THE UNIVERSITY OF CALGARY

Phenotypic Plasticity of Stem Elongation in *Stellaria longipes*: anatomical and biochemical studies

by

Simon Dich Xung Chuong

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

DEPARTMENT OF BIOLOGICAL SCIENCES

CALGARY, ALBERTA

JUNE, 1998

National Library of Canada
Acquisitions and Bibliographic Services
395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque nationale du Canada
Acquisitions et services bibliographiques
395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence

Our file Notre référence

0-612-34949-7

Canada

Abstract

This thesis examines and compares the morphological and biochemical aspects of stem elongation in the context of phenotypic plasticity in *Stellaria longipes*. Two populations of *Stellaria longipes* with differing degrees of plasticity, an alpine ecotype with low levels of plasticity and a prairie ecotype with high levels of plasticity, were used for this comparative investigation. Histological studies revealed that both cell division and cell elongation were responsible for stem elongation and that it was the epidermal cells that regulated the rate of stem elongation. Furthermore, histochemical studies of stem sections indicated that the timing of deposition of phenolic compounds such as suberin and lignin was important in the control of stem elongation plasticity. The development of an endodermis was observed in both mature internodes and elongating internodes under exposure to stress conditions. The endodermis may serve to provide structural support and adaptive functions to the plants under low temperature stress. The role of phenylalanine ammonia-lyase (PAL) was also examined under different photoperiod and temperature treatments. PAL mRNA expression and enzyme activity was induced when plants were subjected to low temperature and this induction was a light-dependent process. The two ecotypes showed similar results in all studies with only differences being in the timing, duration and magnitude of the response to the treatment, which may partially account for their differences in plasticity. Overall, these results suggest the possible role(s) of secondary metabolism in the regulation of stem elongation plasticity in *S. longipes*.

Acknowledgements

I would like to thank Dr. C. C. Chinnappa for taking me in as a graduate student. I appreciate his support and encouragement. I would also like to thank Dr. Edward C. Yeung, especially for his advice, guidance throughout the course of my research, for believing in me and for being my interim supervisor. Thanks to Dr. David Reid for his ideas and motivation which made me believe in myself throughout my research and for the permission to use the radioactive facility. A great big thanks to Dr. Kathiresan Arumugam for his advice and suggestions throughout the course of my thesis research. I truly appreciate his perseverance and work ethics. Grateful thanks to Dr. Daniel Chong for getting my feet wet with molecular techniques and to Dr. Richard Joy IV for supplying the *Phytolacca* PAL cDNA.

Special thanks go to Siew Hwee, Dr. Jin-Hao Liu, Nicole Ramesar-Fortner, Dr. Roger Mandel, and Cindy Pidgeon for being ready to offer help whenever it is needed. Thanks to Ken for always helping and fixing the growth chambers. Thanks to Dr. Jim for his everyday advice. Thanks to Dr. Peter Facchini for allowing me to use the equipments in his lab. Then there are the members of Dr. Moloney's lab, thanks to Janis for all her help; Carol for the use of radioactivity, Steve Z for help with the occasional software jams. Finally, there are, Dr. Thorpe, Tino, Lee, Val, Sang-Un, Sonny, Claudio, Dave, Scott R, and Soheil for their everyday help.

Thanks to my family for their support and accepting the fact that I will be in school forever. A special thanks goes to Thomas, Kalee and Aaron Tran for putting their support behind me. An ultimate thanks goes to my wife, Jessica, for her support, encouragement, patience and understanding. None of my academic achievements are possible without the biggest support of my wife.

With greatest gratitude I dedicate this thesis to my wife, Jessica Park-Chuong, who has provided continuous and unconditional love and support throughout my academial pursuit

Table of Contents

List of Figures

List of Plates

Abbreviations

CA	= *trans* cinnamic acid
CCoAMT	= caffeoyl Co A methyltransferase
DC	= dark and cold temperature
DIF	= difference between DT and NT
DT	= day temperature
DW	= dark and warm temperature
LDW	= long day and warm temperature
NT	= night temperature
PAL	= phenylalanine ammonia-lyase
PAS	= periodic acid and Schift reagent
SDC	= short day and cold temperature
TBO	= toluidine blue-O
XET	= xyloglucan endotransglycosylase

CHAPTER ONE
General Introduction

Phenotypic plasticity, the ability of genetically identical organisms to display distinct morphological and physiological characteristics in response to different environmental cues, is an important adaptive feature that can significantly contribute to the success of a population (Schlichting, 1986). However, the degree of plasticity exhibited by a population may vary. Therefore, individuals of defined genome will not likely be very successful in a constantly changing environment whereas one with a more flexible or plastic developmental program will. However, high plasticity is not always complemented with greater fitness and being more plastic does not always guarantee species a selective advantage (Gottlieb, 1977).

Most of the evidence on phenotypic plasticity is related to morphology rather than physiology because it is easier to observe the former than the latter (Bradshaw, 1965) and examples of morphological plasticity of plants in response to the changing environment have been well recognized. Studies examine the link between environmental signals and phenotypic plasticity and have demonstrated that morphological plasticity exhibited by the organism is a consequence of ecological adaptive strategies (Schmitt et al., 1995; Dudley and Schmitt, 1996; Schmitt and Dudley, 1996). Examples of morphological plasticity include: 1) the rapid elongation of the internodes of rice in response to the rising water levels (Eiguchi et al., 1993) and wheat in response to deep-seedling (Suge et al., 1997), 2) the observation that *Impatiens capensis* stems elongate in high density but not in low density (Dudley and Schmitt, 1996) and 3) the changes in vegetative variegation in *Dracaena sanderana* in response to different light intensities (Vladimirova et al., 1997).

These examples support the adaptive importance of phenotypic plasticity during the course of evolution; thus a better understanding the evolutionary framework of phenotypic plasticity may enhance our knowledge of the interaction between plants and their environment.

A plant phenotype is governed by its genome and the surrounding environment. In nature, plants respond to the changing environment by modifying their physiology and morphology which are under the control of gene expression. These changes may be harmful to plants or they may enhance their survival. Plants of the same genotype grown under different environmental conditions are morphologically distinct indicating that during their existence in a particular habitat, environmental pressure can cause some changes at the gene level (Kuhlemeier et al., 1987). During plant growth and development, the expression of specific gene products is involved. Therefore, studies of developmental regulations of gene expression are essential for the understanding of phenotypic plasticity in plants. Although the importance of plasticity at evolutionary, ecological and developmental levels are well known (Bradshaw, 1965 and Smith, 1990), our understanding of the molecular mechanisms of this phenomenon remains elusive. Thus, insights into these mechanisms can also help bring about a better understanding of the interaction between plants and the environment.

Stellaria longipes subsp *longipes* belongs to the Caryophyllaceae family. It is a herbaceous perennial plant with circumpolar distribution. It is found in such diverse areas as prairie, boreal, montane and alpine throughout the northern hemisphere (Chinnappa and Morton, 1976). *S. longipes* has been shown to exhibit a large degree of phenotypic plasticity through distinct morphological changes such as stem elongation, leaf shape and flower number (Chinnappa and Morton, 1984). These plastic characters are

responsible for the species' success in colonizing a wide range of habitats. Among the populations that colonized these numerous habitats, the alpine and prairie ecotypes have been widely used in comparative studies of phenotypic plasticity (Macdonald, 1988; Emery, 1994; Kathiressan, 1997). Therefore, the species is a good model system to examine plasticity.

Using a range of controlled growth chamber conditions, Macdonald and Chinnappa (1989) were able to quantify phenotypic plasticity and document morphological variation among individuals of *S. longipes*. The individuals from prairie, montane and boreal habitats were indistinguishable from each other while the tundra form was morphologically distinct. Macdonald et al. (1988) also found that the amount of phenotypic plasticity varied among populations from different types of habitats. When plants were grown under short days (8 hours photoperiod) and cold (night 5°C and day 8°C) (SDC) conditions, both alpine and prairie ecotypes developed short stems and ovate leaves. However, when these plants were transferred to long day (16 hours photoperiod) and warm (night 18°C and day 23°C) (LDW) conditions, plants of both ecotypes elongated their leaves and stems. The individuals from the arctic and tundra environments showed the least amount of plasticity and a more stable morphology in terms of the magnitude of stem and leaf elongation than those of the prairie ecotype which exhibited a greater and more rapid response under LDW conditions.

The physiological aspects of stem elongation in *S. longipes* were examined by Emery et. al. (1994) and the role of ethylene and gibberellins on stem elongation plasticity of *S. longipes* was investigated. Their results showed that ethylene plays a key role in regulating stem elongation plasticity of the prairie ecotype but not the alpine ecotype of *S. longipes*. Kathiresan et al. (1997) also examined the role of ethylene biosynthesis in stem elongation

plasticity of alpine and prairie ecotypes of *S. longipes*. They suggested the differential regulation of ACC synthase gene families as a possible basis for phenotypic plasticity in the two ecotypes. Furthermore, Kathiresan (1997) also showed that γ-aminobutyric acid (GABA) stimulates ethylene biosynthesis and stem elongation of *S. longipes in vitro*. Despite the number of physiological studies of stem elongation in *S. longipes*, our knowledge on the anatomical changes that transpire as a result of the environmental factors is lacking. In this study an attempt has been taken to understand the anatomical changes as well as the biochemical changes that are involved in stem elongation of *S. longipes* under different environmental conditions.

Plant growth is strictly controlled internally by its developmental program and externally by the environment (Fry, 1988). Stem elongation is a complex developmental process involving the growth of stem tissues which are under the actions of growth hormones such as auxins, gibberellins, cytokinins, abscisic acid and ethylene. Environmental stimuli such as temperature, photoperiod, light intensity and quality also play major roles in controlling stem elongation (Erwin et al., 1992). These internal and external factors are believed to affect the subapical meristematic region which is the major site of cell multiplication and elongation giving rise to stem elongation (Sach, 1965). In meristematic and differentiating cells, walls have the ability to endure turgor pressure exerted by the protoplasm. Noncovalent and covalent bonds between wall polymers must exist to achieve this stability (Talbot and Ray, 1992).

Cell division is composed of duplication and distribution of organelles and partition of cellular volume with a new wall. Cell elongation involves the irreversible increase in cell volume which can occur by expansion (McCann and Roberts, 1994). Cell expansion occurs only in the primary walls

which are laid down while a cell is expanding. The plant primary cell wall is made up of a network of polymers that is comprised mainly of cellulosic microfibrils embedded in a matrix of interwoven noncellulosic polysaccharides and proteins (Fry, 1988; Carpita and Gibeaut, 1993). This tough, yet flexible, polymeric structure determines cell shape, withstands turgor pressure exerted by the protoplasm, controls cell expansion and provides cell to cell communication (Brett and Waldron, 1996). Biochemical and biophysical examinations of cell walls indicate the matrix as the most significant part for regulating the growth properties of the walls (Cosgrove, 1993). Turgor pressure is undoubtedly a driving force of cell expansion (Cosgrove, 1993). But this process requires modifications of existing cell wall architecture to allow extensibility and insertion of newly synthesized wall polymers. Without such modifications, wall thinning and rupture would take place (McCann and Roberts, 1994).

In plants, the final shape and size of an organ is a result of cell division and cell expansion. Expansion of an organ is a highly coordinated process because the cells of adjacent layers may have different growth rate. For example, in oat coleoptile, epidermal cells undergo no cell division while cortical cells divide twice, yet all cell layers must grow at the same rate without detectable cell wall thinning (Varner and Lin, 1989). The peripheral cells generally consist of thick walls which constrain the extension of the inner tissues and thus control the rate of organ elongation (Kutschera, 1992). This indicates that there is coordination in the synthesis, secretion and assembly of wall components between cell layers in a growing organ.

