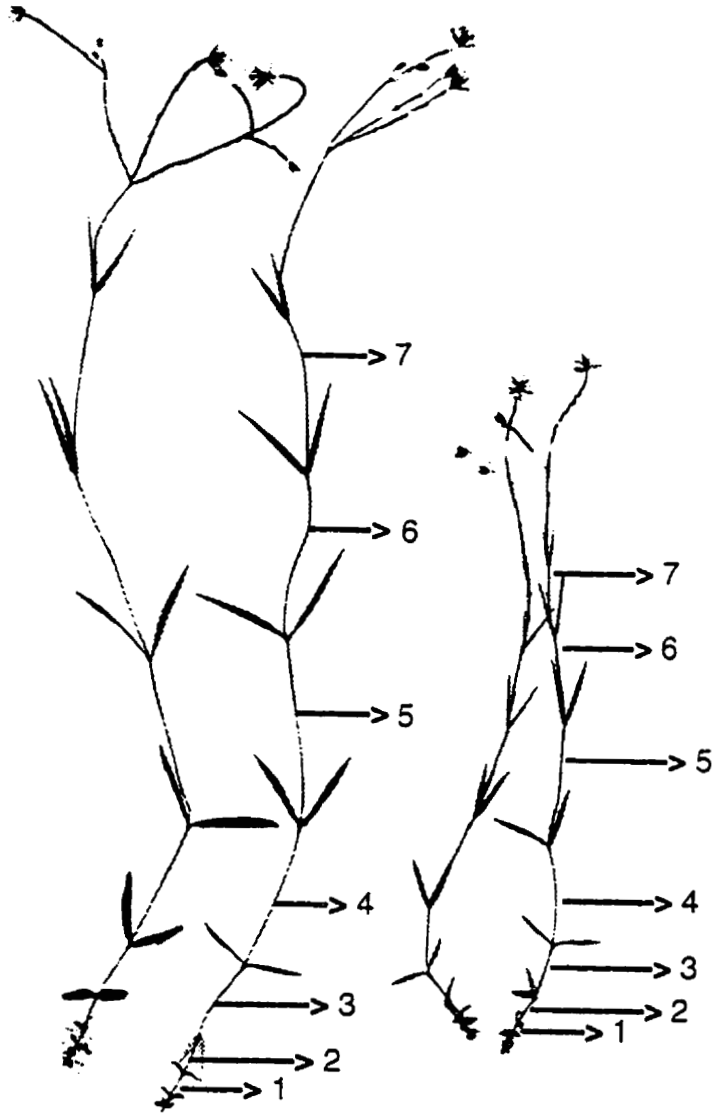

16
12
8
4
0
Anthocyanin (Ab 535 / g fr wt)
R/FR 1.9
R/FR 0.7
0 3 6 9 12 15 18 21 24 27 30
# of days

7B

Figure 2.16: Ramets of alpine (1D) and prairie (7B) plants grown under low and high R/FR showing numbering of the internodes used for anthocyanin measurements.

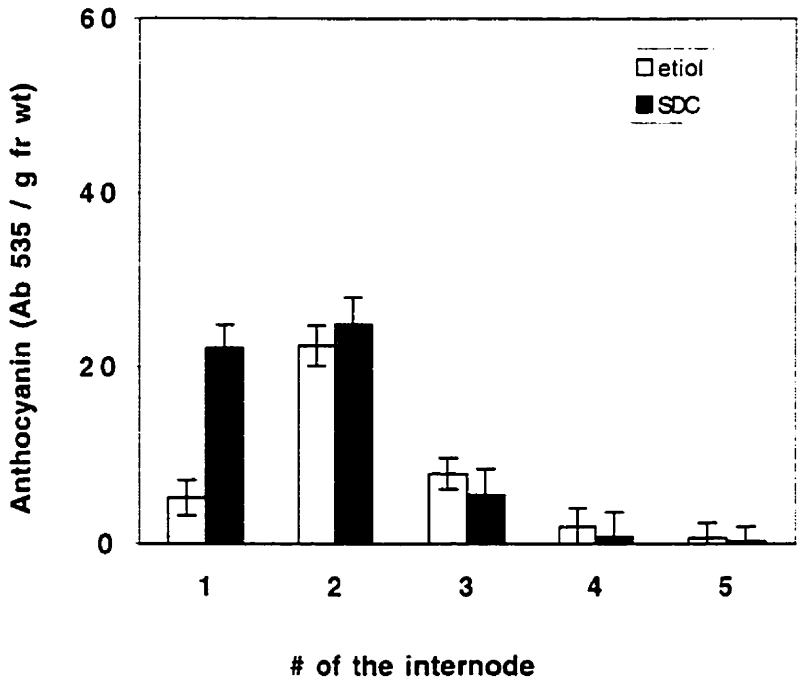
1D

7B

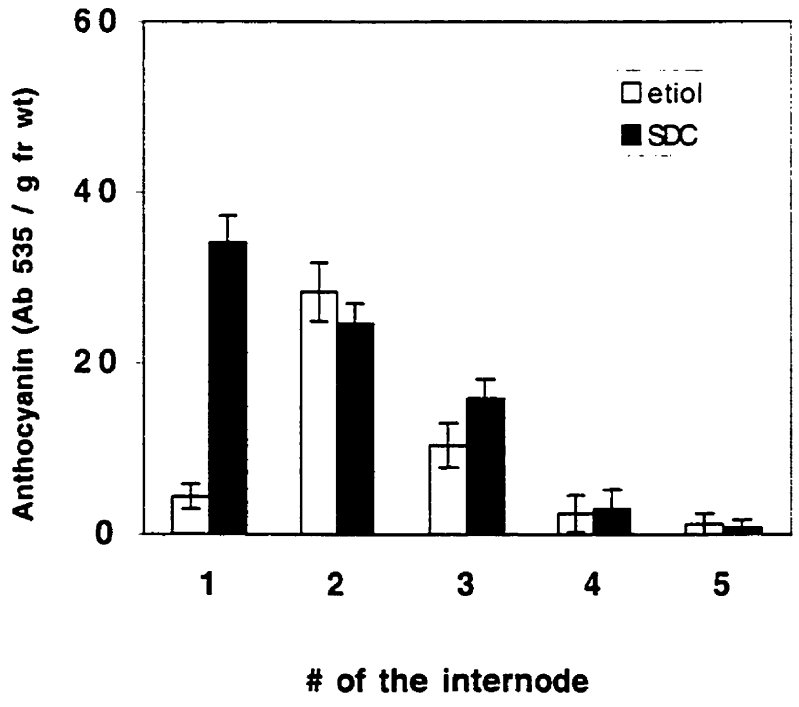


low R/FR high R/FR

Figure 2.17: Amount of anthocyanin accumulated in the various internodes after 30 days in the etiolated (etiol) 1D plants as compared to 1D plants transferred directly from short day cold conditions (SDC) and grown under low R/FR, 0.7 (A) and high R/FR, 1.9 (B). Data plotted are means of at least three values and the vertical bars indicate ± standard error (SE).

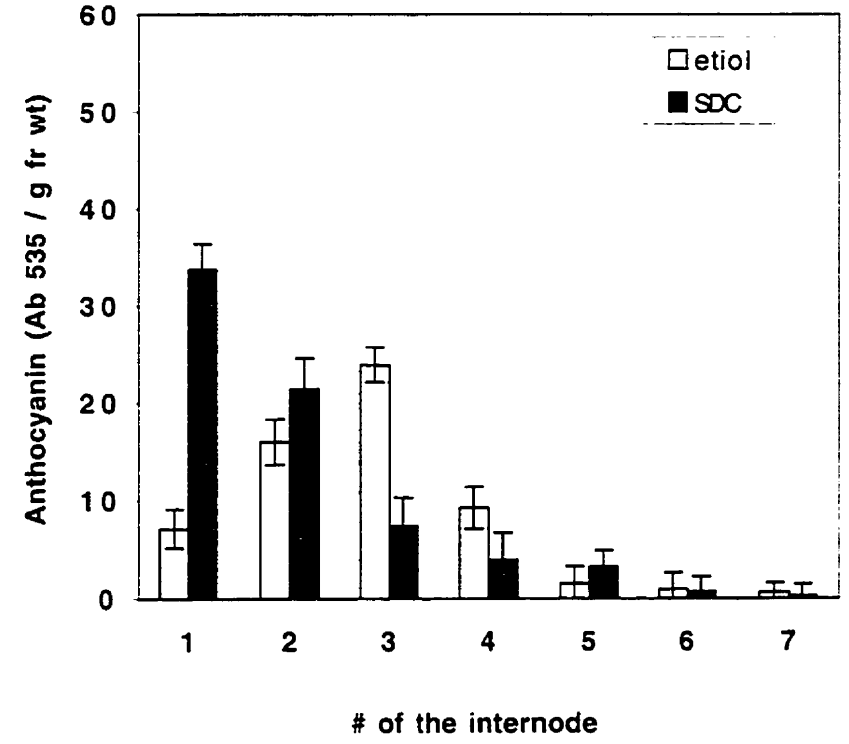

60
40
20
0
Anthocyanin (Ab 535 / g fr wt)
etiol
SDC
1
2
3
4
5
of the internode

A

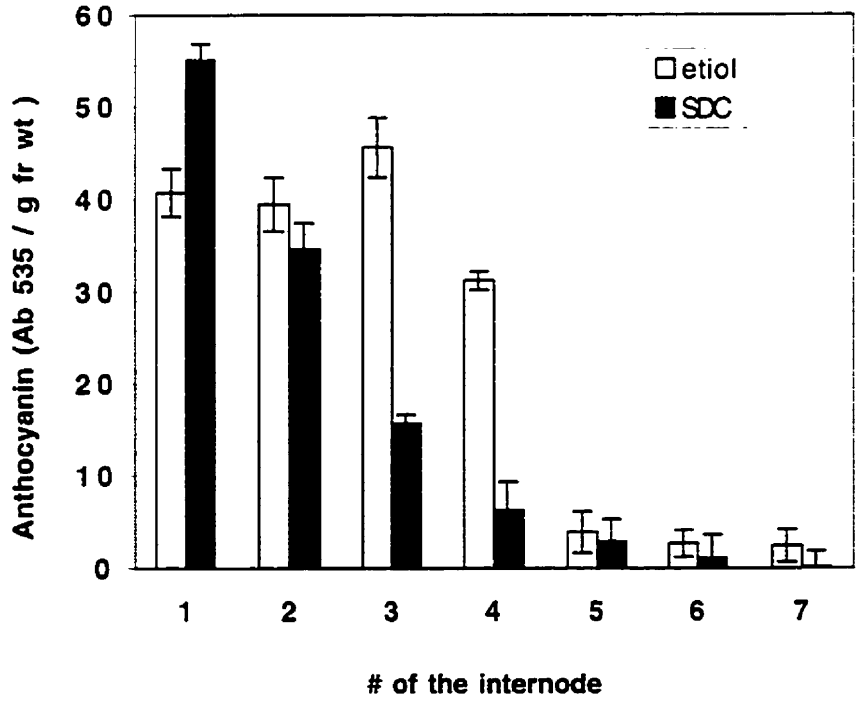

60
40
20
0
Anthocyanin (Ab 535 / g fr wt)
etiol
SDC
1
2
3
4
5
of the internode

B

Figure 2.18: Amount of anthocyanin accumulated in the various internodes after 30 days in the etiolated (etiol) 7B plants as compared to 7B plants transferred directly from short day cold conditions (SDC) and grown under low R/FR, 0.7 (A) and high R/FR, 1.9 (B). Data plotted are means of at least three values and the vertical bars indicate ± standard error (SE).


60
50
40
30
20
10
0
Anthocyanin (Ab 535 / g fr wt)
etiol
SDC
1
2
3
4
5
6
7
of the internode

A


60
50
40
30
20
10
0
Anthocyanin (Ab 535 / g fr wt)
etiol
SDC
1
2
3
4
5
6
7
of the internode

B

Both alpine and prairie ecotypes transferred directly from SDC as compared to etiolated plants showed a significant difference in anthocyanin accumulation in different internodes under varied R/FR. The accumulation of anthocyanin in the first five elongating internodes for alpine ecotype and first seven elongating internodes for prairie ecotype on day 30 after their transfer to the respective chambers were measured. The numbering of the internodes for alpine and prairie ecotypes is shown in Fig. 2.16. Alpine and prairie ecotypes when transferred directly from SDC showed highest levels of anthocyanin in the first and second internode under low or high R/FR and these levels were in the decreasing order from lower to the upper internodes (Figs. 2.17 & 2.18). Etiolated 1D plants showed highest levels of anthocyanin in the second internode under both low and high R/FR whereas etiolated 7B showed highest levels of anthocyanin in the third internode under the same conditions. In addition etiolated 7B under high R/FR showed high levels of anthocyanin in first and second internodes.

Discussion

Both alpine and prairie ecotypes of *Stellaria longipes* could detect the changes in the quality of light and initiate shade avoidance responses when exposed to reduced R/FR as reported in other plants (Ballare etal., 1990; Casal and Smith, 1989). A significant difference in the stem elongation, total biomass of the ramets, leaf area and anthocyanin accumulation was observed for both the ecotypes under varied R/FR, although the prairie ecotype responded more dramatically. The present results show that sensitivity to low R/FR may vary between the two ecotypes. As compared to alpine ecotype, the prairie ecotype are from habitat where there is an intense competition for light from crowded neighbouring plants. Spectral distribution of partially filtered light by neighbouring plants

in the natural prairie habitat (Fig. 2.6) shows that there is a great depletion in the visible region (400 to 700nm), with more far-red light (700-800nm). Under these circumstances, the increase in stem elongation provides the prairie plants with an adaptive advantage of receiving maximum light for photosynthesis.

The stem elongation in both the alpine and prairie ecotypes when transferred from SDC to LDW with varied R/FR started on day 4 (Fig. 2.9). Similar results have been reported by Emery et al., (1994) and Tatra (2000). Histological studies showed that the cell number is relatively constant during the first 4 days of LDW and the cell number in the second internode begins to increase by day 5 (Chuong, 1998). Aslo methylation studies demonstrated that both the ecotypes irrespective of R/FR ratios, showed maximum genomic cytosine demethylation on day 4, possibly indicate the role of demethylation in triggering stem elongation (Tatra, 2000). 7B ramets showed greater stem elongation under low R/FR as compared to 1D. This indicates that 7B is more plastic for shade avoidance response than 1D. The stem elongation response of *S.longipes* to light quality has a potential consequence on the overall fitness of the plant as shown by Emery et al (1994) in 'reciprocal transplantation' studies, that alpine plants were unable to survive in a prairie habitat while prairie plants were able to survive in an alpine habitat. These results indicate that the "shade avoidance syndrome" is an adaptive response controlled by R/FR in the environment (Smith, 1990). In non-competing vegetation wild types of *Brassica rapa* have a fitness advantage as compared to their *ein* mutant lines deficient for phytochrome and constitutively expressing the shade avoidance response (Schmitt et al, 1995). Also wild type tobacco when grown in the presence of competing neighbours, has a fitness advantage over transgenic tobacco which constitutively express

PHYA with non-elongated phenotypes (Casal etal., 1994). Similar experiments by Dudley and Schmitt (1995; 1996) showed the adaptive significance of shade avoidance stem elongation in competitive and non-competitive environments.

In addition to stem elongation, effects of low R/FR on other characters such as shape of the leaf and total biomass of the ramets were also studied. Both alpine and prairie ecotypes showed only a slight increase in the total area of the leaf under low R/FR as compared to their counterparts under high R/FR (Fig. 2.10A). However both 1D and 7B plants showed marked increase in the length of the leaf lamina (more lanceolate) under low R/FR (Fig. 2.10B). These results are similar to the previously published shade avoidance responses in tomato (Kerckhoffs et al., 1992) and different species of *Crotalaria* (Wulff, 1998). Although both the ecotypes of *S.longipes* showed significant increase in the elongation of stem and leaf under low R/FR, the prairie ecotype showed greater increase for both these characters as compared to the alpine ecotype, indicating that the shade avoidance responses are more dramatic in prairie ecotype. Like shoot elongation, increase in the length of the leaf may play an important role in gaining adaptive advantage when the plants are grown under low R/FR. As shown in some species of *Trifolium*, that through petiole extension the leaf lamina can raise over the neighbouring vegetation and expose to the unfiltered light (Thompson and Harper, 1988). Thus the length of the leaf may be an important factor in light perception and survival of the plant under shade.