In order to promote growth, existing wall material must expand. However, this expansion may or may not require cell division because cell division results in an increase in the number of cross walls. Permanent

growth of a plant cell requires that the cell wall undergoes permanent expansion. Growth cannot simply occur from the synthesis and accumulation of cytoplasmic components such as proteins unless it is accompanied by a process of irreversible wall extension (Ray, 1987). Elongation of a stem is normally the combination of expansion of existing cells and the production of newly formed cells resulting from division. The ability of a growing cell wall to mechanically extend has been referred to as platicity (this term is not use in the same context as described previously). Cell wall plasticity generally results from stress relaxation in the walls, leading to water uptake by the cell and allowing expansion of the wall (Cosgrove, 1993). Stress relaxation in a wall is possibly caused by the mechanical weakening of the walls by the action of wall-loosening enzymes such as expansins, xyloglucan endotransferase (XET) and glucanases to allow turgor-driven extension (Cosgrove, 1997). The loss of cell wall plasticity generally occurs when phenolics cross link with wall polymers and wall peroxidases are possibly associated in this wall stiffening process (Golgberg et al., 1987). Therefore cross-links of wall polymers are significant contributing factor of cell growth.

Environmental conditions not only affect the overall growth of the plant but the formation of secondary metabolites as well, as change in environmental conditions normally brings about a shift in the metabolic pathways in plants. The formation of secondary metabolites is strongly influenced by environmental factors such as light, temperature, minerals and soil moisture (Waller and Norwacki, 1978). In plants, secondary metabolites are not waste products but instead are phenolic polymers that play important roles such as signal molecules in signal transduction, and providing structural support and protection against abiotic and biotic agents.

Anthocyanins have been shown to accumulate in leaves and stems of many plants under exposure of high irradiance and low temperature (Christie et al., 1994; Leyva et al., 1995). Suberization of cells in potato tuber in response to wounding has been demonstrated (Griffith et al., 1985). In general, deposition of lignin to walls of cells at the site of fungal or pathogen attack is a regular plant defense phenomenon (Liang et al., 1989). Crosslinking of phenolics to wall polysaccharides has been shown to be a factor involved in loss of cell wall plasticity (Golberg et al., 1987) and changes in cell wall phenolics may also be under environmental control. The study of plant secondary metabolism not only identifies the role of secondary metabolites in plant growth and development, but also provides important information on the interaction of plants with the environment.

Phenylalanine ammonia-lyase (PAL), the key enzyme that catalyzes the first metabolic step from primary metabolism into secondary metabolism, has been studied intensively and has been shown to be associated with many aspects of plant growth and development (Jones, 1984). Figure 1.1 summarizes the general phenylpropanoid pathway leading to the production of various secondary metabolites. The deamination of L-phenylalanine to *trans*-cinnamic acid by PAL is considered a key step because cinnamic acid is the precursor of phenylpropanoid compounds. Once formed cinnamic acid is further modified by the actions of hydroxylases, O-methyltransferases and other enzymes of specific branch pathways for the biosynthesis of diverse compounds. In plants, these products have many important functions in structural support, defense and signaling (Hahlbrock and Scheel, 1989).

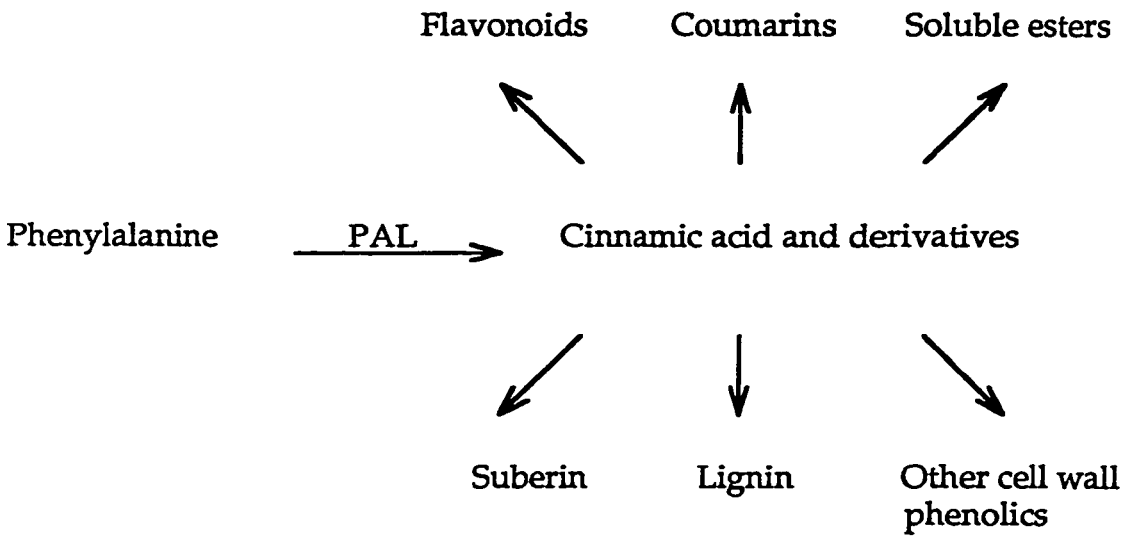


Figure 1.1. A simple schematic diagram of phenylpropanoid metabolism.

Numerous studies have shown that growth regulators such as auxin and cytokinin (Ozeki et al., 1990), and environmental stress such as elicitors (Dixon, 1983), injury (Tanaka et al., 1989) and light (Loschke et al., 1981) induce PAL activity. Of all the environmental stimuli studied, most research has focused on the induction of PAL by various light qualities (Camm and Towers, 1977). Although PAL induction has been clearly demonstrated under various stress conditions, there is very little evidence clearly correlating its induction with a specific secondary product accumulation. In general, the increase in PAL activity may not necessarily bring about an increase in any particular secondary metabolite formation because of the complex nature and the numerous products generated from the pathway.

Much work on phenotypic plasticity of *S. longipes* emphasized on the ecological and physiological aspects. Little is known about anatomical and biochemical changes during stem elongation and how these changes are

regulated. In this thesis, the working hypotheses are that during the rapid stem elongation, both cell division and cell elongation must be involved. Beside growth hormones, temperature, photoperiod, and secondary metabolites also play key roles in controlling stem elongation plasticity. In order to examine these hypotheses, structural and biochemical changes of the stems under different growth conditions were monitored. The results were obtained using histological, histochemical, biochemical and molecular techniques. By using both alpine and prairie ecotypes, detailed changes in the plasticity of this species can be shown to exist among populations from different types of habitats.

Chapter 2 presents an anatomical study which examined the hypothesis that both cell division and elongation of stem tissues are involved in stem elongation. Chapter 3 describes the changes in cell wall components that were detected during different developmental stages of the internode and examines the roles of phenolic compounds in stem elongation plasticity. These results provide excellent anatomical and histological foundations of stem elongation and the factors that possibly control stem elongation. The goal of Chapter 4 was to determine what role, if any, that phenylalanine ammonia-lyase (PAL) has in the production of phenolic compounds in response to environmental cues such photoperiod and temperature. These environmental factors were consistently applied to earlier studies of phenotypic plasticity on *S. longipes* (Macdonald, 1988; Emery, 1994; Kathiresan, 1997).

The important issue of this thesis is not just anatomical and biochemical changes, but rather how these changes may control adaptive morphological plasticity. Therefore, the findings from these studies are important for the elucidation of evolutionary significance of phenotypic

plasticity of *S. longipes*. Thus a better understanding and knowledge, in addition to the previous genetics, molecular and ecophysiological contributions, of the anatomical and biochemical controls of stem elongation plasticity of *S. longipes* can be gained.

CHAPTER TWO

Stem elongation in alpine and prairie ecotypes of *Stellaria longipes*.

Summary

The morphological aspects of stem elongation in alpine and prairie ecotypes of *Stellaria longipes* were examined and compared. Genotypes of *S. longipes* that were maintained under SDC conditions and then transferred to LDW conditions showed rapid stem elongation 5 days after the transfer and the most significant growth was observed in the second internode below the shoot apex. The alpine ecotype displayed a smaller degree of stem elongation plasticity as compared to the prairie ecotype. Histological examination indicated that both cell division and cell elongation were observed in cortical cells whereas only cell elongation took place in epidermal cells. Changes in cell width were not observed. These results clearly indicated that both cell division and cell elongation are involved in stem elongation but that the epidermis plays a pivotal role in controlling the rate of stem elongation plasticity in *S. longipes*.

Introduction

One of the most significant response in *S. longipes* is the rapid stem elongation after its transfer from SDC conditions to LDW conditions. Stem elongation is a complex process involving both irreversible cell wall expansion and cell division. Cell expansion is driven by the combination of water uptake and cell wall loosening which is accompanied by deposition of new wall materials and an increase in cell volume (Cosgrove, 1997). In most cases, the process of cell expansion is regulated by phytohormones such as auxins, gibberellins, ethylene and other growth hormones and by external

stimuli such as light, temperature, gravity and water availability (Brett and Waldron, 1996). The control of cell expansion is important because the final shape of an organ is a consequence of both cell division and cell expansion. One of the major interests of this chapter is to understand the anatomical processes and controls of cell expansion that directly or indirectly influence stem elongation.

Temperature and photoperiod are two primary environmental factors controlling growth and development of plants. Macdonald et al., (1984) showed that both of these factors are involved in the regulation of stem elongation in *S. longipes*. The effect of temperature on plant stem elongation has been the object of many studies. Went (1952) observed that plants grown under cooler day temperature (DT) than night temperature (NT) were shorter than those grown under a warmer DT than NT. A large number of studies have demonstrated that plants grown under short day have lower stem elongation rate as compared to those grown under long day. The changes in day and night temperatures have been shown to influence internode length, plant height and other aspects of plant growth and development and that these effects on stem elongation are a result of increased cell elongation not cell division (Erwin et al., 1994; Myster and Moe, 1995). Erwin et al., (1989) quantified the effect of temperature on the morphogenesis of *Lilium longiflorum* and found that the difference (DIF) between DT and NT could be used to quantitatively described stem elongation. Studies have consistently shown that internode elongation increase as the value of DIF increases (Erwin et al., 1989; Erwin et al., 1992). Although temperature is a primary external factor involved in the growth and development of plants, little anatomical information is available regarding the plants' response to temperature.

Light also has profound effects on the morphogenesis of plants throughout their life cycle and has many crucial functions including the stimulation of leaf and chlorophyll differentiation, inhibition of hypocotyl growth, induction of many nuclear- and chloroplast-encoded genes, and the provision of an internal time keeping mechanism that signals plants to switch from vegetative to reproductive growth (Chory, 1997). This light-dependent morphogenesis of plants is a complicated process involving the action of several photoreceptor systems including the phytochromes. Phytochromes are soluble proteins that absorb red (R) and far red (FR) light and control the expression of genes that are involved in a plant growth and development (Quail et al., 1995). In many plants, stem growth is influenced by both light intensity and the red to far red ratio (R/FR). A decrease in R/FR stimulates stem elongation, reduces leaf growth and branching (Hughes and Wagner, 1987). In fact, stem elongation is an important photomorphogenic characteristic commonly used for the screening of phytochrome mutants.

Stem elongation is a complex process involving the coordination of cell division and cell elongation of different cell layers. Numerous studies have demonstrated that the peripheral cell layers or the epidermis control the growth of the entire organ (Kutschera, 1987; Kutschera et al., 1987). In the growing organ the epidermis is under physical tensions exerted by the inner tissues (Kutschera, 1989). Brown et al. (1995) examined the relationship between the processes of stem elongation and the occurrence of tissue stress and concluded that the inner tissue, especially the pith, provides the driving force for stem elongation, whereas the epidermis limits and thus determines the rate of stem elongation. Using excised pea stems and maize coleoptiles, it was also shown that the steady organ growth involves the coordinated action of wall loosening in the epidermis and the constant tension provided by the

inner tissues (Matsuda and Yamamoto, 1972; Kutschera et al., 1987). Changes in the cell wall structures were only detected in the epidermis of *Pisum sativum* internode treated with IAA (Kutschera and Briggs, 1987). Rayle and Cleland (1992), however, concluded that the epidermis does not play a unique role in controlling organ growth based on the response of peeled coleoptiles and epicotyls to auxin.

This chapter investigates the anatomical changes that cause stem elongation plasticity in alpine and prairie ecotypes of *S. longipes* in response to different photoperiod and temperature treatments. Histological analyses of stem sections at different stages of development were examined. The involvement of the epidermis in stem elongation was also investigated in intact internodes.

Materials and Methods

Growth conditions

For all experiments presented plants were maintained as described here-only changes will be noted. Plants from two areas of Kananaskis Country, Alberta were randomly selected and used for all experiments. Population 1 (2,453 m elevation) plants are referred to as the alpine ecotype and population 7 (1,310 m elevation) plants are referred to as the prairie ecotype. A genet of each population was divided into several clones which were planted in 4 cm pots containing a mixtures of peat moss : sand : Terra-green (2:1:1). All plants were maintained in growth chambers (Conviron, Winnipeg, Canada) with Sylvania Gro-flux lifetime fluorescent tubes providing photosynthetically active radiation of about 220 μmoles m^{-2} sec^{-1}. Prior to experimental treatment, plants were placed in a growth chamber under short day cold (SDC) conditions (8°C day, 5°C night, 8 hours

photoperiod) for at least 120 days (This is the time needed to produce maximal growth as reported by Macdonald et al. 1984). These plants were then transferred to a chamber under long day warm (LDW) conditions (22°C day, 15°C night, 16 hours photoperiod). Photosynthetically active radiation taken at plant height with a LI-COR quantum sensor (Model LI-190SB; LI-COR Inc., Lincoln, NE, USA) measured at 220 μmol $m^{-2} s^{-1}$. Measurements of internode length were taken at the time of stem tissue excision for histological examination. Ramet lengths and internode lengths were recorded because they displayed the greatest degree of plasticity.

Macroscopic and microscopic measurements of ramet height, internode length and cell length

Four pots from the same genet were selected for each population. Ten newly emerging ramets were marked at day 0 with coloured threads before these pots were transferred from SDC to LDW. Increments in growth were measured every 2 days for 18 days. Total ramet height was measured from the shoot apex to the soil surface with a ruler to the nearest mm. Since the second internode below the shoot apex was the most responsive after transfer to LDW conditions, measurements for this internode were also recorded. In the same experiment, segments of 2 to 3 mm from the upper, central and lower portions of the second internode were fixed to provide an anatomical record of the internode. These segments were fixed in 1.6 % paraformaldehyde and 2.5 % glutaraldehyde in 0.05 M phosphate buffer, pH 6.8 overnight at 4°C. The sections were dehydrated through a series of graded ethanol (30%. 50%. 70%. 95% and two changes of absolute ethanol), infiltrated and embedded in Historesin (Leica Instrument GmbH, Germany). Sectioning was done with glass knives using a rotary microtome (Reichert-Jung

Microtome 2040, Germany) (Yeung, 1984). Thin sections of 3 μm thick were allowed to float on drops of distilled water on gelatin-coated microscope slides on a hot plate for three hours or until the water is completely evaporated. The sections were then stained with toluidine blue O (TBO) and histochemical stains for insoluble carbohydrates and proteins using the periodic acid-Schiff (PAS) procedure (Yeung, 1984). The slides were then examined under a light microscope and cells were measured from each segment of each internode using a calibrated eye-piece micrometer. Internodes of 10 ramets were used and 10 cortical cells from three random segments were measured. Cell numbers were calculated for each internode by dividing the internode length by the cell length. Each experiment was repeated three times. Photomicrographs of stem cross sections were taken with Kodak Technical Pan film.

Preparation of epidermal peels

Epidermal peels were prepared from the second internode of each time interval after transfer. Peels were made simply by pulling off leaves above the second internode. These peels were incubated in macerating fluid (40% H_2O_2 : 5% glacial acetic acid : 55% water) overnight at room temperature to remove cellular contents. The peels were rinsed thoroughly in water and stained briefly in TBO. The peels were then mounted in water and observed under a microscope. Measurements of epidermal cells were made using a microscope with a calibrated eye-piece micrometer.

Results

Stem elongation of alpine and prairie ecotypes

When SDC-treated plants were transferred to LDW conditions, significant changes in growth occurred in both populations in that rapid stem elongation occurred 5 days after the transfer. The most significant growth was observed in the second internode from the shoot apex (Plate 2.1). The overall growth is typical of the sigmoid pattern in both ecotypes (Fig 2.1). However, alpine and prairie ecotypes showed contrasting degree of stem elongation in response to treatment (Plate 2.1). The prairie ecotype showed a rapid pattern of increase over an extended period whereas the alpine ecotype exhibited a brief and gradual growth pattern. The overall final ramet height of the prairie plants was more than two times that of the alpine ones. When the alpine plants appeared to have stopped their stem elongation, the prairie plants were still in the midst of their period of rapid stem elongation (Fig 2.1).

The length of the second internode below the shoot apex was also compared between alpine and prairie ecotypes of *S. longipes*. Similar sigmoid pattern of the overall ramet height was observed in both ecotypes. The length of second internode was measured at different time intervals from vegetative stage until floral initiation in alpine and prairie ecotypes of *S. longipes* (Fig 2.2). As shown in Plate 2.1, rapid stem elongation occurred after 5 days in LDW conditions in both prairie and alpine ecotypes. Existing or older internodes displayed minimal elongation while neoformed internodes displayed a rapid growth. As the internode continues to increase in length, the center of growth shifts upward toward the middle and then to the upper portion, so that an acropedal pattern of growth occurs throughout the entire internode. Prolonged exposure to LDW conditions resulted in longer internode in both ecotypes. However, the prairie ecotype displayed a more

Plate 2.1. Examples of alpine and prairie ecotypes of *Stellaria longipes*. Shown are ramets of the two genotypes grown until the flowering stage under LDW conditions. The numbers at the bottom represent the number of days in LDW.

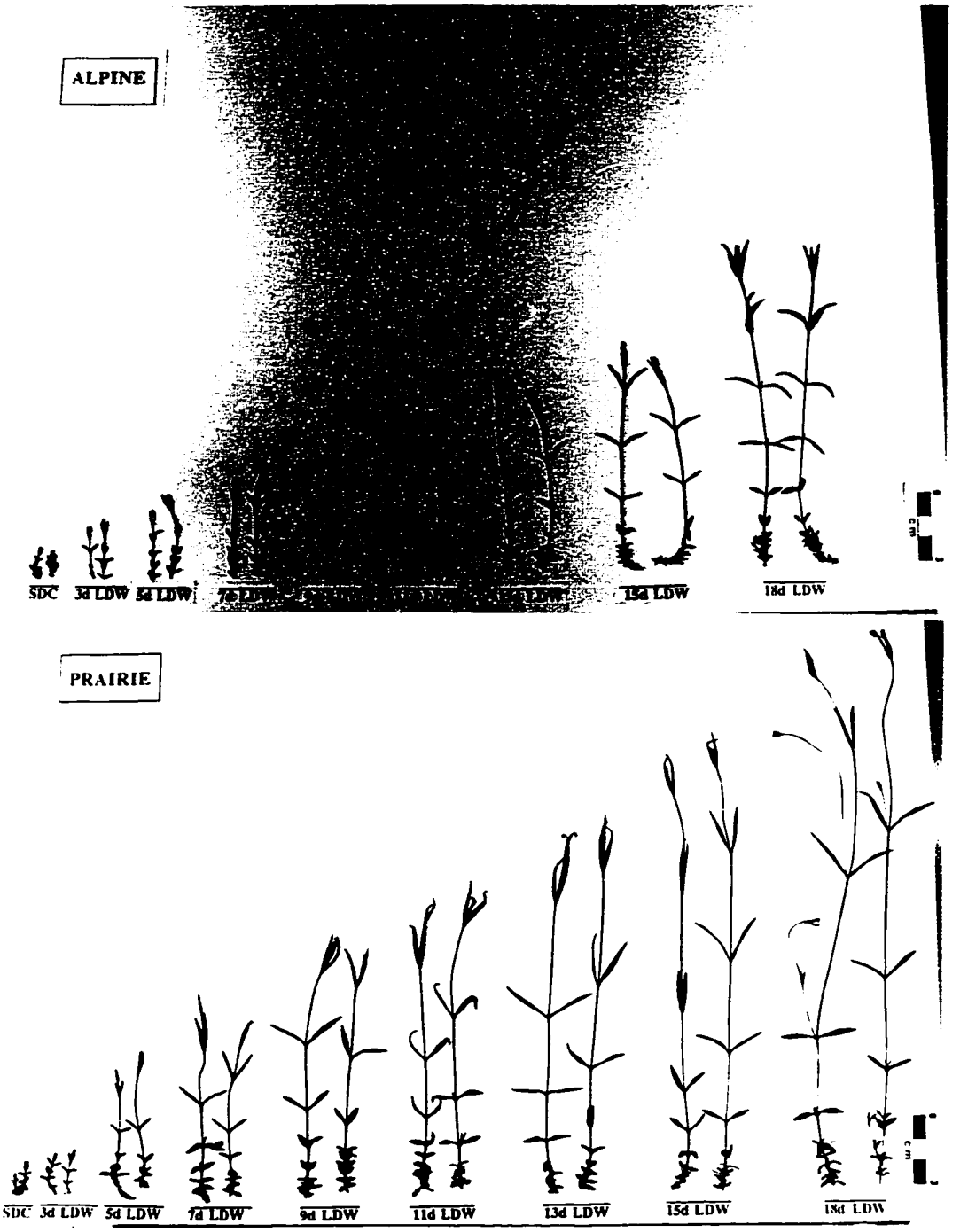

ALPINE
SDC
3d LDW
5d LDW
15d LDW
18d LDW
PRAIRIE
SDC
3d LDW
5d LDW
7d LDW
9d LDW
11d LDW
13d LDW
15d LDW
18d LDW

Figure 2.1. Stem elongation in the alpine and prairie ecotypes of *Stellaria longipes* upon transfer from SDC to LDW conditions. Data are means of at least 20 observed values. Vertical bars indicate ± standard error (SE). If the standard is not shown, it is smaller than the symbol used.