In addition to variation in the stem and leaf elongation, another interesting trend of shade avoidance response observed in both the ecotypes of *S.longipes* was variation in the biomass of the ramets. The ramets of both alpine and prairie ecotypes grown under

high R/FR showed greater dry weight as compared to their counterparts under low R/FR (Fig. 2.11). These results are similar to the total dry matter accumulation recorded in radish (Keiller and Smith, 1989) and Crotalaria (Wulff, 1998) when grown under white light supplemented with far-red light. However for both the ecotypes of *S.longipes* fresh weight of the ramets was slightly higher under low R/FR as opposed to their dry weights. Stem elongation in *S.longipes* occurs through both cell expansion and cell division (Chuong, 1998). So it could be hypothesized that there is an increased number as well as size of the cells in the ramets under low R/FR as compared to high R/FR which could account for their increased fresh weight. On the other hand two-third of the cell's dry weight is made up of wall matrix containing several polysaccharides, glycoproteins, proteins and phenolic compounds (Fry, 1986; Brett and Waldron, 1996). Increased deposition of phenolic compounds may be responsible for the inhibition of stem elongation under high R/FR and account for the increased dry mass. Keiller and Smith (1989) studied supplementary far-red light mediated changes in carbohydrate status and enzymes of sucrose metabolism such as sucrose phosphatase synthase (SPS) and invertase in radish. SPS plays a key role in the synthesis of sucrose and regulation of photosynthate partitioning in higher plants. Their results showed that under the additional far-red light SPS activity increased in leaves while the amount of carbohydrate decreased and invertase activity increased in petiole but decreased in roots. The effect of low R/FR on the dry matter distribution and hexose accumulation have also been reported in a variety of species (Lercari, 1980; Kasperbaur and Hamilton, 1984). Thus the morphological changes within a plant in response to shade appear to be correlated with profound redistribution of growth and dry matter. More biochemical and histological

studies on *S.logipes* under varied R/FR will provide better insight into the possible mechanisms of stem elongation response under shade.

Anthocyanin synthesis appears to be controlled by R/FR ratios in *S.longipes*, since both the ecotypes showed increased accumulation of anthocyanin under high R/F as compared low R/FR (Fig. 2.12). Similar observations in tomato were reported by Kerckhoffs et al., (1992). Also anthocyanin synthesis has been shown to be induced by monochromatic irradiation with red, far-red, blue and UV-light in various plants (Mohr, 1994). The anthocyanin accumulation pattern were similar in alpine and prairie ecotypes either when the plants were transferred directly from SDC to varied R/FR conditions or after the SDC plants were etiolated for 7 days and then transferred to varied R/FR conditions. However etiolated plants of both the ecotypes under both the light conditions showed greater accumulation of anthocyanin as compared to the plants transferred directly from SDC conditions (Figs. 2.12 & 2.15). When the SDC plants were etiolated, the levels of anthocyanin were zero for both the ecotypes by day 6 of etiolation (Fig. 2.14) indicating that light is required for anthocyanin synthesis in *S.longipes* like for many other species reported (Duell-Pfaff and Wellman, 1982; Mancinelli, 1984). An interesting observation made regarding the sites of anthocyanin accumulation is that the accumulation of anthocyanin for both the ecotypes grown under low or high R/FR was greater in stems compared to leaves (Fig. 2.13). Within the stem, the amount of anthocyanin in different internodes was also compared. When the plants were transferred directly from SDC to LDW with low or high R/FR conditions alpine and prairie ecotypes showed highest levels of anthocyanin in the first and second internodes and these levels were in decreasing order from lower to the upper internodes (Figs. 2.17 & 2.18).

However when the SDC plants were etiolated for 7 days and then transferred to varied R/FR light, neither of the ecotypes showed a similar pattern of anthocyanin accumulation gradient from lower to upper internodes. Etiolated 1D plants showed highest levels of anthocyanin in the second internode under both low and high R/FR whereas etiolated 7B showed highest levels of anthocyanin in the third internode under the same conditions. In addition etiolated 7B under high R/FR showed high levels of anthocyanin in first and second internodes. Shichijo et al.. (1993) showed that moderately low temperature stimulates R-induced synthesis of anthocyanin in the first internode of Sorghum seedlings, indicating that the phytochrome system is also influenced by temperature (Van Der Woude and Toole, 1980; Wall and Johnson, 1982). In *Polygonum cuspidatum* seedlings the pattern and sites of anthocyanin accumulation also were dependent on temperature during irradiation (Yamaguchi et al., 2000). The etiolated seedlings of *Polygonum* when irradiated with red light at 5^0C accumulated greater levels of anthocyanin in the upper regions of the hypocotyls while de-etiolated seedlings accumulated more anthocyanin in the lower regions under the same conditions. These results indicate that the sites of anthocyanin accumulation are dependent upon the pre-treatment before the irradiation. When the SDC plants of 1D and 7B were directly transferred to LDW chambers with varied R/FR, there are two independent / inter-dependent factors, ie quality of the light and temperature controlling anthocyanin synthesis in these plants. Unlike the etiolated plants, which were transferred from SDC conditions, when etiolated for 7 days have already been exposed to higher a temperature prior to their transfer to LDW chamber with varied R/FR. So it can be inferred that the difference in the temperature pre-treatment may play a role in the accumulation pattern of

anthocyanin in *S.longipes*, which has been shown to be very sensitive to variations in both temperature and light (Emery, 1994). Furthermore, it could be presumed that etiolation of plants can cause variations in the distribution of phytochrome with varying spectral sensitivity. This could be correlated to the levels of anthocyanin in different regions of the plant parts or the site of anthocyanin synthesis. The control and action of phytochrome(s) on site-specific anthocyanin synthesis mediated through signal transduction pathways is still to be fully elucidated.

Various mechanisms through which phytochromes can mediate shade avoidance stem elongation response may involve alterations in the levels of hormones, cell wall characteristics and changes in the membrane potential and membrane phosphorylation (Cosgrove, 1994). With the help of light-shielding experiments and local irradiations with fibre optics it has been shown that the growing stems and leaves can sense the light stimulus and mediate some unidentified signal to modulate an appropriate response. Earlier work gave indirect evidence that red light inhibited stem growth by causing stiffening of the cell wall. Recent studies by Kigel and Cosgrove (1990) using direct measurements of cell turgor pressure and wall relaxation characteristics showed that red light inhibited stem elongation in pea seedlings by slowing wall loosening. Parallel studies by Van Volkenburgh and co-workers (1987) showed that light induces expansion of leaves in *Phaseolus* by increasing wall extensibility. Thus it seems light regulates stem and leaf growth by modulating the wall loosening properties of the growing cells. Although through membrane depolarization studies it is suspected that light controls cell wall expansion by altering H^+ extrusion and H^+ -ATPase activity, detailed biochemical

analysis of these changes remain elusive (Spalding and Cosgrove, 1992; Memon and Boss, 1990).

In spite of the possibility of various mechanisms involved in the transmission of light mediated stimulus, hormone-mediation of light response has attracted considerable attention from past and present researchers. Inhibition of mesocotyl growth in *Avena* seedlings was attributed largely to reduction in the supply of auxin from the coleoptile (Iino, 1982). Although there is much less evidence for photomodulation of stem growth *via* auxin in dicots, studies by Behringer et al., (1992) showed that auxin levels increased by 40% in the epidermis of pea stems in response to 'end-of day far-red light' treatment (EODFR) unlike a photomorphogenic mutant which lacked EODFR growth response did not show any increase in the auxin levels. These results suggest that far-red light mediated stem elongation might be the result of increased auxin levels in the stem epidermis. Although gibberellins (GAs) are well known for mediating stem elongation response in many plants, occurrence of gibberellins in many forms has complicated the studies on their light mediated regulation. In spinach long day (LD) mediated bolting (rapid elongation of the rosette stem) is associated with increases in the active forms of GAs and this effect could be suppressed with GA biosynthesis inhibitors (Talon et al., 1991). In addition to auxin and gibberellins, ethylene may also play a major role in the light mediated stem elongation response. Ethylene has been shown as one of the major growth factors responsible for controlling stem elongation plasticity in both alpine and prairie ecotypes of *S.longipes* (Emery et al., 1994). In both the ecotypes, ethylene production increased during rapid elongation when the plants were transferred from SDC to LDW conditions. Also the amount of ethylene produced by alpine plants is lesser than

that of prairie plants, which show greater elongation. Similar studies involving comparison of the amount of ethylene produced and the expression of ethylene biosynthetic genes such as ACC synthase and ACC oxidase in both alpine and prairie ecotypes under varied R/FR may yeild better insights into the role of ethylene in mediating the shade avoidance stem elongation in *S.longipes*.

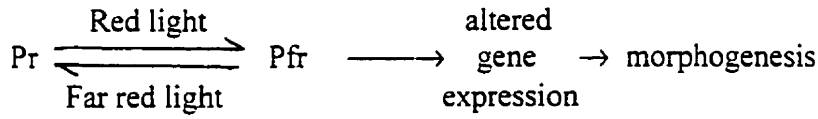
The use of mutants, transgenic plants and different ecotypes with contrasting degree of shade avoidance response will help to unravel the detailed mechanisms of shade mediated modulation of plant growth.

Chapter three

Role of phytochrome species in the stem elongation response under varied R/FR

Introduction

The photomorphogenetic action of red light (R) involves the conversion of the R-absorbing form of phytochrome (Pr) to the far-red (FR)-absorbing form of phytochrome (Pfr). Signal perception of light by Pr triggers Pfr formation initiating a transduction process, which culminates in the altered expression of selected genes resulting in an altered phenotype. This can be represented schematically as follows (Quail, 1997):



The equilibrium between Pr and Pfr is markedly effected by the relative amounts of R and FR, light with high R/FR yields high phytochromephotoequilibriuum (φ) value, represented as Pfr/P, where P = Pr + Pfr. An inverse relationship between φ and shade avoidance avoidance internode extension has been observed (Morgan et al., 1980; Casal and Smith, 1989). Immunological studies have shown that the Pfr form of light labile phytochrome PhyA (PfrA) is very unstable and rapidly disappears when the dark-grown tissues are exposed to light, resulting in loss of total phytochrome (Furuya and Schafer, 1996). However, Pfr degradation is known to occur in dark also through 'dark reversion' (Vierstra, 1993; 1994).

Phytochrome A (PhyA), light labile and Phytochrome B (PhyB), light stable, are the best-characterized members of this family (Furuya, 1989; 1993). To elucidate the physiological functions of different phytochrome species involved in the shade avoidance response, various mutants and transgenic plants transformed with individual *phy* genes driven by constitutive promoters have been used extensively. Cucumber *lh* (Kendrick and Nagatani, 1991; Ballare et al., 1991a), *Arabidopsis hy3* (Whitelam and Smith, 1991) and *Brassica rapa ein* (Devlin et al, 1992) mutants lacking Phytochrome B and tomato *au* mutant (Kerckhoffs et al., 1992) lacking light labile phytochrome (PhyA) were used. In addition, two different PHY B over expressors, the RBO (McCormac et al. 1993) and the ABO (Wagner et al, 1991) and potato PHYA over expressors (Yanovsky et al., 1998) have been investigated in detail. Studies by Kendrick and Nagatani (1991) on cucumber *lh* mutant indicated that it did not respond to EODFR and has massively elongated hypocotyls. It was further reported that, these mutants when grown in white light of high R/FR take on the phenotype of wildtypes treated with white light with very low R/FR, in other words, *lh* appears to behave as if it is in a constitutive shade avoiding mode. Spectrophotometric and immunoblot analyses have shown that *hy3* contains wild-type levels of phytochrome A but lack phytochrome B (Koornneef et al., 1980; Parks et al., 1989; Somers et al., 1991). The Arabidopsis *hy3* mutant lacking phytochrome B is also almost completely blind to low R/FR, correspondingly these mutants either do not show an extension growth response to a reduction in R/FR ratio or display very small enhancement of extension growth (Whitlam and Smith, 1991), while Arabidopsis phyB over expressors exhibit a short hypocotyl phenotype and the responses to R/FR are very subtle (Wagner et al., 1991). These data seem to indicate that the absence of PhyB

disables the capacity for R/FR perception to a greater extent. However over expression of PHYA genes in tobacco, tomato, and *Arabidopsis* have been shown to inhibit stem or hypocotyls elongation and PhyA appears to be able to play a role normally performed by PhyB (Boylan and Quail, 1989, 1991; Keller etal., 1989; Nagatani et al., 1991; Cherry et al., 1992). More careful characterization of the light responses in these transgenic plants has suggested that the responses seen could be due to unusually high levels of the PhyA and might not reflect the effect of the molecule in homologous background. Thus it appears that PhyB is likely to be the principal photoreceptor involved in the perception of changes in R/FR and the mediation of shade avoidance stem elongation, while PhyA activity enhances the extent of response to reductions in R/FR perceived by PhyB. Not all shade avoidance responses are mediated by the same phytochrome (Smith, 1994; Casal, 1996). In an effort to study the role of PHY B in mediating differential shade avoidance stem elongation in alpine and prairie ecotypes, a partial PHY B cDNA was amplified from *S.longipes*.

Materials and methods

Plant material:

Genotypes 1D and 7B grown in SDC conditions were transferred to the chambers with varied R/FR ratios as described in chapter two.

DNA extraction:

DNA from stem and leaf tissues was extracted using Doyle and Doyle (1987) method with slight modification. Fresh tissue was frozen in liquid nitrogen, ground to powder form and homogenised in the 2 x CTAB extraction buffer containing hexadecyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 100 mM Tris-HCl (pH

8.0), 20 mM EDTA and 0.2% (v/v) ß-mercaptoethanol. After incubating at 60^{0}C for 1 hour with occasional mixing the homogenate was then extracted with chloroform-isoamyl alcohol (24:1; v/v) and centrifuged at 10,000 g at room temperature. The aqueous phase was separated and 2/3 volumes of cold isopropanol was added to precipitate the nucleic acid. The DNA was spooled with a glass Pasteur pipette and washed in buffer containing 76% ethanol and 10 mM ammonium acetate. The DNA pellet was dried and dissolved in distilled water. DNA was treated with RNase A to a final concentration of 10 µg/ml for 30 min at 37^{0}C and reprecipitated with an equal volume of 5 M ammonium acetate and 2.5 volumes of cold absolute ethanol at -20^0 C for at least 1 hour, followed by centrifugation at 10,000 g for 15 minutes. The pellet was washed with 70% ethanol, dried and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). DNA concentrations and purity were determined using SMART SPEC 3000 (Bio-Rad, U.S.A.).

RNA extraction:

Total RNA from stem and leaf tissues grown under varied R/FR light was isolated using the guanidinium thiocyanate method (Strommer et al., 1993). One gram of frozen tissue was ground to powder in liquid nitrogen, homogenized in 6ml of extraction buffer (4 M guanidinium thiocyanate and 50 m β-M mercaptoethanol pH 7.0) and 1.2 ml 2 M sodium acetate pH 4.0. After the homogenate was vortexed for 30 seconds, 6 ml of water saturated phenol and 1.2 ml of chloroform: isoamyl alcohol (24:1 v/v) were added and the sample was vortexed again for 30 seconds. The slurry was centrifuged at 10,000 g for 15 minutes at 4^{0}C. The supernatant was extracted twice with an equal volume of phenol : chloroform (1:1 v/v) and once with an equal volume of chloroform. To the aqueous phase an equal volume of prechilled isopropanol was added and the mixture was left at -20^{0}C

for at least one hour to precipitate RNA. The RNA was pelleted by centrifugation at 15,000 g for 15 minutes at 4^0C and the RNA pellet was resuspended in 500 µl DEPC-treated water. An equal volume of 4 M LiCl was added and the RNA was precipitated once again on ice for at least 1 hour. After centrifugation at 14,000 rpm for 15 minute at 4^0C, the RNA pellet was washed in 70% ethanol twice, air dried and dissolved in DEPC-treated water. RNA concentrations and purity were determined using SMART SPEC 3000 (Bio-Rad, USA).

Polymerase chain reaction (PCR):

Sequence specific primers were synthesized using Pharmacia DNA synthesizer (Pharmacia LKB Biotechnology, Uppsala, Sweden). All PCRs were carried out using a Robocycler (Stratagene, LaJolla, CA, USA) in 35 cycles of denaturation (1 min) at 95^0C, annealing (1 min) at 56^0C, and primer extension / enzymic reaction (2 min) at 72^0C, using Taq DNA polymerase (Pharmacia).

Reverse transcription:

Three microgram of total RNA was denatured at 70^0C for 10 min and kept on ice for 10 min. The reaction mixture (50 µl) contained 5 µl of 10 x reverse transcription buffer, 1 µl of 10 mM dNTPs, 1 µl of AMV reverse transcriptase (200 units/ml), 1 µl of RNase inhibitor (40 units/µl), 1µl of Oligo dT, denatured RNA and RNase free water (DEPC) to make up the volume to 50 µl. The reaction was carried out at 42^0C for 50 min.

Cloning of PHYB cDNA fragment:

Messenger RNA (mRNA) was extracted from the plants and the corresponding first strand cDNA was synthesized using AMV reverse transcriptase kit and this was used as template in PCR along with the designed primers. Primers were designed from the

conserved regions of phyB, and used in the polymerase chain reaction to amplify a partial 947 bp cDNA from *Stellaria longipes*. The nucleotide sequence of the primers is as follows:

Sense primer: 5 ATN, GGN, TTA/G, CAT/C, TAT/C, CCN, GCN, AC 3 (23 bp)

Antisense primer: 5 AG, A/G/T A T, C/T AA, T/C TG, C/T AA, GC, A/G TG, A/G/T AT 3 (22 bp).

The RT – PCR reaction mixture (50 µl) contained 5 µl of 10 x Taq buffer, 2.5 µl of cDNA first strand, 1 µl of 10 mM dNTPS, 0.2 µl of Taq enzyme (5 units/µl), 2.5 µl of 10 nmol sense and anti sense primers each and sterile distilled water to make up the volume to 50 µl.

The PCR amplified partial cDNA was purified, cloned into a pGEM-T-EASY plasmid vector at EcoR I site and sequenced.

Elution of DNA fragments from agarose gel:

DNA fragments required for cloning and probe synthesis were either eluted from the gel using 'Glass milk powder method' as described by Davis et al (1986) or excised and LMP purified (Sambrook et al., 1989).

DNA sequencing:

The PCR amplified partial cDNA were cloned and sequenced using the automatic sequencing facility available at Department of Medical Biochemistry, University of Calgary. Upon conceptual translation, amplified sequence showed a high degree of homology to other cloned PHY B sequences.

Nucleotide and amino acid sequence analysis:

Homology searches for the DNA sequence were made at Gen Bank using the Advanced

BLASTX search program (Altschul et al., 1990). Characterization of the nucleotide sequences and conceptual translation of nucleotide sequences were done using DNA strider ™ 1.2 (Centre d'Etudes de Saclay, France) and Mac Vector ™ 6.0 DNA sequence analysis (Oxford Molecular group Inc., Campbell, CA, USA) programs. The alignments of homologous nucleotide and amino acid sequences were done using CLUSTAL X multiple sequence alignment program.

Southern blotting and hybridization:

Ten µg of genomic DNA was digested with specific restriction endonucleases (Pharmacia, Uppsala, Sweden) overnight at 37^{0}C. The digested DNA was separated on a 1.2% agarose gel overnight at 25 V and blotted onto a nylon membrane (Hybond-N+, Amersham, UK) in 10 x SSC (Sambrook et al., 1989). After blotting the membranes were baked at 80^{0}C for 2 hours and prehybridized in Rapid-hyb buffer (Amersham, UK) at 58^{0}C for 4 hours. Hybridization was performed at 58^{0}C in the same buffer for at least 5 hours using a 900 bp (1kb) insert of a cDNA clone encoding for PHYB gene in *Stellaria longipes* as a probe, radiolabeled by random primers (Sambrook et al., 1989). Membranes were washed once in 2 x SSC plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 58^{0}C for 20 min, twice in 1 x SSC plus 0.1% SDS at 58^{0}C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 58^{0}C for 20 min. The membranes were then exposed to Kodak XAR 5 film –80^{0}C.

Northern blotting:

For northern analysis 15 µg of total RNA was denatured by 50% formamide and 6% formaldehyde at 70^{0}C for 15 minutes and separated by 1.2% agarose gel electrophoresis using 6% formaldehyde and 1 x MOPS buffer (40 mM 3-morpholino propanesulfonic

acid, 10mM sodium acetate, 1 mM EDTA, pH 7.0). The RNA was blotted onto Hybond N+ nylon membranes using 20 x SSC (Sambrook et al., 1989). After blotting the membranes were baked at 80^0C for 2 hours and prehybridized in Rapid-hyb buffer (Amersham, UK) at 65^0C for 4 hours. Hybridization was performed in the same buffer at 65^0C, for at least 5 hours using a 900-bp (1-kb) insert of a cDNA clone encoding for PHYB gene in *Stellaria longipes* as a probe, radiolabeled by random primers (Sambrook et al., 1989). Membranes were washed once in 2 x SSC plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 65^0C for 20 min. twice in 1 x SSC plus 0.1% SDS at 65^0C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 65^0C for 20 min. The membranes were then exposed to Kodak XAR 5 film -80^0C. The same membranes were stripped off for reprobing by washing several times in 0.01 x SSC and 0.05% SDS at 95^0C and reexposed to Kodak XAR 5 film to confirm complete probe removal without any background activity.

Results

PCR amplification and cloning of partial PhyB phytochrome B cDNA from *Stellaria*:

A 945-bp fragment was PCR amplified from the cDNA of prairie ecotype using the primers corresponding to the conserved regions of Phytochrome B, cloned into pGEM-T-EASY plasmid vector at EcoR I site and sequenced (Fig. 3.1). Conceptual translation of this sequence showed 85 to 95 % homology to the predicted amino acid sequences from *Solanum*, *Lycopersicon*, *Nicotiana*, Soya bean, *Pisum*, and *Arabidopsis* (Fig. 3.2).

Southern hybridization:

The genomic DNA of alpine and prairie ecotypes was digested with BamH I,

Figure 3.1: The nucleotide sequences of partial PhyB cDNA from *Stellaria*. This 945bp fragment was PCR amplified from the cDNA of prairie ecotype of *Stellaria longipes* and encodes 315 amino acids.

```
  1 ATG GGC TTG CAT TAT CCT GCT ACG GAT ATT CCA CAG GCG TCT AGG TTT TTG TTT AAG CAG   60
  1  M   G   L   H   Y   P   A   T   D   I   P   Q   A   S   R   F   L   F   K   Q   20
 61 AAT AGG GTT AGG ATG ATT GTT GAT TGT CAT GCT GAT TCT GTT TCT GTG GTG CAA GAT GAA  120
 21  N   R   V   R   M   I   V   D   C   H   A   D   S   V   S   V   V   Q   D   E   40
121 CGG TTA CGG CAA CCT CTT TGT TTG GTT GGG TCT ACG CTT AGG GCG CCT CAC GGG TGT CAT  180
 41  R   L   R   Q   P   L   C   L   V   G   S   T   L   R   A   P   H   G   C   H   60
181 TCT CAG TAT ATG GCT AAT ATG GGT TCT ATT GCT TCG TTG GTA ATG GCG GTT ATT ATT AAT  240
 61  S   Q   Y   M   A   N   M   G   S   I   A   S   L   V   M   A   V   I   I   N   80
241 GGT AAT GAC GAT GAA GGT AGT ACT AGG AAT GCT ATG AGG TTA TGG GGT TTG GTT GTG TGT  300
 81  G   N   D   D   E   G   S   T   R   N   A   M   R   L   W   G   L   V   V   C  100
301 CAT CAC ACT TCT CCA CGG TCA ATC CCT TTT CCT CTT CGG TAT GCT TGT GAG TTT CTA ATG  360
101  H   H   T   S   P   R   S   I   P   F   P   L   R   Y   A   C   E   F   L   M  120
361 CAG GCT TTT GGG CTT CAA TTA AAT ATG GAA CTG CAA TTG TCA GCA CAA GTG TTG GAG AAA  420
121  Q   A   F   G   L   Q   L   N   M   E   L   Q   L   S   A   Q   V   L   E   K  140
421 CGT GTG TTG CGG ACT CAA ACC CTT TTA TGT GAT ATG ATT CTG AGG GAA TCT CCG ACG GGA  480
141  R   V   L   R   T   Q   T   L   L   C   D   M   I   L   R   E   S   P   T   G  160
481 ATC GTT ACC CAA AGT CCG AGT ATA ATG GAT TTA GTT AAG TGT GAT GGG GCT GCT CTT CTG  540
161  I   V   T   Q   S   P   S   I   M   D   L   V   K   C   D   G   A   A   L   L  180
541 TTC TGT GGT AAG TAC TAC CCA TTA GGG GTG ACC CCA ACG GAA TTG CAG TTG AAG GAC ATC  600
181  F   C   G   K   Y   Y   P   L   G   V   T   P   T   E   L   Q   L   K   D   I  200
601 GTG CAG TGG TTA TTG AGT AAC CAC GGA GAC TCA ACA GGG TTG AGC ACA GAT AGT TTA GCT  660
201  V   Q   W   L   L   S   N   H   G   D   S   T   G   L   S   T   D   S   L   A  220
661 GAT GCG GGT TAC CCT GGT GCA TTA GCT TTG GCC GAT GCC GTT TGT GGT ATG GCG GTA GCT  720
221  D   A   G   Y   P   G   A   L   A   L   A   D   A   V   C   G   M   A   V   A  240
721 TTT ATC ACT CGA AGT GAT TTT TTA TTT TGG TTT AGA TCA CAC CCC GCA AAG GAA ATC AAA  780
241  F   I   T   R   S   D   F   L   F   W   F   R   S   H   T   A   K   E   I   K  260
781 TGG GGT GGT GCA AAA CAT CAC CCT GAA GAT AAG GAT GAT GGG CGA ATG CAT CCC CGG TCT  840
261  W   G   G   A   K   H   H   P   E   D   K   D   D   G   R   M   H   P   R   S  280
841 TCT TTC AAA GCC TTT CTT GAA TGT GGA AGT TAC AAG CCG GAG CTA GCC TGG GAG AAC GCC  900
281  S   F   K   A   F   L   E   C   G   S   Y   K   P   E   L   P   W   E   N   A  300
901 GAA ATG GAT GCA ATT CAT TCC TTG CAA CTT ATT TTA CGA GAC TCG                       945
301  E   M   D   A   I   H   S   L   Q   L   I   L   R   D   S                     315
```

Figure 3.2: Partial amino acid sequence alignments of PhyB from *Stellaria longipes*, *Arabidopsis*, *Solanum*, *Pisum*, tomato, tobacco, and Soya bean. Identical amino acid residues have asterisks above.

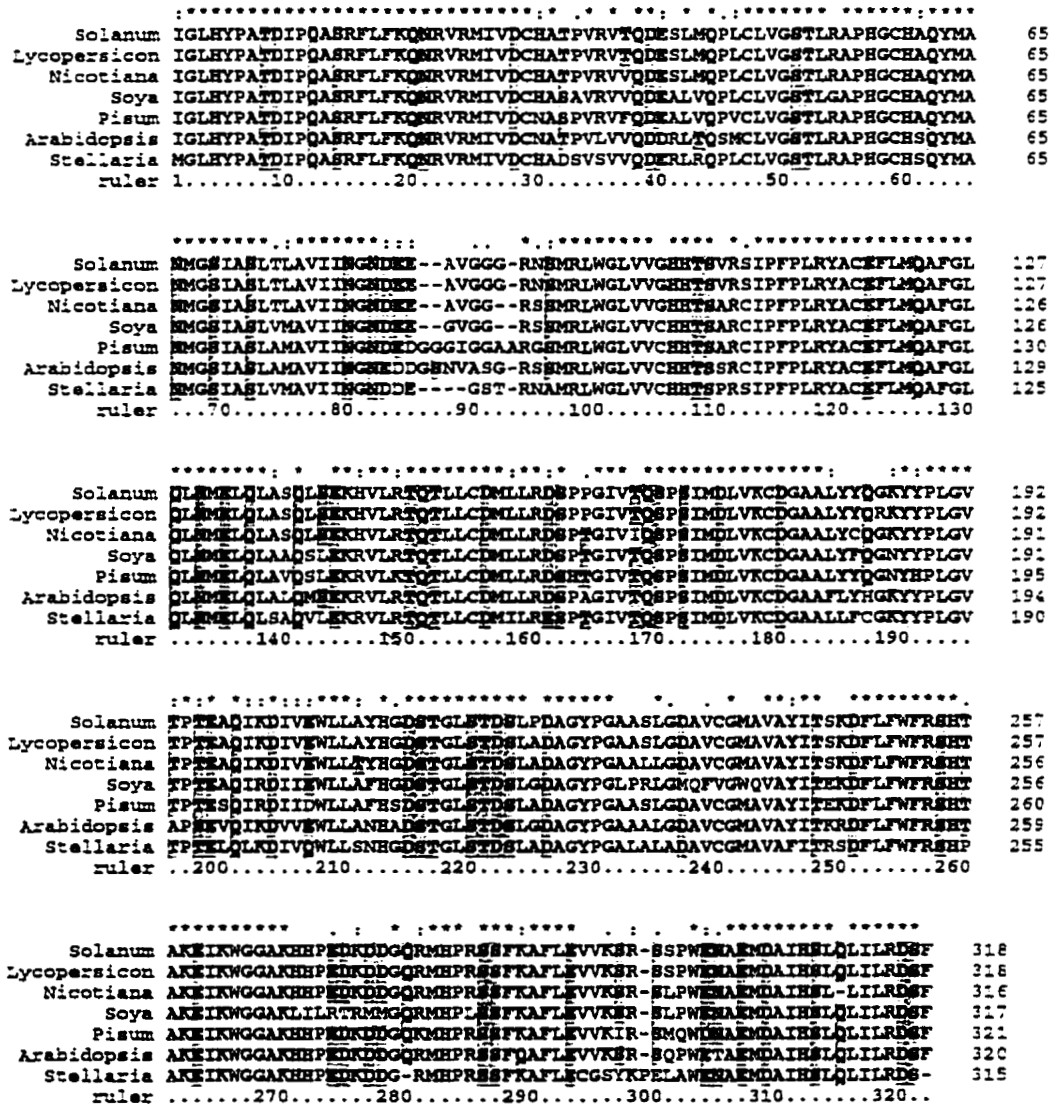

Solanum
Lycopersicon
Nicotiana
Soya
Pisum
Arabidopsis
Stellaria
ruler

Figure 3.3: Southern blot analysis of PhyB in alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* . 10 µg of genomic DNA was digested with BamHI (1), EcoRI (2), EcoRV (3), Hind III (4), XbaI (5), PstI (6), separated on 1.2% agarose gel, blotted onto a nylon membrane and hybridized with 945 bp PhyB cDNA fragment from *Stellaria* . The numbers to the right represent the sizes of λ 1-kb ladder fragments (M).

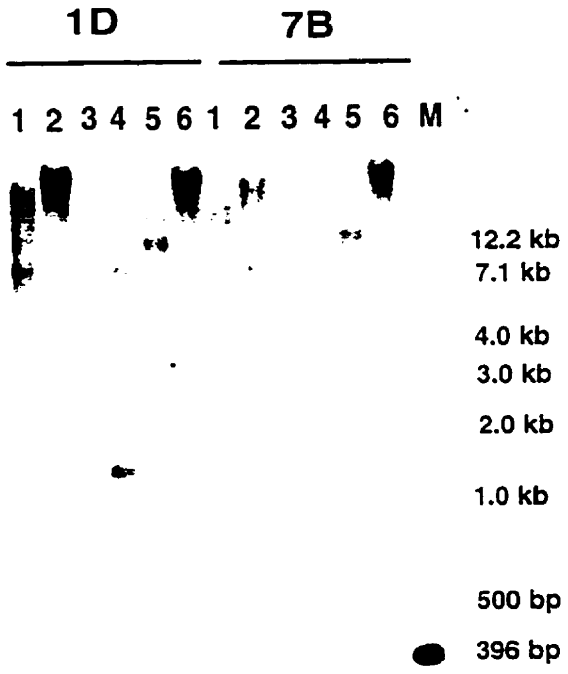
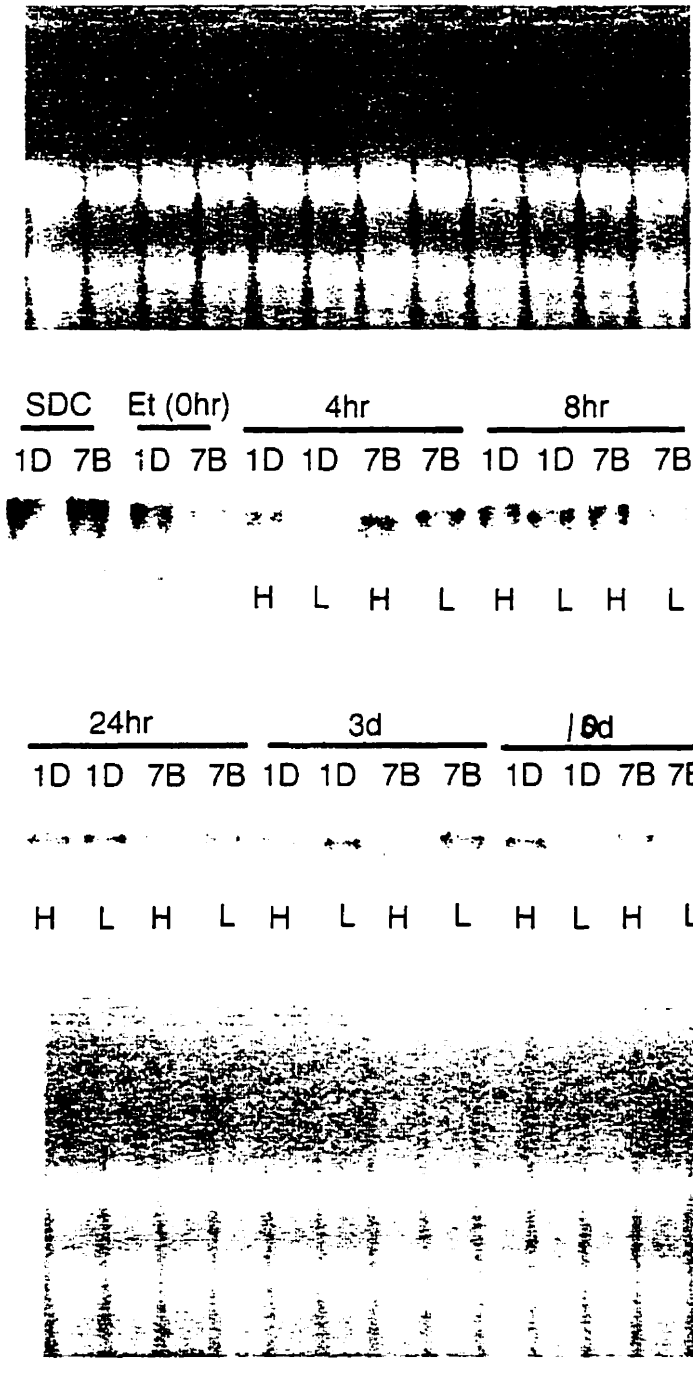

1D
7B
1 2 3 4 5 6 1 2 3 4 5 6 M
12.2 kb
7.1 kb
4.0 kb
3.0 kb
2.0 kb
1.0 kb
500 bp
396 bp

Figure 3.4: Expression of PHYB mRNA in alpine and prairie ecotypes of *Stellaria longipes*. Each lane in the northern blot contains 15 µg of total RNA extracted from stem and leaf tissues. The different lanes containing RNA from alpine (1D) and prairie (7B) plants grown for three months under SDC conditions, etiolated for seven days (Et 0 hr) and 4 hr, 8 hr, 24 hr, 3 d and10 d after their transfer to chambers with high R/FR (H) or low R/FR (L) are probed with random prime labeled 945 bp PHYB cDNA fragment from *Stellaria*.