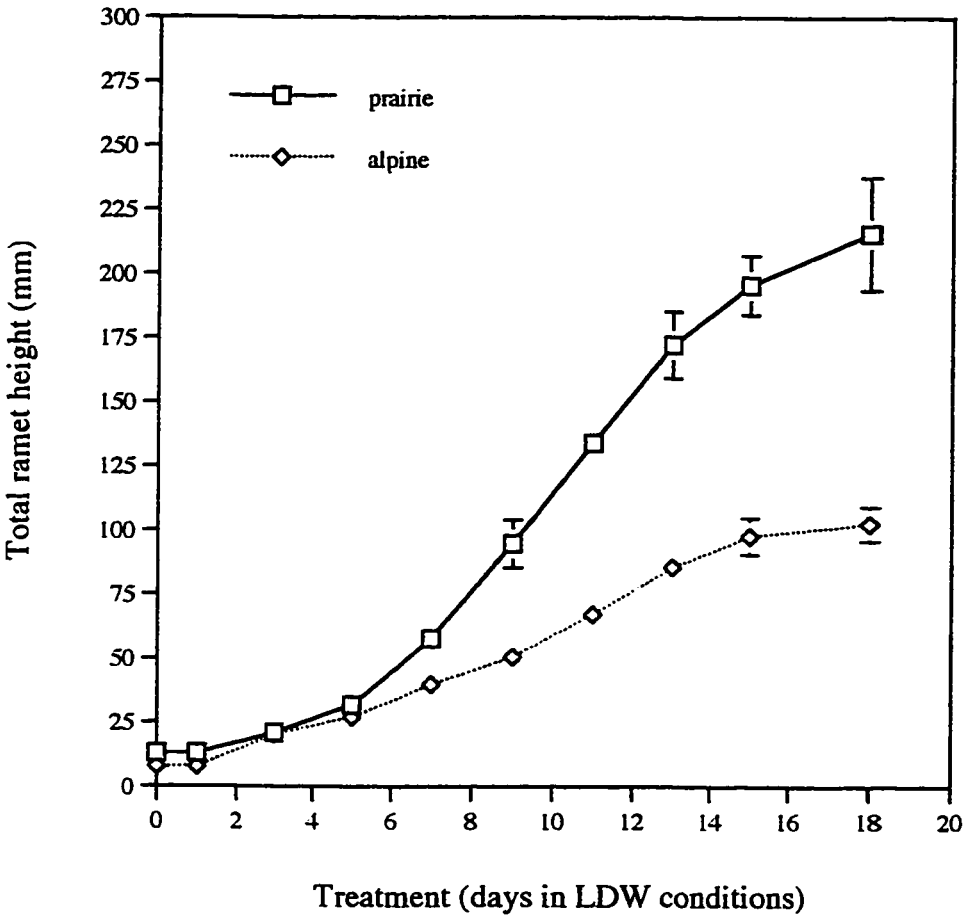
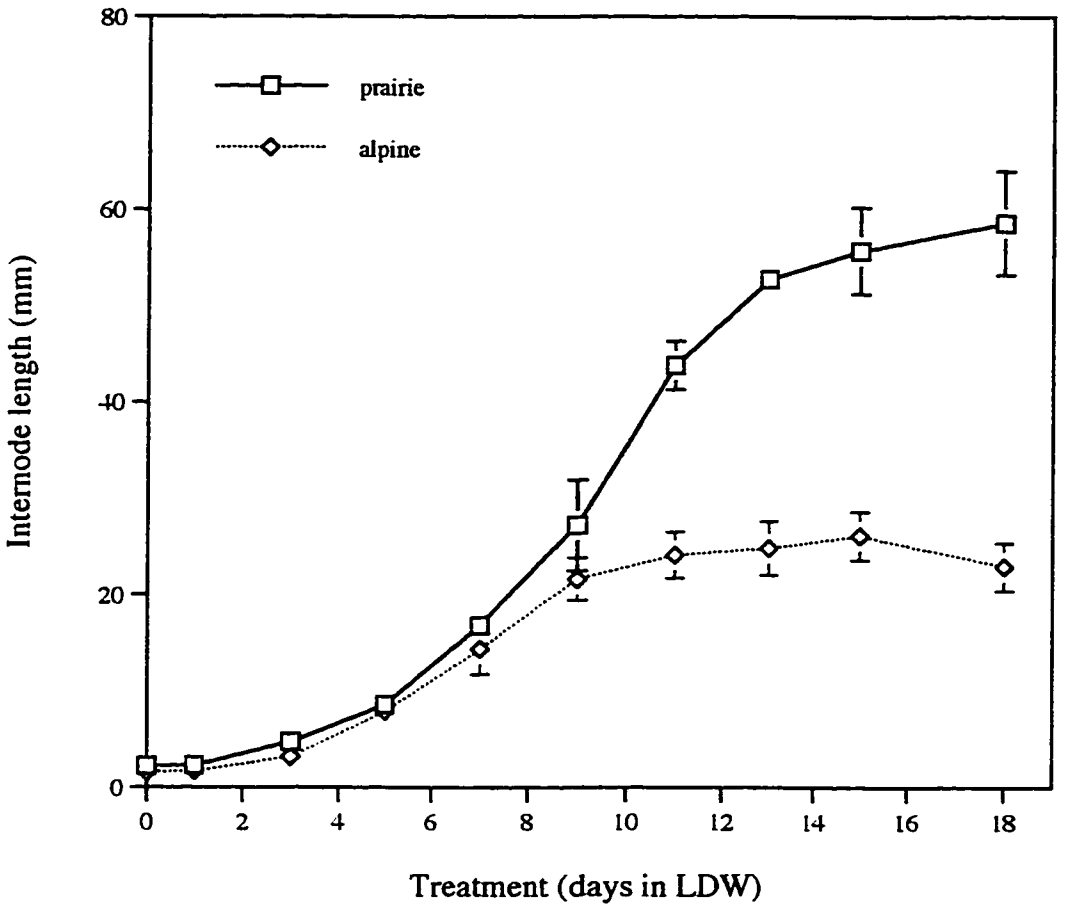

300
275
250
225
200
175
150
125
100
75
50
25
0
prairie
alpine
Total ramet height (mm)
0
2
4
6
8
10
12
14
16
18
20
Treatment (days in LDW conditions)

Figure 2.2. Effect of LDW conditions on the length of the second internode of alpine and prairie ecotypes of *Stellaria longipes*. The internode was measured based on its order of appearance below the shoot meristem at that particular stage. Data are means of 30 measurements. Vertical bars represent ± standard error (SE). If the standard error is not shown, it is smaller than the symbol used.


80
60
40
20
0
prairie
alpine
Internode length (mm)
0
2
4
6
8
10
12
14
16
18
Treatment (days in LDW)

prominent and extended rate of stem elongation as compared to alpine ecotype (Fig 2.2). The length of the second internode between the two ecotypes remained fairly equal for the first 5 days. As the ramets underwent the period of rapid elongation, the second internode of the prairie ecotype increased rapidly over an extended period as compared to the brief and gradual increase of the alpine ecotype (Fig 2.2). The period of rapid elongation continues for 14 days in LDW conditions until which floral buds emerged (Plate .2.1).

Changes in epidermal and cortical cells during stem elongation

The second internode below the shoot apex was also used to examine the role of epidermis in controlling stem elongation. Cortical and epidermal cell lengths and widths were measured in stem tissue. As the internode length increased in both ecotypes, epidermal cell length increased linearly while cortical cells remained relatively constant (Fig 2.3). In both ecotypes, cortical cell numbers increased as internode length increased indicating that cell division occurred (Fig 2.4). Epidermal cell number, on the other hand, remained constant suggesting cell elongation. Temperature and photoperiod appeared to have no effect on widths of cortical cells and epidermal cells (Fig 2.5 A and B). During growth in LDW conditions, a positve correlation between epidermal cell length and cortical number and the increase in internode length was observed in both ecotypes.

Figure 2.3. Effect of LDW conditions on the second internode epidermal and cortical cell length of alpine and prairie ecotypes of *Stellaria longipes*. Data are means of 30 measurements. Vertical bars indicate ± standard error (SE). If the standard error is not shown, it is smaller than the symbol used.

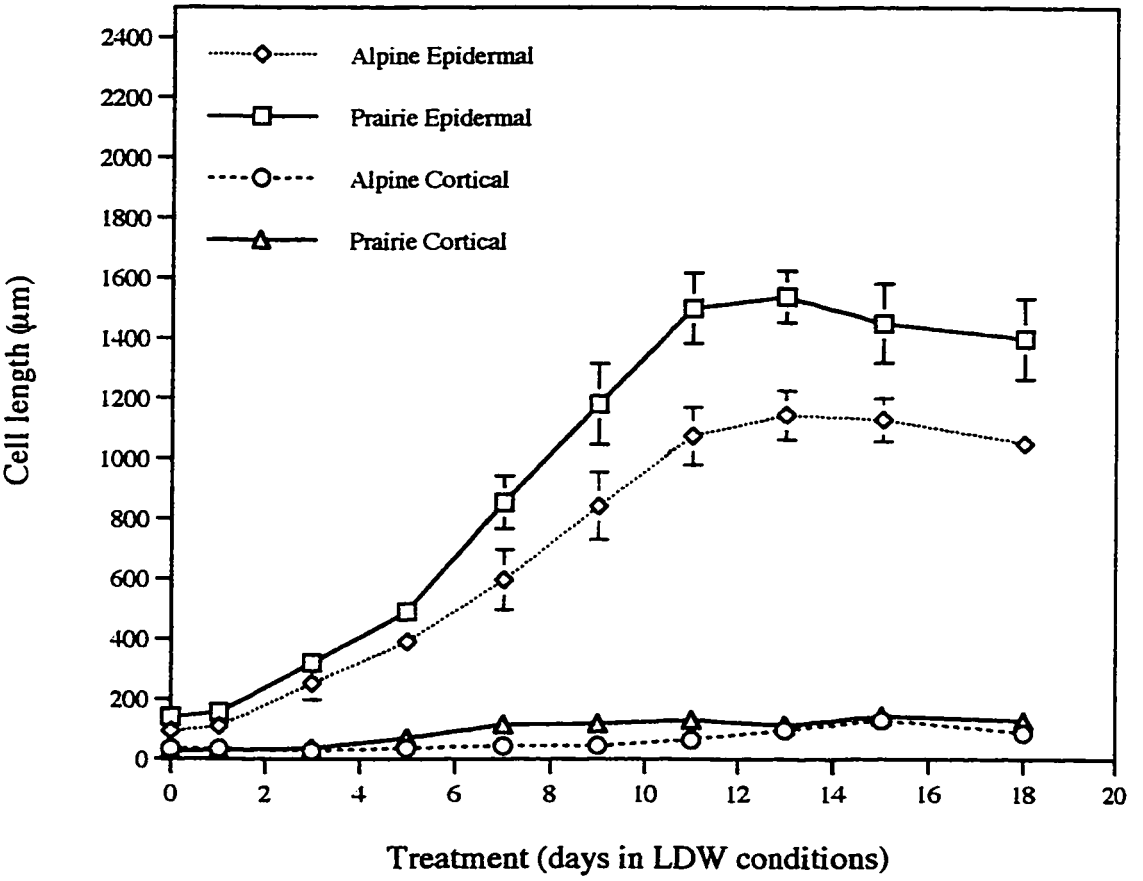

Alpine Epidermal
Prairie Epidermal
Alpine Cortical
Prairie Cortical
Cell length (µm)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
Treatment (days in LDW conditions)

Figure 2.4. Effect of LDW conditions on the number of epidermal and cortical cells from the second internode of alpine and prairie ecotypes of *Stellaria longipes*. Cell number was calculated by dividing the internode length by the cell length. Data are means of 30 values. Vertical bars represent ± standard error (SE). If the standard error is not shown, it is smaller than the symbol used.