Ethidium bromide stained gel pictures corresponding to each of the blot are also shown for equal loading of RNA in each lane.


SDC
Et (0hr)
4hr
8hr
1D 7B 1D 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L
24hr
3d
8d
1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L H L H L

EcoR I, EcoR V, Hind III, XbaI, or PstI and hybridized with PhyB cDNA fragment from *Stellaria.* The presence of one hybridizing band in all these enzyme digested genomic DNA suggests that the genome may contain only one copy of *PHYB* gene (Fig. 3.3). Also genomic DNA of alpine and prairie ecotypes did not reveal any polymorphism for any of the enzymes used.

mRNA expression of PHYB in the alpine and prairie ecotypes:

The expression of PHYB mRNA in alpine and prairie ecotypes was examined under varied R/FR light. Total RNA was extracted from the alpine and prairie plants grown under SDC for 3 months and from SDC plants etiolated for 7 days (0hr). The etiolated plants were transferred to low (L) or high (H) R/FR conditions and total RNA was extracted from 4 hr, 8 hr, 24 hr, 3 d, and 10 d samples. The blots containing 15 μg RNA from all these samples were hybridized with 945 bp PHYB cDNA from *Stellaria* (Fig. 3.4). Both the alpine and prairie ecotypes showed higher levels of transcript expression in SDC plants. Upon transfer of the etiolated plants to low R/FR or high R/FR light both the ecotypes showed stable levels of expression from 4 hr to 10 d. Although the stem elongation and accumulation of anthocyanin varied significantly in both the ecotypes under high R/FR as compared to low R/FR, neither of the ecotypes showed any difference in the transcript abundance of phyB between low R/FR and high R/FR, at any of the time intervals from 0 hr to 10 d.

Discussion

Phytochrome cDNAs and PHY genes have been cloned from several monocots and dicots such as oat (Hershey et al., 1987), pea (Sato, 1988), rice (Kay et al., 1989), *Arabidopsis* (Sharrock and Quail, 1989), maize (Christensen and Quail, 1989). Most of

the phytochrome genes that have been cloned appear to correspond to Type I or light labile phytochrome. However genes corresponding to Type II or light stable phytochromes have also been sequenced from *Arabidopsis* (Sharrock and Quail, 1989), tomato (Pratt et al., 1997) and potato (Heyer and Gatz, 1992). Sequence analysis shows that *Arabidopsis* PHYA is more closely related to sequences corresponding to Type I phytochrome from other dicots than to PHY B or PHY C from *Arabidopsis*. The limited homology between Type I and Type II genes can also be explained on the basis of the inability of Type I phytochrome gene probes to hybridize to more than one sequence in genomic Southern blot analysis in rice (Kay et al., 1989). The different members of phytochrome family may be encoded by divergent sub-families of genes. The partial cDNA fragment amplified from *S.longipes* also showed similar results, exhibiting very high homology to known PHY B sequences of other plant species (Fig. 3.2). Southern analysis done in the present studies suggests that PhyB is encoded by a single gene in *S.longipes* (Fig. 3.3). Also alpine and prairie ecotypes did not exhibit any polymorphism.

It has been showed that PHYA, PHY B and PHY C genes of *Arabidopsis* are differentially regulated quantitatively and qualitatively (Sharrock and Quail, 1989). The PHYA transcript is the most abundant species in etiolated seedlings and is down-regulated in continuous white light, while PHY B and PHY C mRNAs are present at relatively low levels in the dark and are not significantly regulated by light. In the present study expression of PHY B mRNA in alpine and prairie ecotypes was examined under varied R/FR light using partial PHY B cDNA probe from *S.longipes*. Both the alpine and prairie ecotype showed higher levels of transcript expression under SDC, which decreased slightly upon etiolation for seven days (Fig. 3.4). This slight decrease in the

mRNA levels could be attributed to variation in temperature or light or both (Runkle and Pearson, 1998). Upon transfer of the etiolated plants to low R/FR or high R/FR light both the ecotypes showed stable levels of expression. In spite of the significant difference in the stem elongation and anthocyanin accumulation under varied R/FR, there is no difference between the two ecotypes in the mRNA levels under high or low R/FR. These results indicate that shade avoidance stem elongation may not be directly related to the PHY B transcript levels but may be regulated at post-transcriptional or translational levels by altering the phytochrome photoequilibrium. Immunoblot analysis by Somers et al., (1991) demonstrated that the phytochromeB levels in *hy3* are reduced to an amount that is at most 2% to 5% of wild-type levels while the decrease in PHY B mRNA is less marked (only twofold to threefold). Further studies involving spectrophotometric and immunoblot analyses using phytochrome type-specific molecular probes will help to provide better insights into the functions of the different phytochromes in shade avoidance responses mediated by low R/FR. Also using the partial PHY B cDNA obtained, full-length clones from *Stellaria longipes* can be isolated and characterized.

Chapter four

Photoregulation of Phenylalanine ammonia-lyase in the accumulation of anthocyanin

Introduction

The anthocyanin biosynthetic pathway, as part of the larger phenyl propanoid pathway, has been well established and a generalized anthocyanin biosynthetic pathway is shown in fig 1.1 (Mol et al., 1989; Forkmann, 1991). Phenylalanine ammonia-lyase (PAL) is the key enzyme which catalyses the initial committed step of deamination of L-phenylalanine to *trans*-cinnamate in the phenylpropanoid pathway. The *trans*-cinnamate is used as a precursor for many plant phenylpropanoid derivatives such as lignin, suberin, flavonoids, coumarins and stilbenes. After a series of reactions *trans*-cinnamate is converted to 4-coumaroyl-CoA which through the flavonoid pathway, results in the production of a range of flavonoid compounds, the most common being flavones, flavonols, isoflavonoids, proanthocyanidins and anthocyanins. Several of the genes encoding enzymes involved in anthocyanin synthesis have been cloned and were shown to be controlled by various developmental and environmental factors. Anthocyanins are responsible for most of the red, pink, purple and blue colours found in plants. They function to attract animals for pollination, seed dispersal and fungal deterrants and they are believed to protect plant cells from UV radiation. Anthocyanin accumulation in response to many different stimuli such as wounding, nutrient deficiencies, pathogen attack, UV irradiation, elicitors and hormones has been reported in various plants (Mol et al., 1996). PAL activity has been shown to increase in response to low temperature in

tomatoes (Rhodes and Wooltorton, 1977), *Brassica napus* (Parra et al., 1990), *Arabidopsis thaliana* (Leyva et al., 1995) and maize (Christie et al., 1994), correlating to the increased anthocyanin accumulation. These results indicate that phenolic compounds may be involved in plant responses to mechanical and environmental stresses.

Light is one of the most important environmental factors in the control of anthocyanin synthesis. In several systems the photoinduction of anthocyanin is preceded by an enhancement in the PAL transcription as well as PAL activity (Dong et al., 1998). All the three photoreceptors, phytochrome, blue-light photoreceptor and UV-B photoreceptor either interacting or acting independently are involved in the photoregulation of anthocyanin production (Mohr and Drumm-Herrel, 1983; Beggs and Wellmann, 1985; Oelmuller and Mohr, 1985; Sponga etal., 1986, Mancinelli et al., 1991; Mohr, 1994). Anthocyanin production in response to R and FR is mediated by phytochrome and the extent of response is a function of light quality, fluence rate and exposure duration (Mancinelli, 1985). Phytochrome regulation of anthocyanin accumulation and PAL activity has been well established in mustard cotyledons (Lange et al., 1971; Beggs et al., 1987).

The alpine and prairie ecotypes of *Stellaria longipes* when grown under varied R/FR, showed significantly higher levels of anthocyanin under high R/FR as compared to their counterparts under low R/FR. Also this response was more dramatic in the prairie ecotype as compared to the alpine ecotype. Anatomical and histochemical studies by Chuong (1998) revealed that the deposition of suberin and lignin in the epidermal and endodermal cells play a possible role in the control of stem elongation plasticity in *S. longipes*. In an attempt to understand the involvement of phenolic compounds

accumulation in stem elongation plasticity and regulation of anthocyanin biosynthesis in the two ecotypes of *S.longipes* under varied R/FR, the mRNA expression and enzyme activity of the first gene in the phenylpropanoid metabolism, PAL has been investigated.

Materials and methods

Plant growth conditions:

Genotypes 1D and 7B grown in SDC conditions for at least three months were etiolated for seven days and transferred to the chambers with varied R/FR ratios as described in chapter two.

Southern hybridization:

The blot containing 10μg of digested DNA per lane was hybridized at 58^{0}C in the Rapid-hyb buffer (Amersham, UK) for at least 5 hours using a 2.2 kb PAL cDNA fragment from *Stellaria longipes* as a probe. A full length PAL cDNA (2.2 kb) from *Stellaria longipes* was obtained by Dr. Q. Cai and cloned into pBS plasmid at EcoR I and Xho I sites.

Membranes were washed once in 2 x SSC plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 58^{0}C for 20 min, twice in 1 x SSC plus 0.1% SDS at 58^{0}C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 58^{0}C for 20 min. The membranes were then exposed to Kodak XAR 5 film at –80^{0}C.

RNA extraction:

Total RNA from stem and leaf tissues of plants grown under SDC conditions, from SDC plants after etiolation for seven days and the etiolated plants grown under

varied R/FR light was isolated using the guanidinium thiocyanate method (Strommer et al., 1993) as described in chapter three.

Northern hybridization:

The blot containing 15 µg of total RNA per lane was hybridized in Rapid-hyb buffer at 65°C for at least 5 hours using a 2.2 kb PAL cDNA from *Stellaria longipes* as a probe. The membranes were washed once in 2 x SSC plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 65°C for 20 min, twice in 1 x SSC plus 0.1% SDS at 65°C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 65°C for 20 min. The membranes were then exposed to Kodak XAR 5 film at –80°C.

Phenylalanine ammonia-lyase (PAL) extraction and assay:

PAL enzyme was extracted and assayed spectrophotometrically by the method of Khan and Vaidyanathan (1986). One gram of frozen stem and leaf tissues were homogenized in a pestle and mortar with 200 mg polyvinylpolypyrrolidone (PVPP) and 3 ml 150 mM Tris-HCl buffer (pH8.8) containing 12 mM ß-mercaptoethanol. The homogenate was centrifuged at 10,00 g for 10 minutes at 4°C to remove the cell debris. The resulting extract was precipitated with ammonium sulfate (70% saturation), centrifuged at 10,000 g for 10 minutes at 4°C and the precipitate was dissolved in 2 ml of 50 mM Tris-HCl (pH 8.8). The dissolved pellet was applied to a Sephadex G-25 column (2 cm diameter,10 cm length) equilibrated with 20 ml elution buffer containing 50 mM Tris-HCl (pH 8.8) and 12 mM ß-mercaptoethanol. Once the dissolved precipitate was completely adsorbed on to the column, the protein was eluted with 4 ml elution buffer. The eluant was used for PAL assay and determining the protein concentration. The PAL assay was performed at 37°C for 30 to 60 minutes in an assay mixture (500 µl) containing

225 µl of 50 mM Tris-HCl (pH 8.8), 250 µl of enzyme extract and 25 µl of 100 mM L-phenylalanine. The reaction was terminated by the addition of 100 µl of 5 M HCl and the reaction mixture was centrifuged at 10,000 g for 10 min to remove the precipitate that may influence the absorbance readings. PAL activity was determined spectrophotometrically by measuring the amount of *trans*-cinnamic acid formed at 290 nm. The PAL activity was expressed as µmol *trans*-cinnamic acid formed (CA) per mg protein per hour.

Total protein concentrations were estimated by Bradford dye-binding assay method (Bradford, 1976), using bovine serum albumin (BSA) as a standard. All Spectrophotometric measurements were done using Ultrspec 2000 UV/Visible spectrophotometer, Pharmacia Biotech Inc, Quebec, Canada.

Statistics:

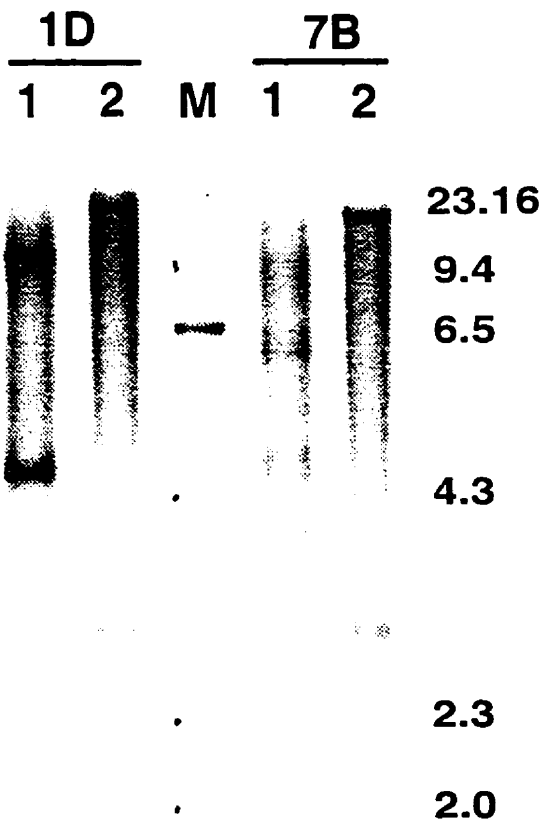
All the values in figures represent the means of at least three independent replicates and the standard errors of the means (SE) were calculated.

Results

Southern hybridization:

The genomic DNA of alpine and prairie ecotypes was digested using EcoRI or Hind III and hybridized with 2.2 kb cDNA PAL from *Stellaria*. The presence of two hybridizing bands in the genomic DNA of both the ecotypes digested with either of the enzymes suggests that the genome may contain about two copies of the PAL gene (Fig. 4.1). Also genomic DNA of alpine and prairie ecotypes did not reveal any polymorphism for EcoR I or Hind III.

Figure 4.1: Southern blot analysis of PAL in alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* . 10 μg of genomic DNA was digested with EcoRI (1) and Hind III (2), separated by electrophoresis in 1.2% agarose gel, blotted onto a nylon membrane and hybridized with the 2.2 kb PAL cDNA fragment from *Stellaria* . The numbers to the right represent the sizes of HindIII digested λ DNA fragments in Kbp marker (M).


1D
7B
1
2
M
1
2
23.16
9.4
6.5
4.3
2.3
2.0

Differential expression of PAL in the alpine and prairie ecotypes:

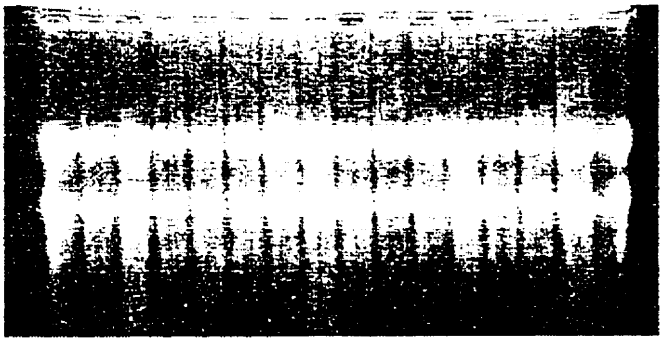
The expression of PAL mRNA in alpine and prairie ecotypes was examined under varied R/FR light. Total RNA was extracted from the alpine and prairie plants grown under SDC for 3 months and from SDC plants etiolated for 7 days (0 hr). The etiolated plants were transferred to low (L) or high (H) R/FR conditions and total RNA was extracted from 4 hr, 8 hr, 12 hr, 24 hr, 2 d, 5 d, 10 d and 20 d samples. The blots containing 15 µg RNA from all these samples were hybridized with 2.2 kb PAL cDNA from *Stellaria* (Fig. 4.2). Both the alpine and prairie ecotype showed minimum levels of transcript expression under SDC and no expression after 7 days of etiolation (0 hr). Upon transfer of the etiolated plants to low R/FR or high R/FR light there was an induction within 4 hr in both the ecotypes. 12 hr to 20 d samples of both the ecotypes grown under low or high R/FR showed stable levels of expression. Although the accumulation of anthocyanin was significantly higher for both the ecotypes under high R/FR as compared to low R/FR, neither of the ecotypes showed any significant differences in the transcript abundance of the samples between low R/FR and high R/FR, at any of the time intervals from 0 hr to 20 d. However 4 hr and 8 hr samples of prairie ecotype grown under both low and high R/FR showed increased levels of expression as compared to alpine ecotype.

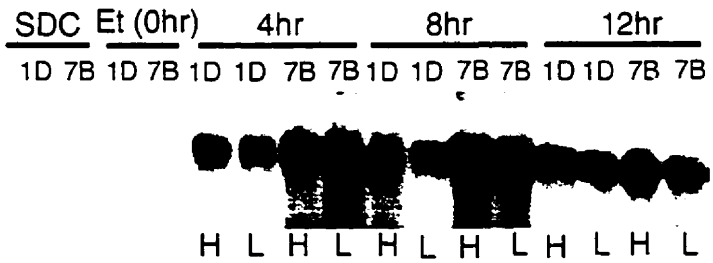
PAL activity in the alpine and prairie ecotypes under varied R/FR:

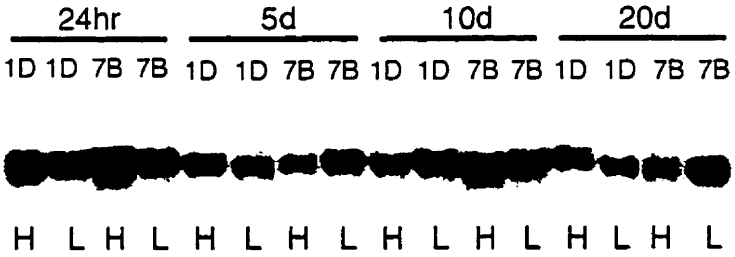
The effect of varied R/FR ratio on PAL activity was studied in both alpine and prairie ecotypes. The plants grown under SDC for 3 months were etiolated for 7 days (0 hr) and then transferred to the LDW chambers with low and high R/FR. Both alpine and prairie SDC plants when etiolated for 7days (0 hr) showed no PAL activity (Fig. 4.3). Both the ecotypes under both low and high R/FR conditions showed similar trends with

Figure 4.2: Expression of PAL mRNA in alpine and prairie ecotypes of *Stellaria longipes*. Each lane in the northern blot contains 15 µg of total RNA extracted from stem and leaf tissues. The different lanes containing RNA from alpine (1D) and prairie (7B) plants grown for three months under SDC conditions, etiolated for seven days (Et 0 hr) and 4 hr, 8 hr, 24 hr, 5 d, 10 and 20 d after their transfer to chambers with high R/FR (H) or low R/FR (L) are probed with random prime labeled 2.2 kb PAL cDNA fragment from *Stellaria*.

Ethidium bromide stained gel pictures corresponding to each of the blot are also shown for equal loading of RNA in each lane.




SDC
Et (0hr)
4hr
8hr
12hr
1D 7B 1D 7B 1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L H L H L


24hr
5d
10d
20d
1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L H L H L H L H L

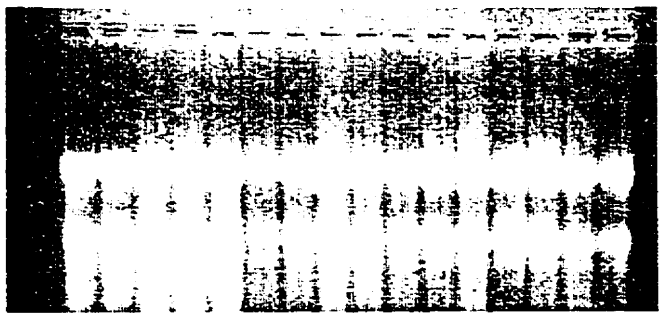
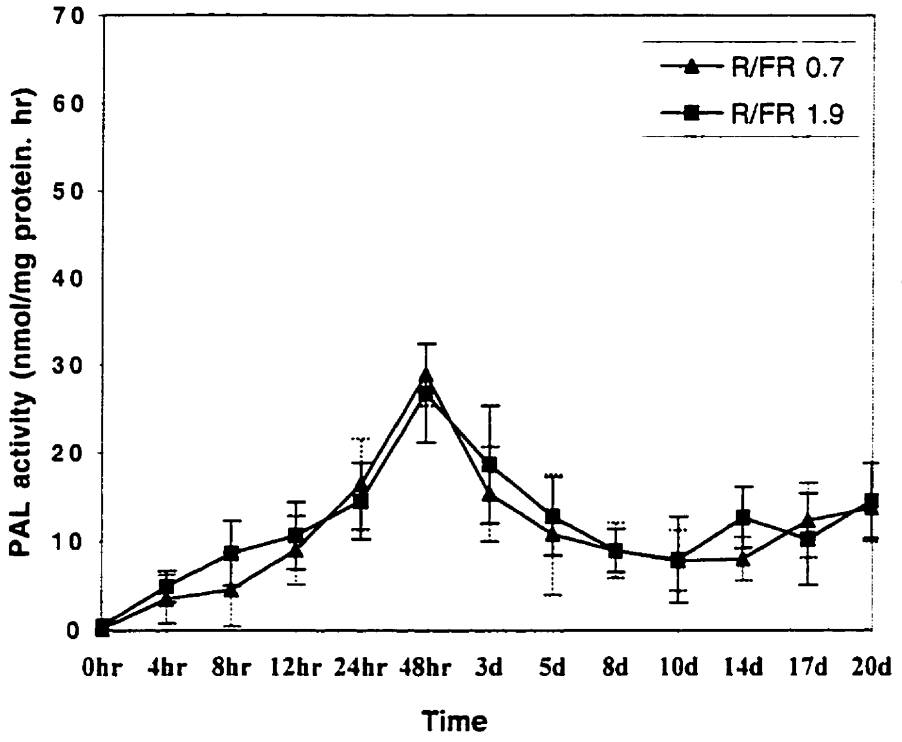
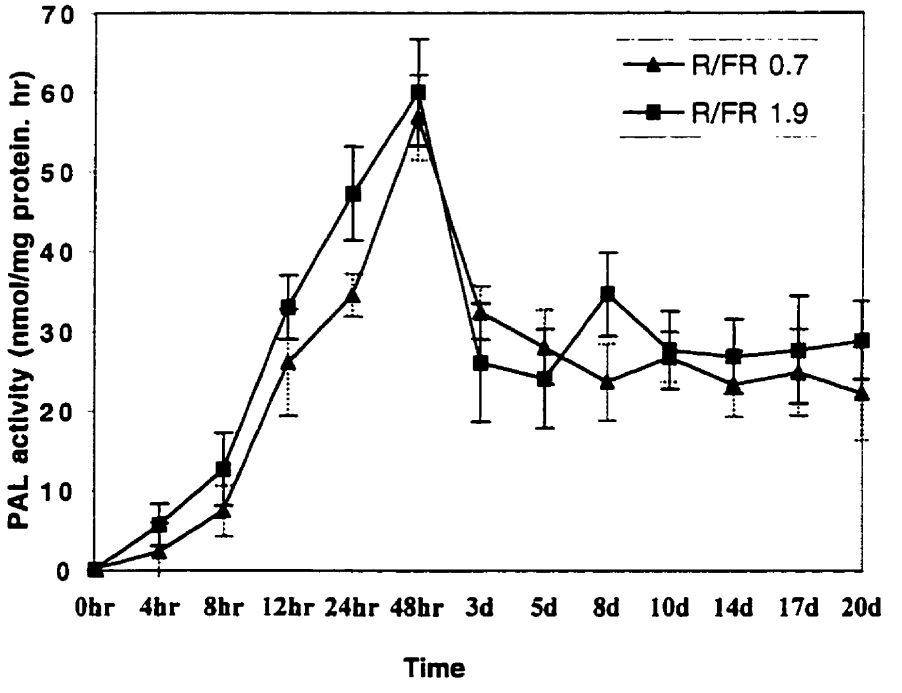


Figure 4.3: PAL activity in alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* upon transfer to varied R/FR conditions after etiolation of SDC plants for 7 days. Data are means of at least three individual experiments and the vertical bars indicate ± standard error (SE).


70
60
50
40
30
20
10
0
PAL activity (nmol/mg protein. hr)
R/FR 0.7
R/FR 1.9
0hr 4hr 8hr 12hr 24hr 48hr 3d 5d 8d 10d 14d 17d 20d
Time

1D


70
60
50
40
30
20
10
0
PAL activity (nmol/mg protein. hr)
R/FR 0.7
R/FR 1.9
0hr 4hr 8hr 12hr 24hr 48hr 3d 5d 8d 10d 14d 17d 20d
Time

7B

peak activity at 48 hr, although the activity in the prairie ecotype is much higher as compared to the alpine ecotype. Also both the ecotypes showed similar levels of activity up to 8 hr and from 8 hr to 20 d prairie ecotype showed significantly higher levels of PAL activity under both low and high R/FR than the alpine ecotype. R/FR ratio did not show any significant effect of on PAL activity in alpine or prairie ecotype.

Discussion

In order to study the regulation of PAL in the biosynthesis of anthocyanin in alpine and prairie ecotypes of *S.longipes* under varied R/FR, the mRNA expression and enzyme activity of PAL was studied. Southern blot analysis suggested that the PAL gene is present as a small gene family in *S.longipes* (Fig. 4.1) and no RFLP was detected between alpine and prairie ecotypes, suggesting possible similar genomic organization in the two ecotypes. These results are similar to the Southern analysis obtained by Chuong, (1998), for PAL in *S.longipes*. PAL has been reported to be encoded by multigene families in many plant species such as potato containing 40 to 50 PAL genes (Joos and Hahlbrock, 1992), bean and parsley containing three to four copies unlike in loblolly pine, which has only a single copy of PAL gene (Whetten and Sederoff, 1992). It has been shown that in *Arabidopsis thaliana* the multiple copies of PAL gene are expressed differentially during development and in response to environmental stimuli such as temperature, light, UV irradiation, fungal elicitor, wounding (Hahlbrock and Scheel, 1989; Lois et al., 1989; Ohl et al, 1990, Christie et al., (1994).

PAL activity is a prerequisite for the anthocyanin biosynthetic pathway. Inhibition of PAL activity by α-aminoxy β-phenylpropionic acid strongly reduces the photoinduction of anthocyanin (Goud et al., 1991; Amrhein and Godeke, 1977). The

involvement of phytochrome in the photoinduction of PAL was confirmed by R/FR reversibility experiments (Goud et al., 1991). In tomato red light initiates strong photoinduction in PAL activity, which can be reversed by a subsequent pulse of FR (Lercari et al., 1982). However, an increase in PAL activity has not always been correlated with the production of a specific phenylpropanoid compound (Camm and Towers, 1977). Studies by Brodenfeldt and Mohr (1988) showed although phytochrome-mediated induction of PAL and CHS is a prerequisite for the appearance of anthocyanins, there in no close correlation between enzyme levels and the rates of synthesis of the end products. Drumm and Mohr (1974) determined a linear relationship between Pfr and the amount of anthocyanin synthesised. Based on the lack of coordination in the kinetics of photoinduction of PAL and anthocyanin accumulation, they concluded that, "some Pfr-dependent mechanism beyond enzyme induction was considered necessary to explain the precisely linear relationship between the amount of Pfr established by a light pulse and the amount of anthocyanin produced by that light pulse". Similar studies by Adamse et al., (1989) demonstrated that photoinduction of PAL in tomato seedlings does not show good correlation with stimulation of anthocyanin biosynthesis. This was further confirmed with the use of *au* and *au, hp* tomato mutants, which produce little anthocyanin in spite of the induction of PAL level in response to red light which is similar to wild type (Goud et al., 1991). Their results indicate that the deficiency in bulk phytochrome in tomato mutants impairs anthocyanin biosynthesis but not through the photoregulation of PAL. This suggests a possible role of another enzyme or co-factor in regulating anthocyanin synthesis.

Both ecotypes of *S. longipes* showed basal level of PAL expression in the plants grown under SDC for three months, suggesting the existence of a low rate of secondary metabolism under cold temperatures. According to the studies by Chuong (1998), there was an induction of PAL transcript upon transfer of plants from LDW to SDC conditions. Peak levels were observed within a few hours of transfer, after which the transcript levels remained at basal level. The present results also showed similar trends. The etiolated plants (0 hr), of both the ecotypes prior to their exposure to varied R/FR showed no expression of PAL mRNA (Fig. 4.2), indicating that light is a prerequisite for the induction of PAL expression in *S.longipes*. Upon transfer of the etiolated plants to LDW chambers with varied R/FR, there is induction of PAL expression within 4 hr, which attained peak levels by 8 hr, followed by a slight decrease and stable levels of expression in both the ecotypes. This suggests the existence of the normal course of secondary metabolism during the growth of the plants under LDW conditions. The increase in the levels of PAL transcripts coincides with the increase in PAL enzyme activity with a lag phase in between (Figs. 4.2 & 4.3). The regulation of PAL activity in response to various environmental stimuli has been studied in several plants and three general mechanisms have been proposed. According to the first two mechanisms the increase in PAL activity is caused by an increased rate of activation of pre-existing zymogen (inactive form of enzyme) and by a decreased rate of degradation of the active enzyme (Lorschke et al., 1981). The third mechanism suggested *de novo* synthesis of PAL mRNA upon the receipt of stimulus (Jones, 1984; Leyva et al., 1995). The present results seem to support the first two mechanisms, considering the extended time lag between the appearance of the transcript and the enzyme activity. The significant differences in the enzyme activity

between the two ecotypes can also be explained on the basis of the first mechanism, that the pre-existing zymogen may be activated to a higher extent in the prairie ecotype resulting in higher enzyme activity as compared to the alpine ecotype. The results obtained also support the other proposed possibility that stored PAL-mRNA is released from a ribonucleoprotein particle on receipt of the stimulus followed by the usual ribosomal initiation (Jones, 1984). The immediate induction of PAL in the etiolated tissues (with no PAL expression) within 4 hr upon their transfer to LDW conditions with varied R/FR most likely rules out the possibilities of the third mechanism, which suggested the *de novo* synthesis. Although it is evident that light mediates PAL induction and anthocyanin synthesis, in *S.longipes* PAL induction does not seem to participate in controlling the anthocyanin accumulation under varied R/FR in either of the ecotypes used. Thus it seems phytochrome mediated anthocyanin synthesis may not be regulated through PAL at transcription or translation levels. However the results of the present study indicate that the subtle variation in the expression of PAL mRNA and significant variation in the PAL activity between 1D and 7B can be attributed partially to the ecotypic differences in the accumulation of anthocyanin. Thus it can be expected that the differences in the anthocyanin accumulation between alpine and prairie ecotypes if regulated through PAL, may be mostly at post-transcriptional level, as there are only very subtle variations in the mRNA expression but there is a significant difference in the enzyme activity between these two ecotypes. Furthermore, the variation with in each of the ecotypes in anthocyanin accumulation between low and high R/FR may be mediated by some Pfr-dependent mechanism beyond PAL induction.

Chapter five

Photoregulation of Chalcone synthase and its role in the accumulation of anthocyanin

Introduction

Anthocyanins, the largest subclass of flavonoids, due to their diverse range of functions, have been studied intensively in a number of different species. The precursors for the synthesis of all flavonoids including anthocyanins are malonyl-CoA and p-coumaroyl-CoA. Chalcone synthase (CHS) catalyses the condensation of malonylCoA with p-coumaroyl-CoA to yield tetrahydoxychalcone (Fig. 1.1). Chalcone isomerase (CHI) then catalyses the isomerization of the yellow-coloured tetrahydroxychalcone to the colourless naringenin (Mol et al., 1985). Naringenin upon subsequent steps can be converted to a range of flavonoid compounds. The simple visual detection of mutants defective in anthocyanin synthesis in maize and other plants led to the extensive isolation and characterization of anthocyanin biosynthetic genes. The first flavonoid biosynthetic gene isolated was CHS from parsley (Kreuzaler et al., 1983).