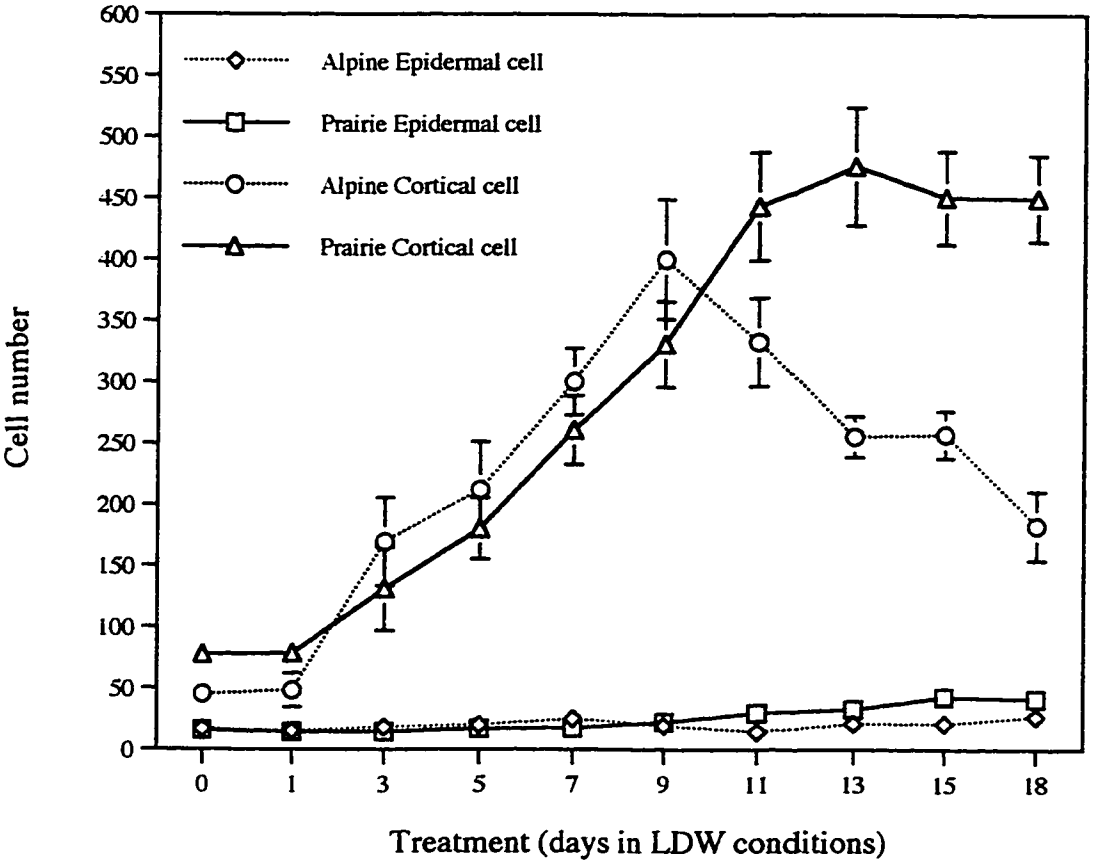
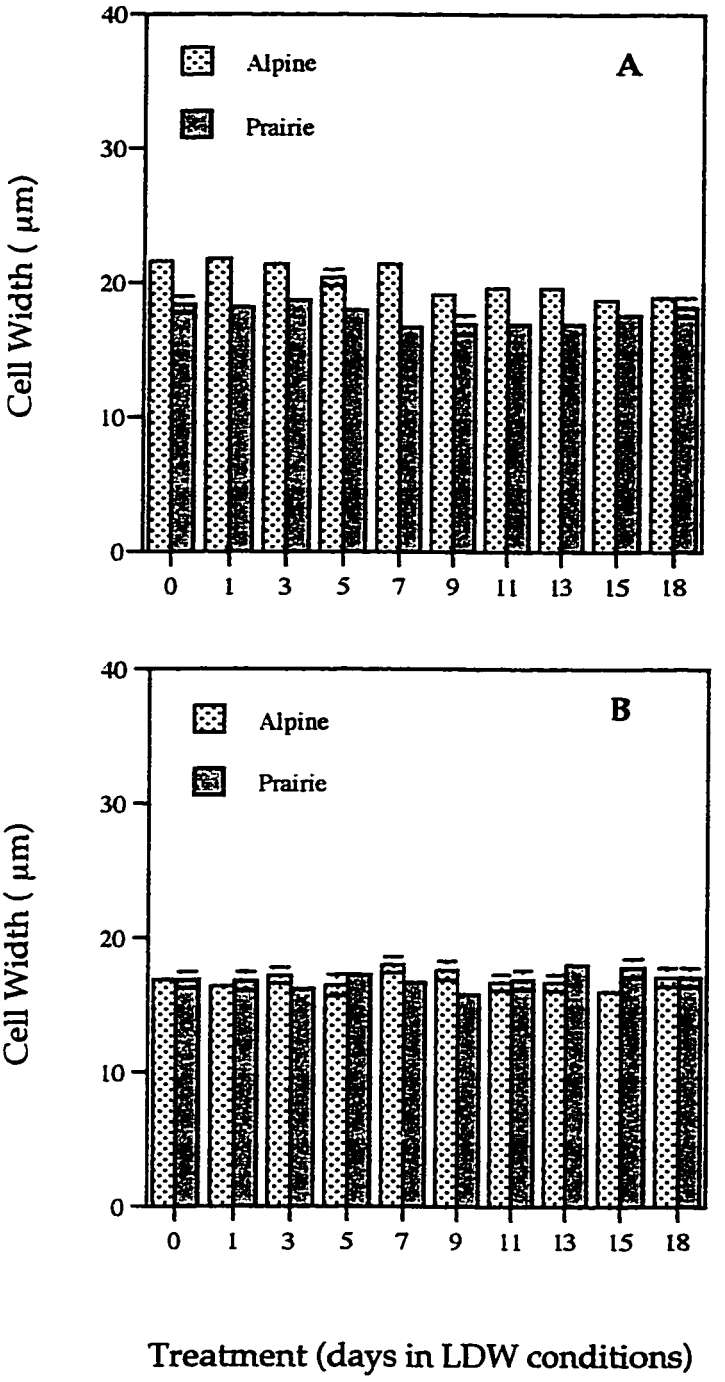

Alpine Epidermal cell
Prairie Epidermal cell
Alpine Cortical cell
Prairie Cortical cell
Cell number
600
550
500
450
400
350
300
250
200
150
100
50
0
0
1
3
5
7
9
11
13
15
18
Treatment (days in LDW conditions)

Figure 2.5. Effect of LDW conditions on the width of epidermal and cortical cells from the second internode of alpine and prairie ecotypes of *Stellaria longipes*. Data are means of 30 values. Vertal bars represent ± standard error (SE). If the standard error is not shown, it is smaller than the symbol used.


Alpine
Prairie
A
B
Cell Width (µm)
Cell Width (µm)
40
30
20
10
0
0 1 3 5 7 9 11 13 15 18
Treatment (days in LDW conditions)

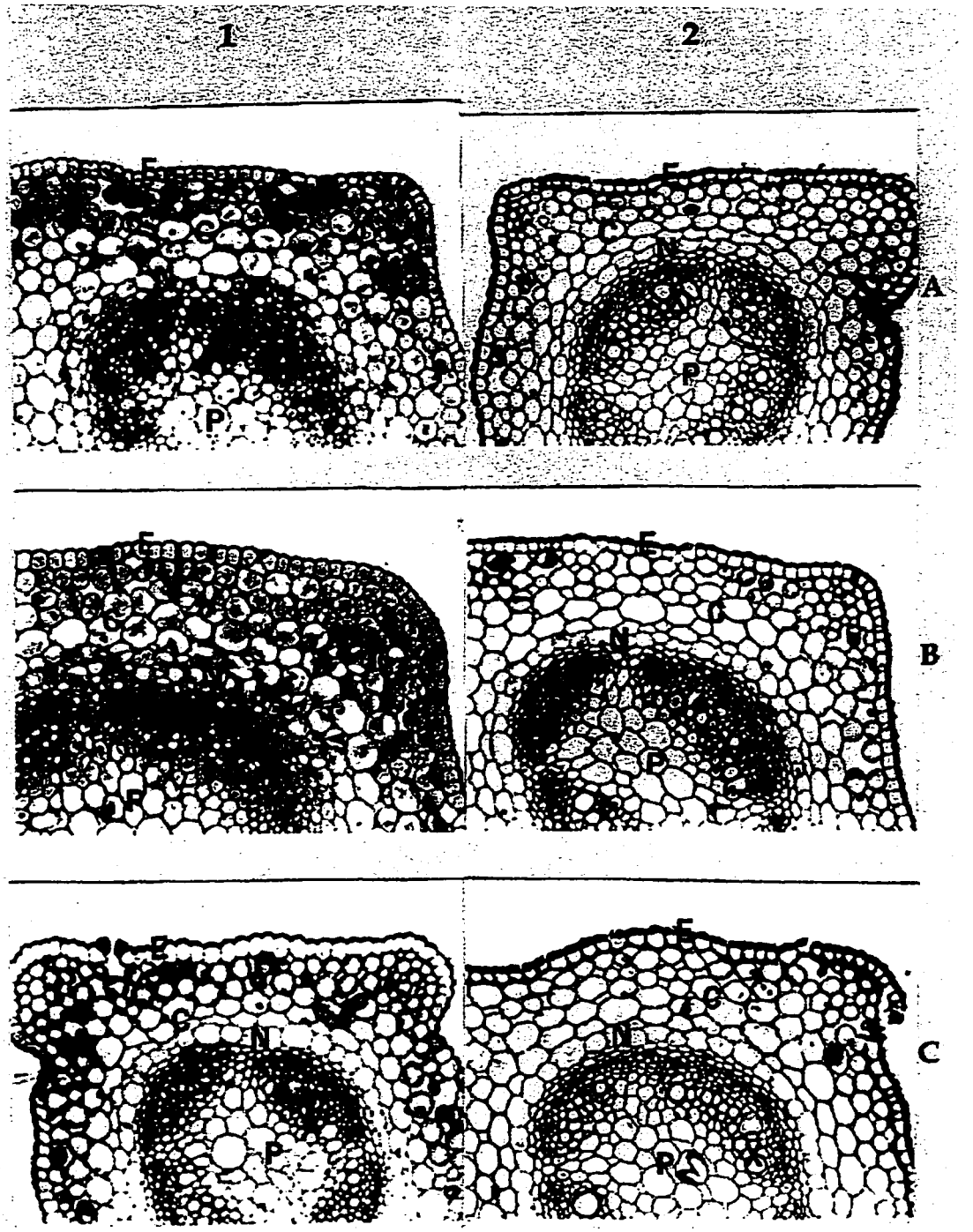
Histological observations of internode under SDC and LDW conditions: cross section description

Histological examination of cross sections of stems of *S. longipes* from SDC conditions and LDW conditions were used. The anatomical features of stem cross section were similar in both ecotypes so only one ecotype is presented (Plate 2.2). Anatomical features of stem cross section consist of epidermis, five layers of cortical cells, vascular tissues and a pith (Plate 2.2). The growing regions of the internode composed of densely stained and nonvacuolated cells (Plate 2.2 1A-C). It is also interesting to note the heavy accumulation of starch grains in the innermost cortical layer (Plate 2.2 1B arrow). In addition, the cuticle and outer walls of epidermis showed gradual thickening as the stem mature and that the innermost cortical cells are distinct in appearance and shape (Plate 2.2 2A-C). The only noticeable difference between the two ecotypes was that alpine ecotype stems mature sooner than those of the prairie ecotype.

Discussion

The effects of photoperiod and temperature on stem elongation were investigated in both alpine and prairie ecotypes of *S. longipes*. Plants grown under SDC conditions showed stunted internode and dwarf overall height while plants in LDW conditions exhibited a marked increase in stem elongation (Plate 2.1). Macdonald et al., (1984) investigated the influence of temperature and photoperiod on stem elongation and indicated that temperature was more important in the induction of stem elongation while photoperiod exerted a lesser effect. However, a combined synergistic effect of photoperiod and temperature is evident. The results shown in this chapter illustrate a sigmoid pattern of stem elongation in both alpine and prairie ecotypes in response to LDW conditions. The only apparent differences in

Plate 2.2. Light microscopy of stem cross sections prepared from two regions (1=upper, 2=lower) of the second internode of prairie ecotypes of *Stellaria longipes* taken at different stages: A) under SDC conditions, B) 9 days in LDW conditions and C) 18 days in LDW conditions. E, epidermis; C, cortical; N, endodermis; X, xylem; P, pith. Cells of the upper region of the internode have densely stained cytoplasm. Note that cortical cells in 1B are rich in starch grains (←). Cells of the lower portion are vacuolated with little stain. The cells of the endodermis have distinct shapes.


1
2
E
P
A
B
C

both ecotypes were the time, duration and magnitude of the response. These differences in stem elongation can be related to the natural climate of these habitats.

Under SDC conditions, plants from both ecotypes were dwarf. This may be attributed to the reduced rates of metabolic functions under suboptimal temperatures. Under low temperatures, plant functions can be dramatically affected. Low temperature also not only caused changes in cellular membranes and biochemical processes but also the inactivation of phytohormones which in turn influence growth and development (Graham and Paterson, 1982). Therefore, the ability of plants to survive in the cold climates often related to their adopted morphological characteristics such as short statue, thick and pointed leaves, high root/shoot ratio and the production of roots near the soil surface. These are important adaptive strategies developed by the plants to deal with environmental stresses. However, in LDW conditions, the plants were then able to attain their normal growth characteristics. When growing conditions are available such that photosynthesis can occur, it is important that the plants can readily take advantage of the opportunities available.

In alpine plants, the delayed response, brief growth period and the shorter overall height could be attributed to the adaptive nature of the plants to their habitats (Plate 2.1). The growing season in this habitat is extremely short and the temperatures rarely exceed 15°C and snow flurries are possible during the growing period (personal observation). Therefore, the alpine plants might have evolved and attained their dwarfness to cope with life in the tundra habitat where they encountered constant climate changes, high winds, and short growing seasons. It appears that these plants have developed efficient and opportunistic strategies that enabling them to take advantage of the limited yet available growth resources. The prairie plants, on the other hand, come from a crowded habitat where they are constantly in competition with the taller neighboring plants. The growing season is much

longer than that of the alpine habitat and the climate is more stable with temperatures of about 20°C. Under LDW conditions, prairie ecotype responds sooner because these signals seem to mark the appropriate conditions for these plants to grow. However, the longer and taller morphology of the prairie plants could also be a result of the shade avoidance response in a competitive environment.

Shade avoidance responses include stem elongation, suppression of branching, reallocation of biomass, and accelerated flowering (Smith, 1982). This process is phytochrome-mediated where the shorter plants of a dense vegetation respond to the reduced ratio of red: far-red light (R:FR) caused by light filtering through the canopy and light reflected by neighboring plants. Phytochromes are well known for their ability to sense and respond to different light intensities. Phytochromes are cytoplasmic proteins that are involved in many aspects of plant growth and development (Quail et al., 1995). The prairie plants come from a dense habitat where plants are constantly competing for light. In order to survive and be sucessful in this type of habitat, these plants must be able to detect future competition through phytochrome determination of light quality and thus trigger morphological responses to increase their ability to compete for light. The hypothesis that phytochrome-mediated shade avoidance response is adaptive has been tested and supported using mutant of *Cucumis sativus* (Casal et al., 1994), transgenic tobacco (Schmitt et al., 1995) and *Impatiens capensis* (Dudley and Schmitt, 1996). These studies all show that the response provides a fitness advantage in a competitive environment and that the expression of the shade avoidance phenotype in a low density environment is disadvantageous (Casal et al., 1994; Dudley and Schmitt, 1996; Schmitt et al., 1995).

Using a 'reciprocal transplantation' study, Emery (1994) clearly showed that alpine plants performed poorly and were unable to survive in a prairie habitat while prairie plants were able to survive in an alpine habitat. Thus it appears that the alpine plants have evolved a number of characteristics that

allow them to survive in extremely harsh environment and restrict their distribution to a colder and non-competitive habitat. This also strongly suggested that stem elongation plasticity is an important adaptive characteristic in *S. longipes'* survival. Phenotypic plasticity is generally believed to have adaptive contribution (Stearn, 1989). Regardless of the morphological differences, both ecotypes must be operating under the similar mechanism by which stem elongation can take place a critical times of a year to maximize their survival in their habitats. These results strongly support the phytochrome-mediated shade hypothesis for adaptive plasticity and that phenotypic plasticity in *S. longipes* is an adaptive mechanism by which plants have acquired through evolution in response to environmental changes.

It is now believed that many of the effects of phytochrome on stem elongation are mediated by plant hormones. The effect of temperature on stem elongation is believed to involve gibberellins (Zieslin and Tsujita, 1988; Moe et al., 1991). Application of GA_{4-7} to *Lilium* bulbs (Zieslin and Tsujita, 1988), *Campanula isophylla* folias (Moe et al., 1991) overcame inhibition of stem elongation when plants were grown in a negative DIF environment. However, Emery (1994) showed no conclusive role for gibberellins that could be accounted for differences in stem elongation between alpine and prairie ecotypes of *S. longipes*. These results in conjunction with the results from this chapter substantially point towards a role of phytochrome in plants' response, particularly stem elongation, to environmental changes and suggest for the indirect involvement of plant phytohormones such as ethylene, auxins and gibberellins are also possible.

Histological study of these ecotypes of *S. longipes* indicated an acropedal pattern of internode growth in which termination of stem elongation along the axis is initiated at the basal region and progressed toward the apical region. The cesation of stem elongation might possibly be due to the mechanical stiffening of the cell walls which is believed to be the primary cause of suppressing wall extensibility. The anatomical observations showed that cell division was confined to the cortical region where an increase in

number was observe (Fig 2.4). However, in the epidermis, stem elongation in response to photoperiod and temperature was elicited mainly through the effects of cell elongation rather than cell division (Fig 2.3 and Fig 2.4). It has been suggested that this response is due to an inhibition of cell elongation without affecting cell numbers (Erwin et al., 1994). Stem elongation in plants has also been demonstrated to be inhibited by white light. Heupel and Kutschera (1997) attributed the inhibitory effect of white light on stem elongation to both the retardation of cell expansion and suppression of cell division in the meristematic region of the hypocotyl. Irradiation of etiolated seedlings caused a rapid inhibition of stem elongation possibly due to a decrease in cell wall extensibility (Kutschera, 1996). This light effect on the reduction of cell-wall extensibility is restricted to the peripheral cell layers (Hodick and Kutschera, 1992).

The observation that the peripheral layers control the rate of stem elongation has been seen in many other systems. In stems, the walls of epidermal cells are many times thicker than the walls of the inner cortical tissues (Kutschera, 1992). The stem cross sections in Plate 2.2 also showed that epidermal cells of the basal region of the internode have thicker walls. This observation correlated with the termination of stem elongation. Thus, thickening of the epidermal cell walls are possibly responsible for the inhibition of stem elongation. Numerous studies have indicated that the peripheral cell walls constrain the extension rate of the internal tissue thereby limiting the rate of stem elongation (Kutschera et al., 1987; Kutschera, 1987). It has also been shown that elongation of epidermal cells in response to auxin is much greater than the inner tissues of bisected stems and coleoptiles (Masusda and Yamamoto, 1972; Firn and Digby, 1977; Kutschera, 1987; Kutschera et al., 1987). Excised segments from the growing region of coleoptiles or stems fail to elongate but the addition of exogenous auxin causes the resumption of growth (Taiz, 1984). Based on the response of the epidermis to auxin, it may be possible to assume that the observed growth is a result of the loosening of the limiting epidermal walls.

This investigation further confirms and extends the possible role of the epidermis in controlling stem growth. The rate of stem elongation in *S. longipes* was found to be dependent on the rate of cell elongation of the outermost cell layer of that tissue. Even though cortical cells showed both cell division and cell elongation (Fig 2.4), cell elongation was primarily observed in the epidermis (Fig 2.3). This could be explained by the thicker epidermal cell walls. Furthermore, the outer wall of the epidermis is covered by a thick cuticle which possibly contains esterified phenolic polymers (Plate 2.2). No such wall thickening was found in the inner walls. These results indicate that the epidermis walls are distinguished from the inner cortical walls by a variety of mechanical, structural and biochemical features. Since plants are subjected to continuous environmental changes, the existence of the rigid peripheral cell walls enable them to withstand many environmental forces.

To date, almost all work on plant organ growth has been studied using excised tissues. The results from this experiment provide evidence for the role of the epidermis in controlling organ growth in intact tissue. Numerous investigations have shown the epidermal cell walls as thick, multi-layered and helicoidal structures which limit elongation of the intact organ (Kutschera, 1992). Observations obtained from this present study also showed that the walls of epidermal cells are much thicker than those of the inner tissues (Plate 2.2). This further supports the conclusion that the control over the rate of stem elongation can only take place by changes in the mechanical properties of the rigid and less extensible epidermal walls. Thus it can be speculated that the sites of sensing and perception of environmental signals are likely to be located in the epidermis or the periphery cell layers. It is also possible to speculate that some unknown factors that terminate cell elongation is also localized in the epidermis of the stem.

Finally, it is pertinent to perform biochemical and biophysical studies so that the underlying mechanisms coordinating the epidermis with the inner tissues to bring about stem elongation can be determined and identified. Other future experiments would include the examination of

different combinations of red and far red light on stem elongation to further strengthen this evidence of the role that phytochromes play in *S. longipes* stem elongation. Physiological investigations to quantify IAA, GA and ethylene in the second internode might also provide insights into the roles of these hormones in stem elongation.

In conclusion, stem elongation is affected by temperature and photoperiod in *S. longipes*. The internode developed and matured in an acropetal manner. The prairie ecotype displayed greater stem elongation as reflected by the longer internode and taller overall plant height. Cell width was not affected by temperature and photoperiod. Although the continuing cell division in inner tissue plays a role, the rate of stem elongation in both alpine and prairie ecotypes of *S. longipes* is probably controlled by the thick-walled epidermal cells where only cell elongation was observed. Clearly, these results substantiate the predominant role of the epidermis in controlling the growth and development of internodes in *S. longipes*.

CHAPTER THREE

Changes in cell wall components during stem elongation of *Stellaria longipes*

Summary

Histochemical studies of stem sections of alpine and prairie ecotypes of *Stellaria longipes* at various developmental stages and environmental conditions were examined. Fluorescence microscopy revealed a number of unusual anatomical features in the epidermal cells and the innermost cortical cell layer. Cross sections examined under a fluorescence microscope showed a strong autofluorescence in the walls of these cells. However, the intense autofluorescence of the cortical cell walls was only detected in cross sections of mature internodes and internodes that were subjected to low temperature treatment. Histochemical examinations revealed that the autofluorescence of the walls of the cortical cells was caused mainly by the deposition of phenolics, namely, suberin and lignin. The characteristics and patterns of development of the innermost cortical cells followed those of an endodermis. The overall patterns and characteristics of these cell wall component changes were similar in both alpine and prairie ecotypes. The only observable difference was the timing of occurrence which correspond with the ecotype stem growth patterns. These indicated that the timing and deposition of cell wall components play crucial a role in stem elongation plasticity of *S. longipes*.

Introduction

The final shape and size of plants is a consequence of cell division and cell elongation. Stem elongation is a complex process involving the co-operation of outer and inner tissues (Kutschera, et al., 1987). The process by

which cells elongate requires cell wall modifications because cell wall components can influence cell elongation. Remarkably, the most important feature of growing plant cell walls is not their rigidity, but their ability to modify during expansion growth and differentiation, and in response to external stimuli. The histological observations from Chapter 2 indicated that stem elongation in *S. longipes* is controlled by epidermal cell elongation in combination with cell division of inner tissues. However, difficulties in histological procedures involving the chemical fixation of stem tissues were found suggesting changes in the cell wall components that prevented improper penetration of fixative causing poor cellular preservation. Thus, histochemical studies using fresh stem cross sections are necessary to determine how these changes were regulated to bring about the changes in stem elongation.