Recently regulatory genes that control expression of the structural genes of anthocyanin biosynthetic pathway have been identified from many plants. These regulator genes encode transcriptional activators that have homology to proteins from mammalian protooncogenes, *Myc* (Ludwig et al., 1989) and *Myb* (Paz-Ares et al., 1987). R and C1 regulatory genes encoding Myc and Myb like proteins respectively control the spatial and temporal accumulation of anthocyanin in different tissues of maize (Holton and Cornish, 1995). It is interesting that the regulation of anthocyanin biosynthesis by these genes differs in different plants. In maize, the R and C1 genes control the

expression of all anthocyanin biosynthesis genes (Dooner et al., 1991) whereas in petunia and snapdragon, the regulator genes control the expression of only a subset of biosynthetic genes (Martin et al., 1991; Quattrocchio et al., 1993).

In many higher plants, including *Arabidopsis thaliana*, maize and grape, transcription of the flavonoid biosynthetic genes in young seedlings is co-ordinately induced by light (Kubasek et al., 1992; Sparvoli et al., 1994; Taylor and Briggs, 1990). Low-temperature stress can enhance the transcript abundance of structural and regulatory genes in maize seedlings (Christie et al., 1994). In *Arabidopsis*, low temperature induces the accumulation of the mRNAs for PAL and CHS in a light-dependent manner (Leyva et al., 1995). An extensive literature is available on the fluence and wavelength requirements for anthocyanin synthesis in many plant species (Mancinelli, 1985; Batschauer and Schafer, 1996; Batschauer et al., 1996; Beggs and Wellmann, 1994). Merkle and co-workers (1994) measured the rate of CHS transcription in parsley cell suspension culture and showed that UV light is the most efficient inducer of CHS mRNA while red and blue light have a limited direct effect and can modulate the UV response. The alpine and prairie ecotypes of *Stellaria longipes* when grown under varied R/FR, showed significantly higher levels of anthocyanin under high R/FR as compared to their counterparts under low R/FR. Also this response was more dramatic in the prairie ecotype as compared to the alpine ecotype. In an attempt to understand the regulation of anthocyanin biosynthesis in the two ecotypes of *S.longipes* under varied R/FR, the mRNA expression and enzyme activity of the first gene, specific to anthocyanin biosynthetic pathway, CHS has been investigated. Since CHS is more specific to anthocyanin biosynthetic pathway, a closer correlation between mRNA expression,

enzyme activity of CHS and accumulation of anthocyanin under varied R/FR was expected.

Materials and methods

Plant growth conditions:

Genotypes 1D and 7B grown in SDC conditions for 3 months were etiolated for seven days and transferred to the chambers with varied R/FR ratios as described in chapter two.

PCR amplification and cloning of a partial CHS cDNA fragment;

Polymerase chain reaction (PCR)

All PCR reactions were carried out using a Robocycler (Stratagene, LaJolla, CA, USA) in 35 cycles of denaturation (1 min) at 95^0C, annealing (1 min) at 58^0C, and primer extension / enzymic reaction (2 min) at 72^0C, using Taq DNA polymerase (Pharmacia).

Reverse transcription

The reverse transcription was carried out as described in chapter three.

Cloning of partial CHS cDNA fragment

mRNA was extracted from the plants and the corresponding first strand cDNA was synthesized using AMV reverse transcriptase kit and this was used as template in PCR along with the designed primers. Primers were designed from the most conserved region of known CHS sequences and used in polymerase chain reaction to amplify a partial 903 bp cDNA from *Stellaria longipes*. The nucleotide sequence of the primers is as follows:

Forward primer: 5 CGA, GCG, GAA, TTC, GAC/T, TAT/C, TAT/C, TTT/C, A/C G/A G/C, ATC, ACA/C, AAG/C, AGT/C, GAA 3 (42 bp)

Reverse primer: 5 GCG, GTG, CTC, GAG, G G/C C, C A/G A, A G/C C, C A/G A, A G/C A, G A/G/C A, C A/G/C C, CCC, A A/G/C T 3 (39 bp)

The RT – PCR reaction mixture (50 µl) contained 5 µl of 10 x Taq buffer, 2.5 µl of cDNA first strand, 1 µl of 10 mM dNTPS, 0.2 µl of Taq enzyme (5 units/µl), 2.5 µl of 10 nmol forward and reverse primers each and sterile distilled water to make up the volume to 50 µl.

The PCR amplified partial cDNA was purified, cloned into a pCR 2.1 (invitrogen) plasmid vector at Xho I / EcoR I site.

DNA sequencing:

The PCR amplified partial cDNA were cloned and sequenced with T_7 and M_{13} reverse primers using the automatic sequencing facility available at Department of Medical Biochemistry, University of Calgary. Upon conceptual translation, amplified sequence showed a high degree of homology to other cloned CHS sequences.

Nucleotide and amino acid sequence analysis:

Homology search for the DNA sequence were made at Gen Bank as described in chapter three.

RNA extraction:

Total RNA from stem and leaf tissues of plants grown under SDC conditions, from SDC plants after etiolation of seven days and the etiolated plants grown under varied R/FR light was isolated using the guanidinium thiocyanate method (Strommer et al., 1993) as described in chapter three.

Southern blotting and hybridization:

The blot containing 10 µg of digested DNA per lane was hybridized at 58^{0}C in the Rapid-hyb buffer (Amersham, UK) for at least 5 hours using a 903 bp CHS cDNA fragment from *Stellaria longipes* as probe. Membranes were washed once in 2 x plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 58^{0}C for 20 min, twice in 1 x SSC plus 0.1% SDS at 58^{0}C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 58^{0}C for 20 min. The membranes were then exposed to Kodak XAR 5 film at –80^{0}C.

Northern blotting:

The blots containing 15 µg of total RNA per lane were hybridized in Rapid-hyb buffer at 65^{0}C for at least 5 hours using 903 bp CHS cDNA fragment from *Stellaria longipes* as a probe. The membranes were washed once in 2 x SSC plus 0.1% SDS at room temperature, twice in 2 x SSC plus 0.1% SDS at 65^{0}C for 20 min, twice in 1 x SSC plus 0.1% SDS at 65^{0}C for 20 min and twice in 0.2 x SSC plus 0.1% SDS at 65^{0}C for 20 min. The membranes were then exposed to Kodak XAR 5 film at –80^{0}C.

CHS extraction:

CHS enzyme was extracted and assayed using HPLC by following the method used by Zuurbier et al., (1993). One g of frozen stem and leaf tissues were homogenized in a pestle and mortar with 10% w/w polyvinylpolypyrrolidone (PVPP) and 5 ml 0.1 M K-Pi extraction buffer (pH 6.8) containing 14 mM ß-mercaptoethanol, 40 mM ascorbic acid, 3 mM EDTA, 10 µM Leupeptin and 0.2 mM Phenyl methyl sulphonyl fluoride. The homogenate was centrifuged at 10,000 g for 20 minutes at 4^{0}C to remove the cell debris. The resulting extract was precipitated with ammonium sulphate (70% saturation),

centrifuged at 10,000 g for 10 minutes at 4^0C and the precipitate was dissolved in 2.5 ml PD 10 buffer (0.1 M K-Pi buffer pH 6.8, 1.4 mM ß-mercaptoethanol, 40 mM ascorbic acid and 5% trehalose). The dissolved pellet was applied to a Sephadex G-25 column (size 2 cm, diameter x 10 cm) equilibrated with 20 ml PD10 buffer. Once the dissolved protein was completely absorbed in the column, the protein was eluted twice with 2.5 ml PD 10 buffer. The eluant was used for CHS assay and determining the protein concentration. Total protein concentrations were estimated by Bradford dye-binding assay method (Bradford, 1976), using bovine serum albumin (BSA) as a standard.

CHS assay using HPLC:

CHS assay was performed at 37 ^{0}C for at least 60 min in an assay mixture containing 50 ul protein extract (7 mg/ml), 50 µl assay buffer (0.5 M K-Pi pH 6.8, 2.8 m M ß-mercaptoethanol and 2% w/v BSA), 5 µl 0.4 mM Malonyl CoA (2 nmol) and 5 µl 0.2 mM p-coumaroyl CoA (1 nmol). At the end of the incubation period 200 µl degassed EtOAc (ethyl acetate) was added, mixed by vortexing and centrifuged at 10.000 g for 5 minutes. The EtOAc layer was transferred into an eppendorf tube and evaporated to dryness using a vacuum concentrator. The residue was then dissolved in 20 µl MeOH and analysed by HPLC. The HPLC system used consisted of an LKB model 2150 HPLC pump, an LKB model 2151 variable wavelength monitor and chromatographic data processor. A guard column packed with octadecyl silica (30 um particle size), a 5 um Rosil C18 seperation column (250 x 4.6 mm id) and an additional seperation column (150 x 4.6 mm id) were used. The eluent consisted of MeOH-H_2O-85% H_3PO_4 (280:136:1), pH 2.6 and a flow rate of 1 ml.min $^{-1}$ was used with pressure, 230 bar.

Synthesis of p-coumaroyl-CoA:

The substrate p-coumaroyl-CoA is not available commercially and was synthesized following the modified method of Meng and Campbell (1997). The clone pQE15, *E.coli* harbouring coumaroyl-CoA ligase plasmid (ligates coumaric acid and CoA in the presence of ATP) was kindly provided by Dr.P.Facchini, Department of Biological Sciences, the University of Calgary. Overnight grown culture of pQE15 in LB medium with 100 μg/ml ampicillin was induced with IPTG to a final concentration of 1 mM. The cells were spun down, resuspended in 2 ml 0.2 M MOPS pH 7.8 and sonicated with three 20-second pulses. The cellular debris was removed by centrifugation at 10,000 g for 10 min and the crude enzyme extract is used for synthesis of coumaroyl-CoA. Synthesis reaction (10 ml) containing 10 mM $MgCl_2$, 1 mM DTT, 0.5 mM coumaric acid, 2.5 mM ATP, 0.25 mM CoA, 0.5 ml enzyme extract and 0.2 M MOPS, pH 7.5, was set up for 1 hr at 30^0C. Sep-Pak column was prepared by washing with 20 ml MeOH, followed with 20 ml 200 mM MOPS pH 7.8. Ten ml reaction mixture was applied to the column, the column was washed with 20 ml water and coumaroyl-CoA was finally eluted with 20 ml MeOH. MeOH was evaporated in a Speed-Vac, the residue was dissolved in filter sterilized double distilled H_2O and quantified using the standard formula, $E = A / bc$. Where E is the molar extinction coefficient, which is 21 mM^{-1}, A is absorption at 333 nm, b is the band width (1 cm) and c is the concentration.

Results

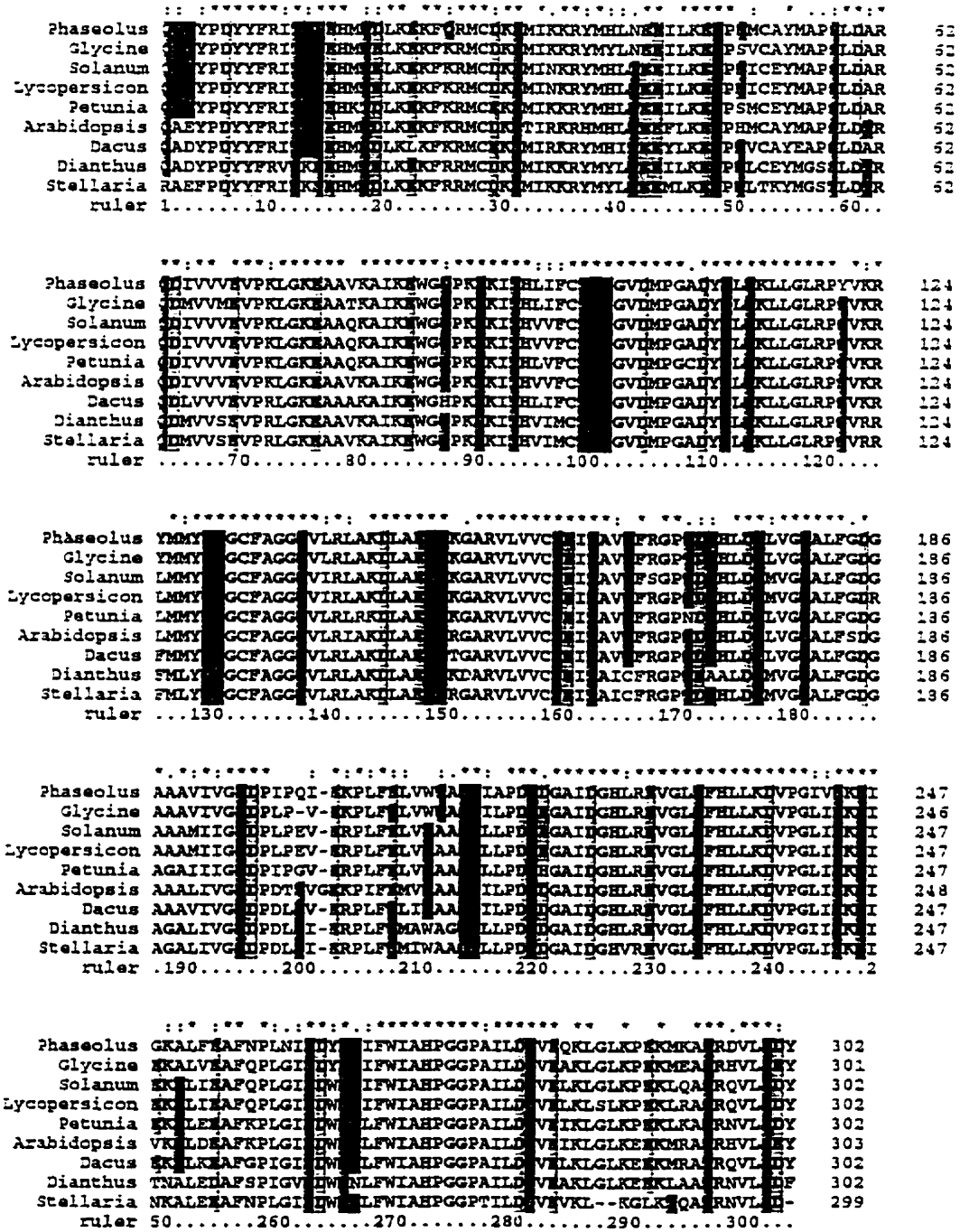
PCR amplification and cloning of partial CHS cDNA from *Stellaria*:

A 903 bp fragment was amplified by PCR using the cDNA of *S.longipes* with the primers corresponding to the conserved regions of chalcone synthase, cloned into PCR^R

Figure 5.1: The nucleotide sequences of CHS cDNA fragment from *Stelalria*. This 903 bp fragment was PCR amplified from the cDNA of prairie ecotype of *Stellaria longipes* and encodes 301 amino acids.

```
  1 CGA GCG GAA TTC CCG GAT TAT TAT TTC CGG ATC ACA AAG AGT GAA CAT ATG  ACT GAT TTG   60
  1  R   A   E   F   P   D   Y   Y   F   R   I   T   K   S   E   H   M    T   D   L    20
 61 AAG GAG AAG TTC AGG CGC ATG TGT GAC AAA TCA ATG ATC AAG AAG CGT TAC ATG TAC TTA  120
 21  K   E   K   F   R   R   M   C   D   K   S   M   I   K   K   R   Y   M   Y   L    40
121 ACC GAG GAG ATG CTT AAG GAA AAT CCC AAT TTA ACC AAA TAC ATG GGT TCA ACA CTC GAC  180
 41  T   E   E   M   L   K   E   N   P   N   L   T   K   Y   M   G   S   T   L   D    60
181 ACG CGG CAA GAC ATG GTC GTG TCC GAG GTC CCT AGG CTA GGC AAG GAG GCT GCT GTC AAG  240
 61  T   R   Q   D   M   V   V   S   E   V   P   R   L   G   K   E   A   A   V   K    80
241 GCC ATT AAG GAA TGG GGT CAG CCC AAG TCC AAA ATC ACC CAT GTT ATT ATG TGC ACC ACT  300
 81  A   I   K   E   W   G   Q   P   K   S   K   I   T   H   V   I   M   C   T   T   100
301 TCC GGT GTC GAC ATG CCC GGG GCC GAC TAC CAA CTC ACC AAA CTC CTC GGT CTT CGT CCC  360
101  S   G   V   D   M   P   G   A   D   Y   Q   L   T   K   L   L   G   L   R   P   120
361 TCT GTC CGT CGT TTC ATG CTC TAC CAG CAA GGT TGC TTT GCT GGA GGA ACG GTC CTT CGT  420
121  S   V   R   R   F   M   L   Y   Q   Q   G   C   F   A   G   G   T   V   L   R   140
421 CTA GCC AAG GAC CTA GCA GAA AAC AAC AGA GGC GCA CGA GTT TTA GTT GTG TGT TCT GAA  480
141  L   A   K   D   L   A   E   N   N   R   G   A   R   V   L   V   V   C   S   E   160
481 ATT ACA GCC ATT TGT TTC CGT GGG CCT ACA GAT ACC CAC CTA GAC TCA ATG GTG GGT CAA  540
161  I   T   A   I   C   F   R   G   P   T   D   T   H   L   D   S   M   V   G   Q   180
541 GCC TTA TTC GGG GAC GGC GCC GGC GCC CTC ATC GTT GGA TCG GAC CCG GAT CTA TCG ATT  600
181  A   L   F   G   D   G   A   G   A   L   I   V   G   S   D   P   D   L   S   I   200
601 GAA CGC CCG CTC TTC CAA ATG ATA TGG GCA GCC CAA ACA CTC CTC CCG GAC TCA GAC GGT  660
201  E   R   P   L   F   Q   M   I   W   A   A   Q   T   L   L   P   D   S   D   G   220
661 GCA ATT GAT GGA CAT GTA CGT GAA GTT GGG CTA ACA TTT CAC CTC CTT AAA GAT GTA CCT  720
221  A   I   D   G   H   V   R   E   V   G   L   T   F   H   L   L   K   D   V   P   240
721 GGG CTT ATT TCT AAG AAC ATT AAT AAG GCC CTA GAA GAA GCA TTT AAC CCA CTT GGT ATT  780
241  G   L   I   S   K   N   I   N   K   A   L   E   E   A   F   N   P   L   G   I   260
781 TCC GAC TGG AAC TCC CTC TTT TGG ATA GCC CAC CCA GGT GGC CCA ACA ATT CTG GAC CAA  940
260  S   D   W   N   S   L   F   W   I   A   H   P   G   G   P   T   I   L   D   Q   280
841 GTT GAG GTG AAA CTA GGC CTT AAA GAA GAA AAA CTC CAA GCA ACT AGG AAT GTG TTG AGT  900
281  V   E   V   K   L   G   L   K   E   E   K   L   Q   A   T   R   N   V   L   S   300
901 GAC                                                                           903
301  D
```

Figure 5.2: Partial amino acid sequence alignments of CHS from *Stellaria longipes*, tomato, carrot, *Solanum* , *Arabidopsis* , *Glycine*, *Phaseolus*, *Petunia* and *Dianthus* . Identical amino acid residues have asterisks above.