The growing plant cell wall is a biphasic structure consisting of a rigid cellulose microfibrilar phase interconnected to a matrix phase (Fry, 1988). Two-third of the wall's dry weight is made up of the matrix which contains several polysaccharides such as pectins and hemicelluloses (Fry, 1986; Brett and Waldron, 1996). The wall matrix also contains glycoproteins, proteins and phenolic compounds (Brett and Waldron, 1996). Biochemical and biophysical evidence suggest the matrix as the major factor regulating cell elongation (Cosgrove, 1993). However, little is known of the cross-links that hold the wall matrix polymers together. Cross-links involve both covalent and noncovalent bonds that hold the wall together (Iiyama et al., 1994). Although not much is known about the details of the cross-links of polymers, it is likely that hydrogen bonds, calcium bridges, ester bonds, and covalent linkages of phenolic compounds all participate in constructing the wall (Brett and Waldron, 1996). Thus knowledge of the chemistry of the matrix cross-

links, especially about the phenolic compounds, would provide a better understanding of the chemical and biochemical processes involved in stem elongation.

The unsaturated double bonds of phenolic compounds autofluoresce under UV illumination. This autofluorescent nature of phenolic compounds makes it particularly amenable to histological study by UV microscopy. Phenolic acids exist as covalently bound cell wall components and are also found within the protoplast throughout the plant kingdom (Wallace and Fry, 1994). Smart and O'Brien (1979) identified the major autofluorescent component of the scutella of both wheat and barley which consists of ferulic acid. Ferulic acid is generally found to be uniformly distributed in the cell wall but it is most abundant in thick walled cells such as epidermis, xylem vessels, bundle sheath and sclerenchyma (Wallace and Fry, 1994).

This chapter examines the changes in cell wall components associated with stem elongation of alpine and prairie ecotypes of *S. longipes* under different developmental and environmental conditions. Histochemical identification of phenolic compounds deposited in walls of epidermal and the innermost cortical cells was made using conventional histochemical techniques in conjunction with fluorescence microscopy. The results revealed the presence of an endodermis in stem sections of both ecotypes and that suberin and lignin are the primary phenolic polymers found in epidemal and endodermal cell walls. Since no observable differences were detected in the characteristics and patterns of development between alpine and prairie ecotypes, results of only one ecotype are presented in this chapter.

Materials and Methods

Plant growth conditions

Alpine and prairie plants were maintained in SDC conditions for a minimum of 120 days as described in Chapter 2. These plants were then transferred from SDC to LDW conditions. They were maintained in LDW for 18 days until the completion of both vegetative growth and floral development. Ten randomly selected ramets were collected during SDC conditions, during the rapid elongation stage (9 day in LDW conditions) and after the completion of stem elongation (18 days in LDW conditions). The second internodes of these ramets were collected and placed in ice. Carrot tissues were used to provide support during freehand sectioning. Sections from the upper, central and basal segments of the internode were hand-sectioned using a sharp double-edged razor blade. These sections were kept in water prior to histochemical staining.

Histochemical procedures

A fluorescent staining procedure to detect suberin, lignin and callose in plants was developed by Brundrett et al. (1988). This procedure was used to monitor the morphological changes in cell walls as the plants were growing under different growth conditions. The freehand sections were first stained in 0.1% (w/v) berberine hemi-sulphate in distilled water for 1 hour, rinsed with several changes of distilled water, stained with 0.5% (w/v) aniline blue in distilled water for 30 minutes, and then rinsed as above. After rinsing, the sections were further stained with 0.1% (w/v) $FeCl_3$ in 50% glycerin for 15 minutes and mounted in the same solution. Lignified walls stained yellowish-white, suberized walls stained blue-white or blue (Brundrett et al., 1988).

Lipid stain

Sudan IV stain was used to determine the presence of suberin and lipid components in cell walls. The Sudan staining solution was prepared by dissolving 0.7 g of the Sudan IV dye in 100 ml of ethylene glycol. The solution was heated to 100°C and stirred thoroughly for 10 minutes. The hot solution was filtered through Whatman No. 2 paper twice (Jensen, 1962). Fresh freehand cross sections of stem were placed in pure ethylene glycol for 5 minutes with constant shaking. These sections were transferred to the staining solution and stained for 5 minutes. The sections were then transferred to 85% (v/v) ethylene glycol in water and gently agitated for 3 minutes. The sections were rinsed in distilled water for 5 minutes and mounted in glycerin-gelatin. Walls containing suberin and or lipidic components stained red.

NaOH treatment

A hot alkaline treatment is the best method for removing some background autofluorescence of the cell walls to reveal the Casparian bands of the endodermis (Peterson et al., 1982). Freehand cross sections were prepared as described above. These sections were treated with 1% (w/v) aqueous NaOH at 60°C for 12 hours. The sections were rinsed with water and mounted in water. The Casparian bands appearred as bright dots on radial walls of the endodermis.

Microscopy and Photography

The stained sections were immediately examined using a Zeiss epifluorescence photomicroscope. Photographs were taken with Kodakrome 400 ASA color slides and print films. The best results were obtained when

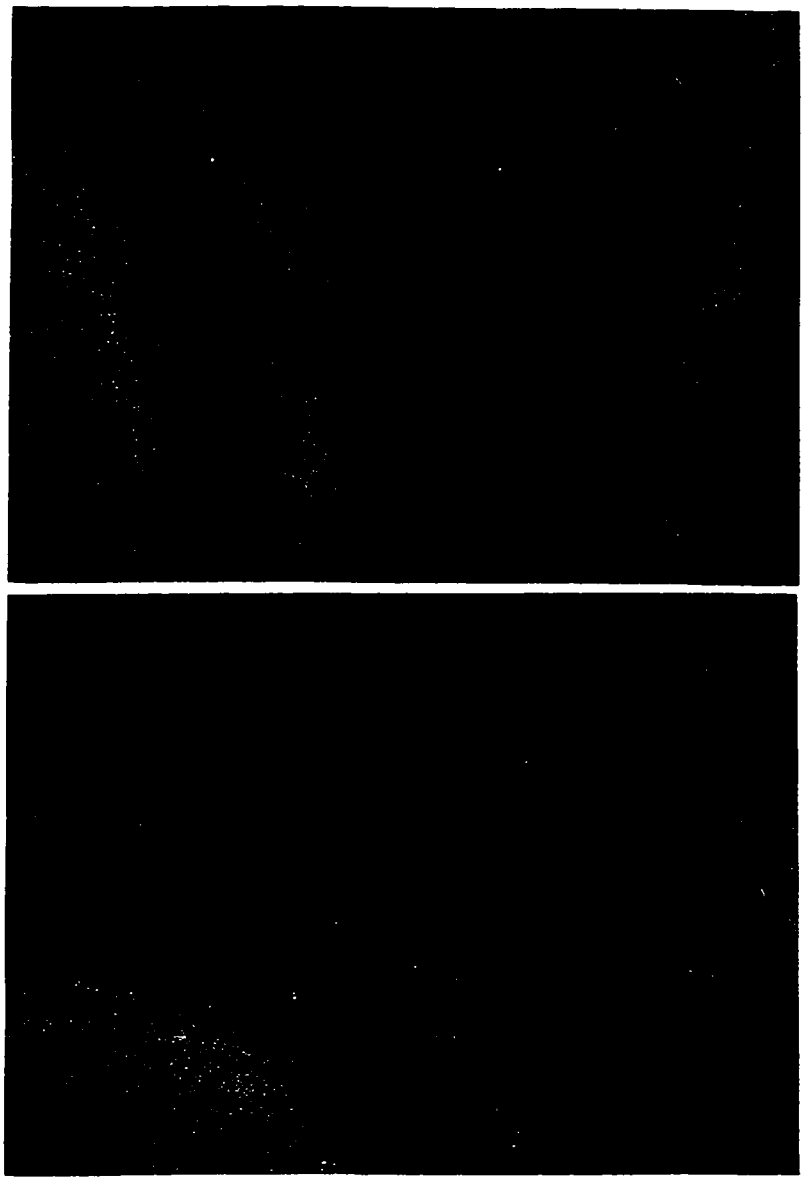
photographs were taken immediately after staining. Minimal bleaching of specimens could be prevented by focusing with brightfield microscopy prior to switching to UV illumination.

Results

Fluorescence Microscopy

Unstained cross sections of fresh stem tissue made from the second internode of alpine and prairie ecotypes of *S. longipes* were examined. Autofluorescence was detected in the cell walls. A bright blue autofluorescence was observed in the inner tangential walls of the epidermis of both SDC and LDW sections. In addition, the tangetial walls of the endodermis and xylem vessels of these sections also fluoresced brightly. Cortical cells showed red autofluorescence (Plate 3.1 A and B). The red fluorescence of the cortical cells is probably caused by the presence of chlorophyll. This made it difficult to assess the wall components showing the autofluorescence. In order to determine the histochemical characteristics of the phenolic compounds accumulated in the walls of these cells, a special fluoresecent staining procedure developed by Brundrett et al., (1988) to detect suberin, lignin and callose was used. In this procedure, the berberine and aniline blue fluorescent stains were used. The berberine serves as the fluorescent fluorochrome while the aniline blue acts to quench the unwanted background fluorescence. This technique permits the different components of the cell wall to be identified. Lignified walls stained bright yellow, Casparian bands stained yellow-white and suberin stained blue-white or blue (Brundrett et al., 1988).

Plate 3.1. Photomicrographs of stem cross sections prepared from the second internode below the shoot apex of *S.longipes* plants from A) SDC conditions and B) LDW conditions. E, epidermis; C, cortex; N, endodermis; X, xylem; →, suberin lamellae. These are unstained sections viewed under UV microscopy. x125.



A

B

In order to determine the pattern of internode development, sections from the upper, central and basal regions of the internode were made and stained. The presence of the autofluorescence in walls of epidermal and endodermal cells was found in sections that were made from the internode of SDC-treated plants and those of the mature internodes from LDW conditions. In sections from the upper region of SDC-treated internode, Casparian bands can be seen as bright dots in the radial walls of endodermal cells (Plate 3.2 A). In the central region of the SDC-treated internode, suberin lamellae have developed on the inner tangential walls but not the outer tangential walls of this layer (Plate 3.2 B Arrow). The deposition of suberin lamellae was more prominent in the basal region of the SDC-treated internode (Plate 3.2 C Arrow). When cross sections were prepared from three regions of rapidly elongating internode under LDW conditions, Casparian bands were absent in the upper region but were visible in the central region while suberin lamellae were deposited in the endodermal cells of the basal region (Plate 3.3 A-C). In mature LDW internodes, cross sections from all three regions showed suberin lamellae deposition in the endodermal cell walls (Plate 3.4 A-C Arrow).

Sudan stains are specific for lipids and stain fats, oils, waxes and free fatty acids (Jensen, 1962). The Sudan IV stain was used to verify that the bright fluorescence of the endodermal cell walls comprised of suberin which is lipidic in nature. Cross sections prepared from the various regions of the second internodes selected from ramets collected during SDC conditions, during the rapid elongation in LDW, and after the completion of stem elongation were used. Casparian bands appeared as dark spots in the upper region of SDC-treated and in the central region of the LDW rapidly elongating internodes (Plate 3.5 A, 3.6 B). The endodermal cell walls of sections from the central and basal regions of SDC-treated internode (Plate 3.5 B,C), the basal

region of LDW rapidly elongating internode (Plate 3.6 C) and all regions of LDW mature internode (Plate 3.7 A-C) stained red suggesting the presence of suberin. The cuticle of the sections of all treatments also stained red indicating the presence of waxes or lipidic substances. However, the inner tangential walls of the epidermis were not stained.

A simple clearing procedure devised to remove unwanted background autofluorescence to allow the identification of Casparian band was used (Peterson et al., 1982). When the sections were treated with hot aqueous NaOH, autofluorescence in all the walls of the cortex, pith cells as well as the inner tangential walls of the epidermis were removed. However, autofluorescence was still observed in the radial walls of the endodermis. This autofluorescence present in the endodermis of sections from the central and basal regions of SDC-treated internode (Plate 3.8 B,C), and mature LDW internode (Plate 3.10 A-C) extended throughout the entire span of the radial walls. Casparian bands appeared as bright dots in the upper region of SDC-treated internode (Plate 3.8 A), and in the central region of rapidly elongating internode (Plate 3.9 B). In the basal region of the LDW internode, there appeared to be a thickening in the walls of the endodermis (Plate 3.9 C).