Phaseolus
Glycine
Solanum
Lycopersicon
Petunia
Arabidopsis
Dacus
Dianthus
Stellaria
ruler

2.1 Invitrogen plasmid vector at 5 Xho I / EcoR I 3 sites and sequenced (Fig. 5.1). Conceptual translation of this sequence showed 90 % homology to predicted amino acid sequences from *Solanum*, *Lycopersicon*, *Nicotiana*, *Glycine*, *Phaseolus*, *Petunia*, *Dacus* and *Arabidopsis* (Fig. 5.2).

Organization of CHS genes in *S.longipes*:

The genomic DNA of alpine and prairie ecotypes was digested with BamHI, EcoRI, EcoRV, Hind III, XbaI, or PstI and hybridized with CHS cDNA fragment from *Stellaria*. The presence of four hybridizing bands with Hind III- or Xba I- digested genomic DNA suggests that there is a multiple *CHS* gene family in the *S.longipes* genome (Fig. 5.3). Also the genomic DNA of alpine and prairie ecotypes did not reveal any polymorphism for any of the enzymes used.

Differential expression of CHS in alpine and prairie ecotypes of S.longipes:

The expression of CHS mRNA in alpine and prairie ecotypes was examined under varied R/FR light. Total RNA was extracted from the alpine and prairie plants grown under SDC for 3 months and from SDC plants etiolated for 7 days (0 hr). The etiolated plants were transferred to low (L) or high (H) R/FR conditions and total RNA was extracted from 4 hr, 8 hr, 12 hr, 24 hr, 2 d, 5 d, 10 d and 20 d samples. The blots containing 15 μg RNA from all these samples were hybridized with 903 bp cDNA from *Stellaria* (Fig. 5.4). Both alpine and prairie plants grown under SDC for three months and SDC plants after 7 days of etiolation (0 hr) showed no expression of the CHS transcript. Upon transfer of the etiolated plants to low R/FR or high R/FR light there is an induction within 4 hr in both the ecotypes. The expression of CHS for 1D under both high and low

Figure 5.3: Southern blot analysis of CHS in alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* . 10 μg of genomic DNA was digested with BamH I (1), EcoR I (2), EcoR V (3), Hind III (4), Xba I (5), Pst I (6), separated by electrophoresis in 1.2% agarose gel, blotted onto a nylon membrane and hybridized with the 903 bp CHS cDNA fragment from *Stellaria*. The numbers to the right represent the sizes of λ 1kb ladder fragments (M).

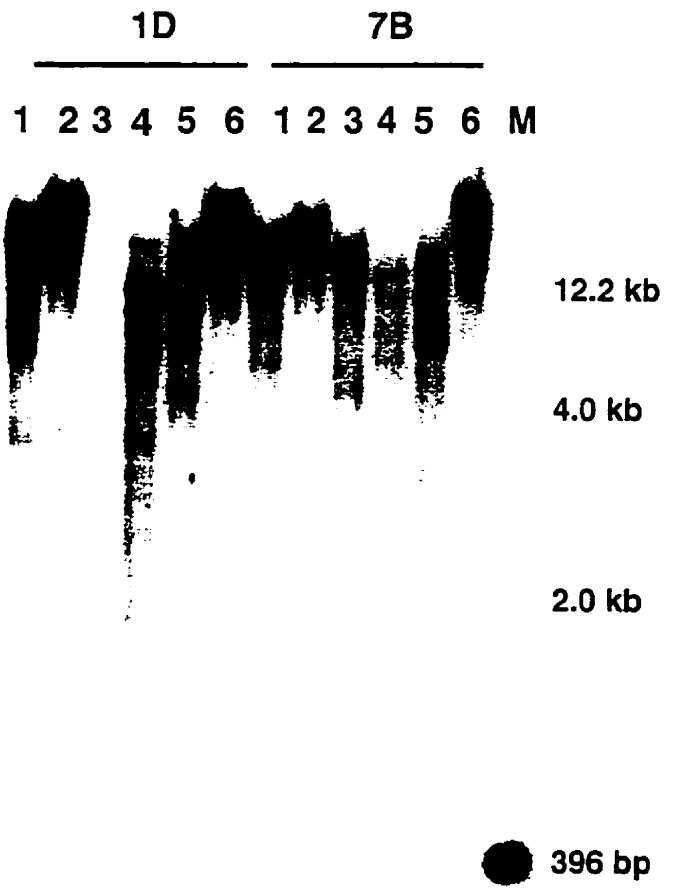
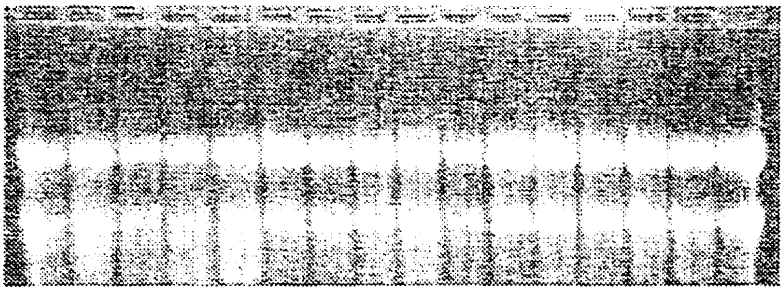
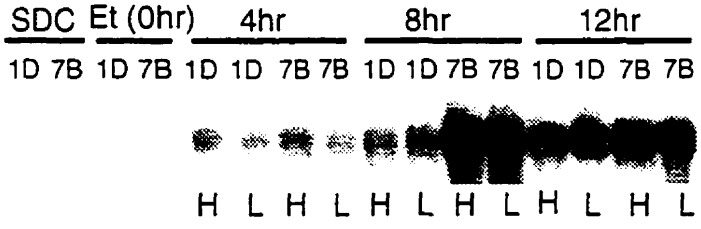
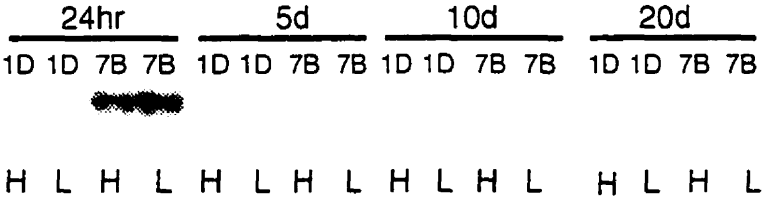

1D
7B
1 2 3 4 5 6 1 2 3 4 5 6 M
12.2 kb
4.0 kb
2.0 kb
396 bp

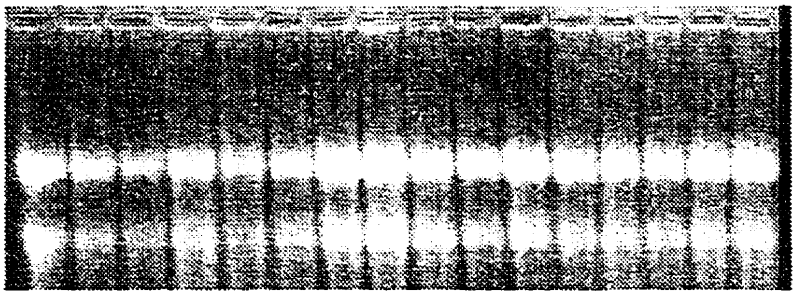
Figure 5.4: Expression of CHS mRNA in alpine and prairie ecotypes of *Stellaria longipes*. Each lane in the northern blot contains 15 μg of total RNA extracted from stem and leaf tissues. The different lanes containing RNA from alpine (1D) and prairie (7B) plants grown for three months under SDC conditions, etiolated for seven days (Et 0 hr) and 4 hr, 8 hr, 24 hr, 5 d, 10 and 20 d after their transfer to chambers with high R/FR (H) or low R/FR (L) are probed with random prime labeled 903 bp CHS cDNA fragment from *Stellaria*.

Ethidium bromide stained gel pictures corresponding to each of the blot are also shown for equal loading of RNA in each lane.




SDC
Et (0hr)
4hr
8hr
12hr
1D 7B 1D 7B 1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L H L H L


24hr
5d
10d
20d
1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B 1D 1D 7B 7B
H L H L H L H L H L H L H L H L



R/FR reached maximum levels by 12 hr unlike 7B, which showed highest levels of expression by 8 hr. Also 7B under both low and high R/FR showed significantly higher levels of expression than 1D from 8 hr to 24 hr. Although the accumulation of anthocyanin was significantly higher for both the ecotypes under 1.9 R/FR as compared to 0.7 R/FR, neither showed significant differences in the transcript abundance of CHS between low R/FR and high R/FR.

CHS assay by HPLC:

To study the activity of CHS based upon HPLC analysis, a naringenin standard curve through HPLC was determined (Fig. 5.5). The detection was performed at 290 nm, the absorbance wavelength of naringenin. In the HPLC system used, naringenin standard in methanol eluted at a retention time of 8.45 min as a sharp peak. The detection response was linear up to 650 nmol. The elution profile for naringenin was also detected in the presence of the substrates malonyl CoA and p-coumaroyl-CoA. Both malonyl-CoA and p-coumaroyl-CoA eluted much before naringenin (Fig. 5.6).

The effect of varied R/FR ratio on CHS activity was studied in both alpine and prairie ecotypes. The plants grown under SDC for 3 months were etiolated for 7days (0 hr) and then transferred to the LDW chambers with low and high R/FR. Both alpine and prairie SDC plants when etiolated for 7 days (0 hr) showed no CHS activity (Fig. 5.7). Both the ecotypes under low and high R/FR conditions showed similar trends with peak activity at 8 hr for 7B and 12 hr for 1D. Both the ecotypes showed similar levels of activity up to 4 hr. The prairie ecotype under high and low R/FR showed higher levels of CHS activity than the alpine ecotype from 8 hr to 2 d. Both alpine and prairie ecotypes

Fig 5.5: Naringenin standard curve through HPLC. Different concentrations of naringenin standard in methanol were eluted through HPLC and the peaks corresponding to naringenin were detected at 290 nm, the absorbance wavelength of naringenin.

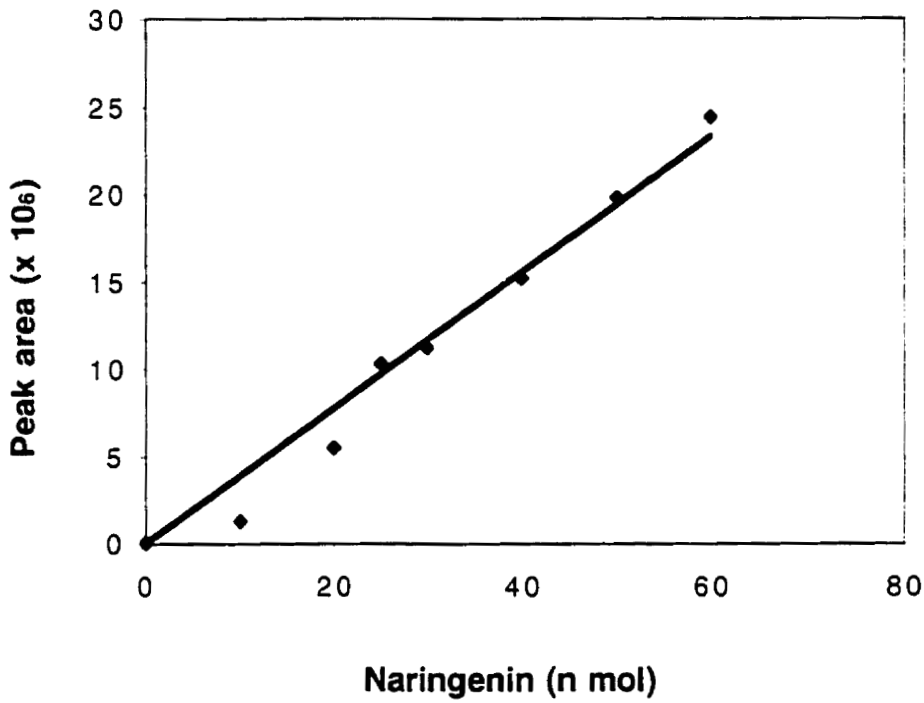

30
25
20
15
10
5
0
Peak area (x 10₆)
0
20
40
60
80
Naringenin (n mol)

Fig 5.6: The elution profile for naringenin, malonyl CoA and p-coumaroyl CoA standards detected at 290 nm through HPLC. The peaks obtained at 4.68 min and 8.45 min correspond to p-coumaroyl-CoA and naringenin, respectively.

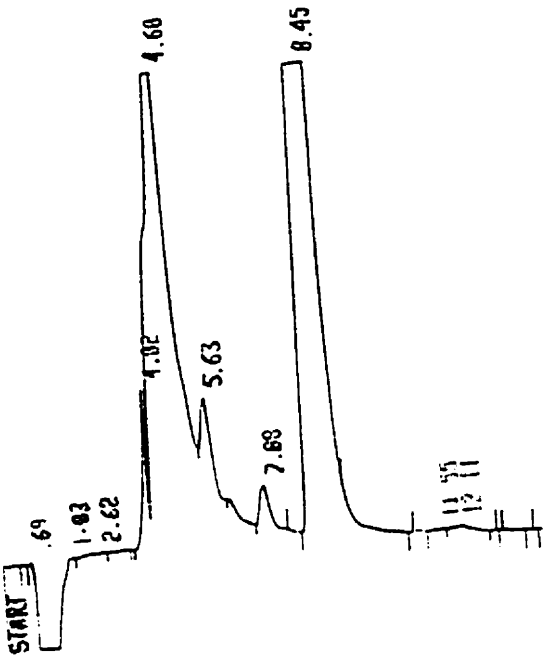
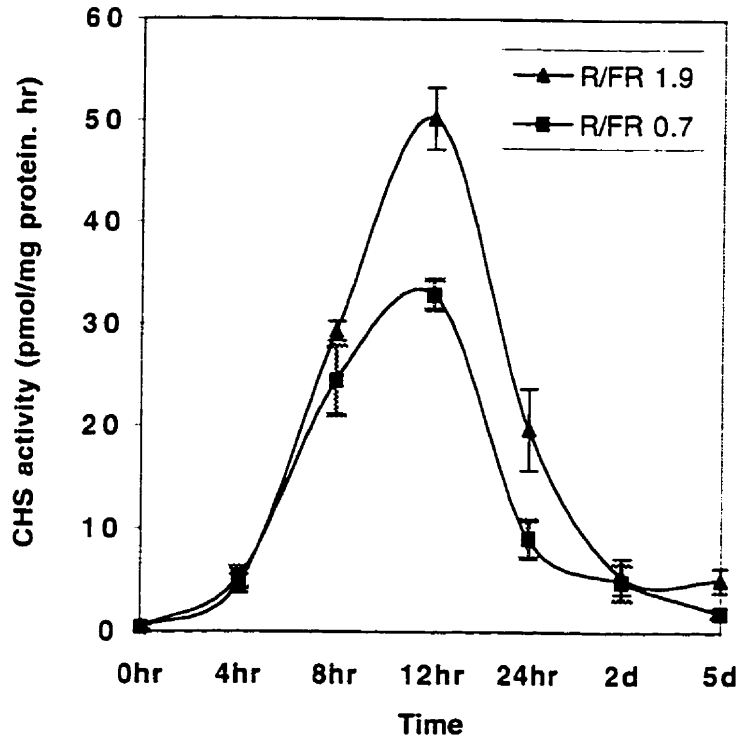
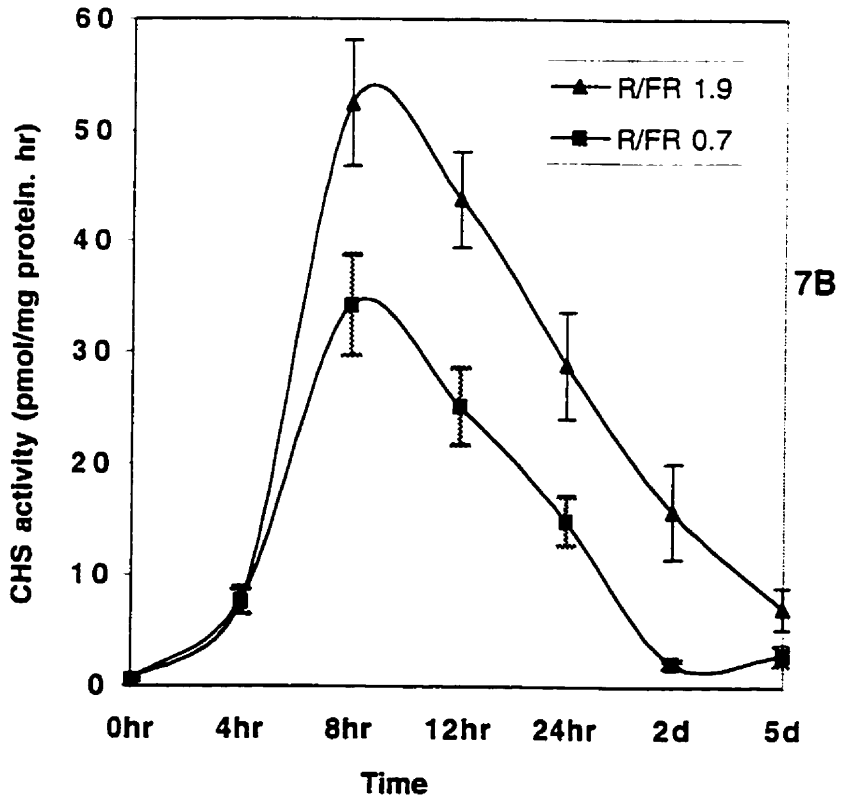

START
.69
1.03
2.82
4.60
5.63
7.68
8.45

Figure 5.7: CHS activity in alpine (1D) and prairie (7B) ecotypes of *Stellaria longipes* upon transfer to varied R/FR conditions after etiolation of SDC plants for 7 days. Data are means of at least three individual experiments and the vertical bars indicate $\pm$ standard error (SE).


60
50
40
30
20
10
0
CHS activity (pmol/mg protein. hr)
R/FR 1.9
R/FR 0.7
0hr
4hr
8hr
12hr
24hr
2d
5d
Time

1D


60
50
40
30
20
10
0
CHS activity (pmol/mg protein. hr)
R/FR 1.9
R/FR 0.7
0hr
4hr
8hr
12hr
24hr
2d
5d
Time

7B

showed higher levels of CHS activity under high R/FR as compared to their counterparts under low R/FR. Also there is strong correlation between the mRNA expression and the enzyme activity in both the ecotypes, in terms of duration of the expression. Although neither of the ecotypes showed any significant difference in the transcript abundance of CHS between low R/FR and high R/FR, both the ecotypes under high R/FR showed higher CHS activity than under low R/FR. Thus in *S.longipes* the CHS activity under varied R/FR corresponds to the accumulation of anthocyanin, with a time lag between the enzyme activity and the accumulation of end product.

Discussion

In order to study the regulation of CHS in the biosynthesis of anthocyanin in alpine and prairie ecotypes of *S.longipes* under varied R/FR, a partial CHS cDNA was amplified (Fig. 5.1). In comparison with previously reported sequences, the amplified CHS sequence from *S.longipes* exhibited 95% identity to the corresponding predicted amino acid sequences (Fig. 5.2). Southern blot analysis suggested that the CHS gene is present as a small gene family in more than 3 copies (Fig. 5.3) and no RFLP was detected between alpine and prairie ecotypes, suggesting similar genomic organization in the two ecotypes. CHS has been reported to be encoded by multigene families in many plant species such as *Petunia* (Koes et al., 1987), pea (Harker et al., 1990) grapevine (Sparvoli et al., 1994) unlike in *Arabidopsis*, which has a single copy of CHS gene (Feinbaum and Ausubel, 1988). Unlike PAL the expression of CHS is very short in both the ecotypes of *S.longipes* (Figs. 4.2 & 5.4).

Studies by Knogge et al., (1986) with oat primary leaves showed that CHS is the rate-limitting enzyme in the pathway leading to anthocyanin accumulation. Similar studies by Chappell and Hahlbrock (1984) showed that the light-mediated appearance of CHS is rate-limiting for the appearance of flavonol glycoside in parsley cell cultures. However studies by Mohr and co-workers (Drumm and Mohr, 1974; Brodenfeldt and Mohr, 1988) do not support the idea that phytochrome mediated anthocyanin synthesis is controlled by PAL or CHS induction and proposed that there may be a second phtochrome-dependent step, beyond the enzyme induction, where the actual rate of flavonoid accumulation is determined. The present data obtained seem to support the latter view for PAL, since neither ecotypes showed any significant difference in the transcript abundance and activity of PAL between low and high R/FR in spite of significantly higher levels of anthocyanin accumulation in both the ecotypes under high R/FR as compared to low R/FR. Unlike for PAL, both the ecotypes showed significantly higher levels of CHS activity under high R/FR, corresponding to their anthocyanin accumulation. These results indicate post-transcriptional regulation of CHS, which is more specific to anthocyanin synthesis than PAL. Also the results do support the idea that light is required for the expression of both the genes, as the etiolated plants did not show any expression of PAL or CHS (Figs. 4.2, 4.3, 5.4 & 5.7). The expression and activity of CHS in alpine ecotype as compared to prairie ecotype under both high and low R/FR is slightly lower for 8 hr, 12 hr and 24 hr samples. Although both ecotypes showed CHS induction within 4 hr of their transfer to varied R/FR, prairie ecotype showed maximum levels of expression and activity within 8 hr unlike, alpine ecotype, which is delayed until 12 hr. These subtle variations in the expression of CHS mRNA between 1D and 7B may

be mediated by differential regulation of CHS regulatory genes and can be attributed to the ecotypic differences in the accumulation of anthocyanin. It has been shown that in pea CHS is encoded by a multigene family and the response of all the members to elicitor and UV irradiation was not the same and varied developmentally and environmentally (Ito et al., 1997). Since in *S.longipes* also CHS is encoded by multigene family, it may be possible that the cDNA clone we have used may not be the member that responds to varied R/FR. This seems less likely because the cDNA used as probe was amplified from the tissue grown under high R/FR. Based on the pattern of mRNA expression in the pigmented and unpigmented Shiraz grape tissues Boss et al., (1996) suggested that UFGT (UDP glucose: flavonoid 3-O-glucosyl transferase) is under a different regulatory regime compared to other flavonoid pathway genes, as the earlier genes of the flavonoid pathway are expressed in both pigmented and most unpigmented tissues unlike UFGT which is not expressed in white grapes. The expression of the earlier genes of the flavonoid pathway in unpigmented tissues may be involved in the synthesis of other flavonols such as proanthocyanindins (colourless). In maize expression of all the genes from CHS to UFGT (Fig. 1.1) is induced when tissues become pigmented, whereas in snapdragon and petunia flowers, the control start points are further on in this pathway, being F3H and DFR respectively (Beld et al., 1989; Huits et al., 1994; Martin et al., 1991). These results suggest that the main point of regulation for anthocyanin synthesis may not be identical in all tissues at all times but rather a number of separate regulatory mechanisms exist. Thus a large number of regulatory and structural genes that are differentially responsive to developmental signals and environmental stimuli can result in sensitive modulation of the flavonoid biosynthetic pathway.

Chapter six

General conclusions and perspectives

The present project involved comparison of shade avoidance stem elongation and anthocyanin accumulation between the alpine and prairie ecotypes of *Stellaria longipes*, with contrasting levels of phenotypic plasticity. When the alpine and prairie plants were grown in chambers with either low (0.7) or high (1.9) R/FR ratio, both the ecotypes responded to the quality of light. However, prairie plants were more responsive. They showed a significant increase in the stem elongation and leaf area with a concomitant decrease in the dry mass and anthocyanin accumulation when grown under low R/FR. In order to study whether the induced shade avoidance responses have strong correlations with the components of fitness, experiments involving reciprocal transplanting of ecotypes between natural habitats with contrasting competitive conditions should be conducted. For example alpine plants, when planted in prairie habitat, need to exhibit greater shade avoidance stem elongation in order to survive in the new habitat with greater competing vegetation from the neighbours. Similarly, the naturally shade avoiding stem elongation plasticity of the prairie ecotype may be disadvantageous when these plants are grown in the alpine habitat with greater wind stress. The survival of these plants in the reciprocal habitats depends upon the flexibility of their genomic expression leading to an appropriate phenotype, often referred to as phenotypic plasticity. This type of study might aid in our understanding of the importance of phenotypic plasticity as a selective force in plant evolution. If some plants survive, one can conduct differential screening in order to explore the molecular basis of phenotypic plasticity and gain insights into the genes responsible for the adaptive changes, which are turned on/off in an

opportunistic manner. Also, it has been argued that the shade avoidance stem elongation response can be deleterious in terms of productivity, because the resources committed to stem growth in dense stands may reduce the amount of assimilates available for leaf or root growth (Smith, 1992; Ballare et al., 1990; Thompson and Harper, 1988; Casal and Smith 1989; Novoplansky et al., 1990). Transgenic manipulation of the relative levels of PHYA and PHYB can markedly modify the allocation of assimilates in crop plants in the field (Smith, 1995). Since for majority of the plants, the relationship between harvestable yield and total biomass is quite constant and if genetic engineering designed to disable the responses of plant to their neighbours results in improved ratios of yield to biomass, then increased productivity may be expected. However, before one arrives at this conclusion, one should ensure that the growth of the stems and the growth of the resource harvesting structures are alternatives in an economic sense for that particular system. It has been shown that for *Amaranthus*, the carbon used for stem elongation in response to low R/FR is not subtracted from a pool that would otherwise be allocated to other organs, but results from an increase in net photosynthesis (Ballare et al., 1991b).

Stem elongation is a complicated process that involves the coordination of cell division and cell expansion. It has been shown that stem elongation in *Stellaria longipes* involves both cell division and cell elongation in cortical cells and only cell elongation in the epidermal cells (Chuong, 1998). Furthermore, based upon the correlation between the thickening of the epidermal cell walls and inhibition of stem elongation, it was speculated that epidermal cells play an important role in controlling the rate of stem elongation. Stem elongation in plants is inhibited by white light, possibly by decreasing cell wall extensibility (Heupel and Kutschera, 1997). The light-mediated reduction of cell wall

extensibility has been shown to be restricted to the peripheral cell layers (Hodick and Kutschera, 1992). When 7B plants from the prairie habitat were planted on top of the Plateau Mountain (alpine habitat) the plants survived showing rapid inhibition of stem elongation and increased accumulation of anthocyanin (personal observation). Accumulation of suberin, lignin and other phenolic compounds such as anthocyanins in the walls of epidermal and endodermal cells in mature and cold-stressed plants of *S. longipes* may play a possible role in the inhibition of stem elongation in these plants (Chuong, 1998). These results indicate that the accumulation of anthocyanins along with the other phenolic compounds could be either the cause or consequence of inhibition of stem elongation. Further research involving biochemical and histochemical analyses on the epidermal and endodermal cells from the plants grown under varied R/FR would help in identifying the phenolic acids that deposit during the termination of stem elongation, thus providing better insights into the physiological mechanisms underlying the stem elongation.

Increase in the rate of ethylene production is one of the several physiological events accompanying stem elongation in *S. longipes*. It is of some interest to note that alpine and prairie ecotypes differ in their ability to produce ethylene in relation to stem elongation under LDW conditions (Emery et al., 1994). This observation provided us a starting point for understanding the molecular events underlying phenotypic plasticity in *S.longipes* (Kathiresan et al., 1996, 1998; Tatra et al., 2000). Fluctuations in the expression of ethylene biosynthesis gene, ACC oxidase were shown to be responsible for generating the ethylene rhythm in both the alpine and prairie ecotypes and the amplitude of the rhythm was smaller in alpine ecotype than in prairie ecotype (Kathiresan et al.,

1996). Since the rhythm was initiated by red light but not blue light pulses, it was suggested that the circadian rhythm of ACC oxidase activity and mRNA accumulation may be controlled by phytochrome. If this is the case, phytochromes may play a possible role in controlling the shade avoidance stem elongation under varied R/FR by modifying ethylene production in *S.longipes*. Evidence for the modification of ethylene production through phytochrome action has been obtained from earlier studies. *Pisum* and *Phaseolus* seedlings showed reduction in ethylene biosynthesis in response to red light treatments (Goeschl et al., 1967; Vangronsveld et al., 1988). Recent studies with sorghum PHY B mutant containing a truncated, non-functional phytochrome B protein (Childs et al., 1997) showed that the amplitude of rhythmic peaks of ethylene in the wild type is only a small fraction of the mutant (Finlayson et al., 1998). These results indicate that a functional PhyB may act as a negative regulator of ACC oxidase oscillations. The link between phytochrome and ethylene production for mediating stem elongation in *S.longipes* can be obtained by measuring the levels of ethylene or the expression and activity of ACC oxidase and ACC synthase in the plants grown under varied R/FR.

It has long been thought that the existence of multiple gene families encoding crucial proteins and novel regulatory mechanisms might serve as a molecular basis of phenotypic plasticity (Smith, 1990; Dover, 1986). The phytochrome and CHS gene families are examples of such multiple gene families within which differential expression in response to environmental and developmental signals is particularly evident. Although phytochrome-regulated responses have been intensively studied, the functions of individual phytochromes remain relatively unknown. In order to evaluate the physiological role(s) played by different phytochromes characterization of entire PHY

family is required. This can be achieved by using heterologous probes from other plat systems for screening cDNA or genomic library or through PCR amplification by designing sequence specific primers corresponding to each of the members of the phytochrome family. Characterization and analysis of individual gene members of the phytochrome family in alpine and prairie ecotypes would reveal any differences in the perception and transduction mechanisms of varied R/FR signals between these ecotypes. Southern analysis suggests that CHS is encoded by a small gene family in *S.longipes*. Similar characterization and analysis of individual members of the CHS gene family in alpine and prairie ecotypes would reveal any differences in the acquisition of regulatory control of the responsive genes between the two ecotypes due to their differential selection pressure.

The results of the present study demonstrate that the intensity of the shade avoidance responses and anthocyanin accumulation in the two ecotypes of *Stellaria longipes* was possibly a result of their origins in two contrasting habitats. In the alpine habitat the plants are not shaded and the R/FR ratio is 1.3, whereas in the prairie habitat the plants are shaded lowering the R/FR to 0.7. Although the results could not precisely explain the molecular mechanisms responsible for the variations in stem elongation and anthocyanin accumulation within the alpine or prairie ecotype under varied R/FR, the results do show that the subtle variations in the mRNA expression of PAL and CHS and significant differences in the PAL activity between the alpine and prairie ecotype may be responsible for variation in the degree of anthocyanin accumulation and stem elongation response between the two ecotypes. Since both the ecotypes showed significantly higher levels of CHS activity under high R/FR than under low R/FR corresponding to their

anthocyanin accumulation, the results indicate that in *S.longipes* regulation of CHS is more specific to anthocyanin synthesis than PAL. A better understanding of the causes and consequences of the shade avoidance responses requires better collaboration between different disciplines such as evolutionary, ecological, genetic, physiological, developmental, biochemical and molecular approaches. Once this is achieved, rapid progress can be made towards revealing the complex regulatory mechanisms that initiate an array of plastic responses, culminating in an appropriate change in the phenotype with overall increased environmental fitness.

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