Plate 3.2. Berberine aniline blue stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown under SDC conditions. E, epidermis; C, cortex; N , endodermis; B, Casparian bands; X, xylem; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.

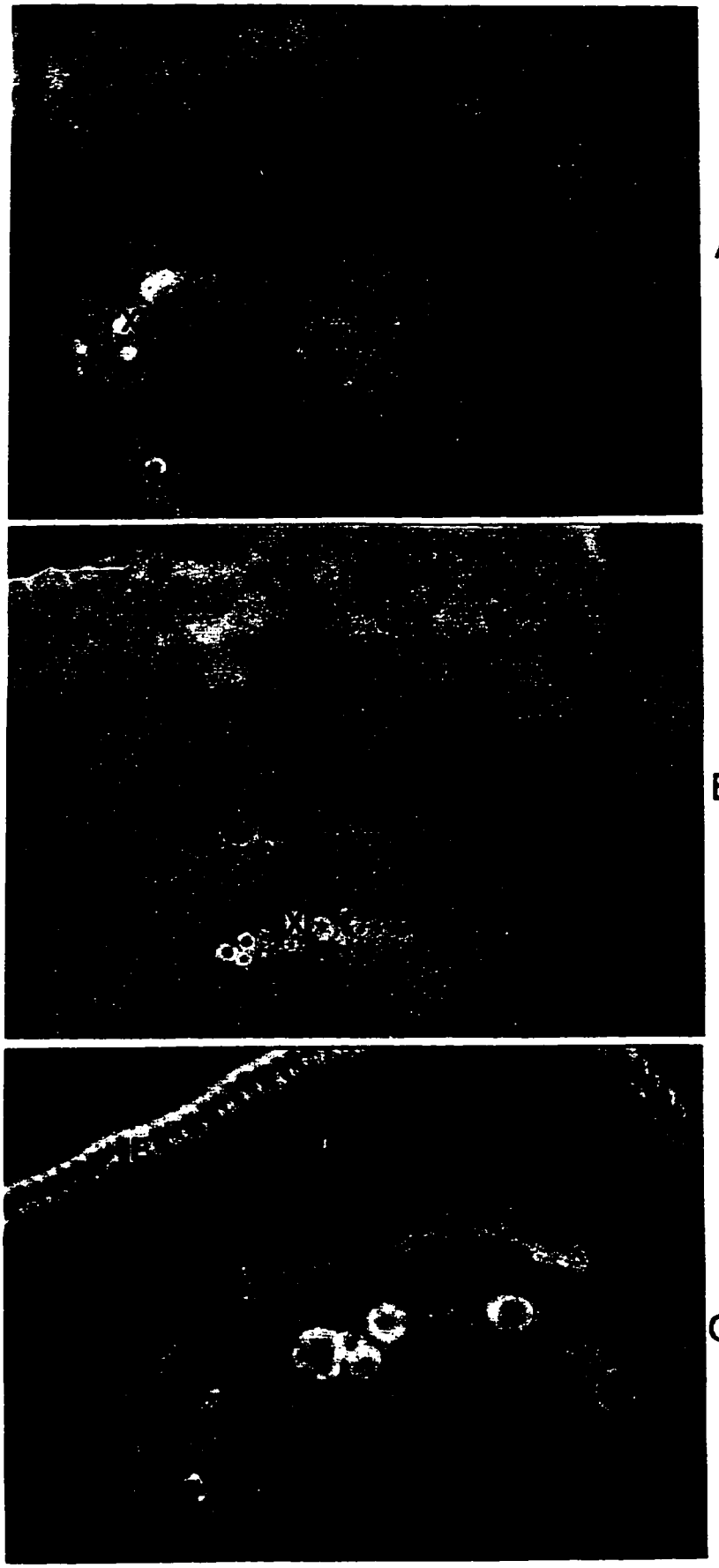

A
B
C

Plate 3.3. Berberine aniline blue stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown for 10 days under LDW conditions. This internode is undergoing a period of rapid stem elongation. E, epidermis; C, cortex; N, endodermis; B, Casparian bands; X, xylem; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.

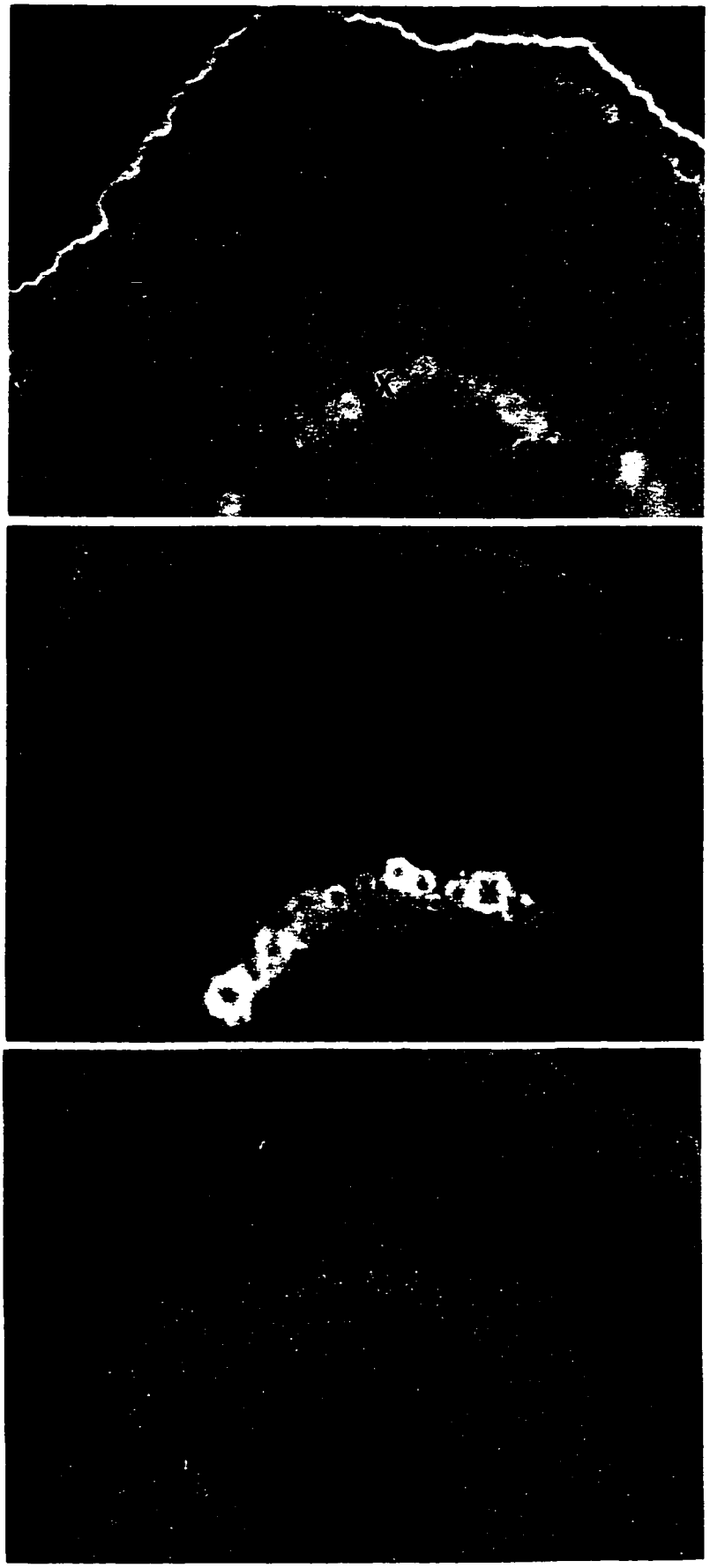

A
B
C

Plate 3.4. Berberine aniline blue stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown for 23 days under LDW conditions. E, epidermis; C, cortex; N, endodermis; X, xylem; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.

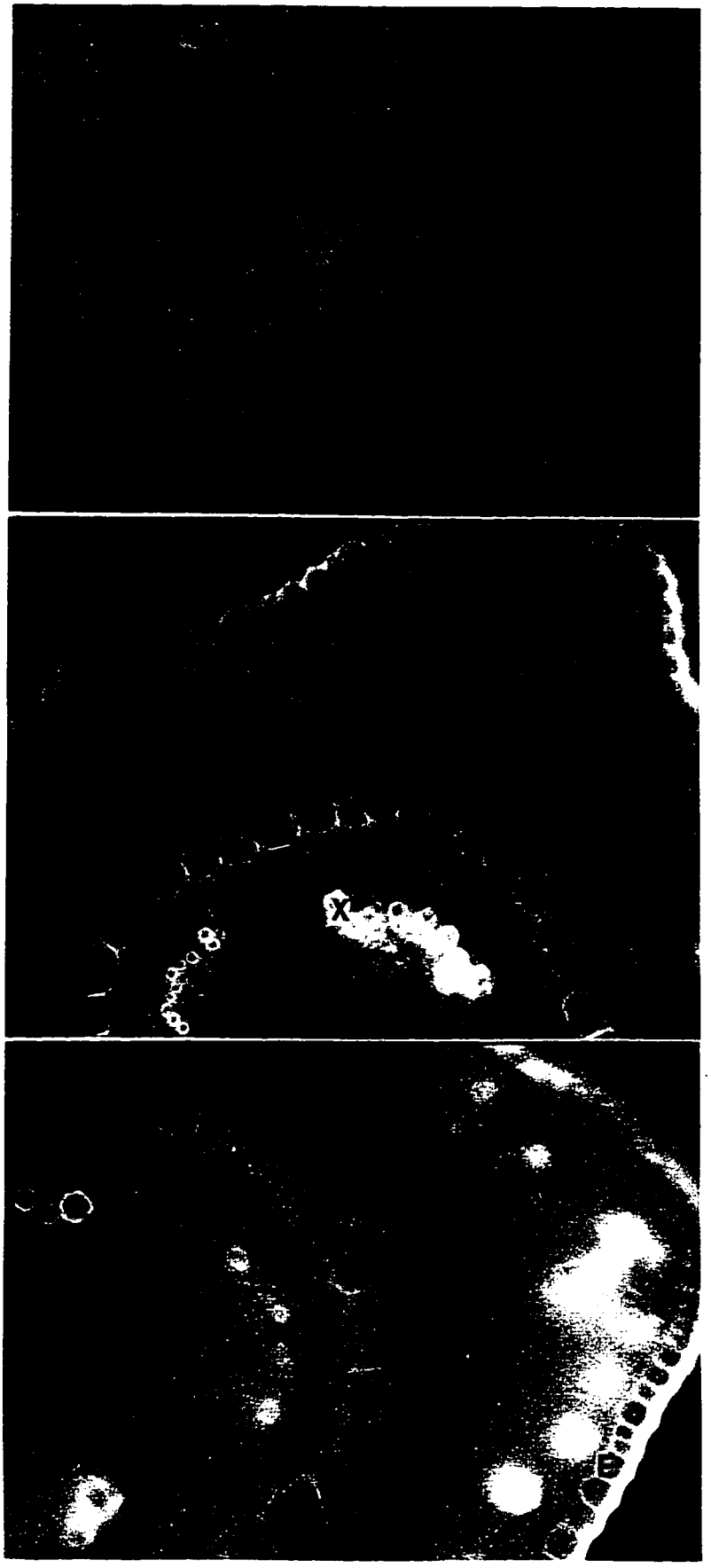

A
B
X
C

Plate 3.5. Sudan stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown under SDC conditions. E, epidermis; C, cortex; N, endodermis; B, Casparian bands; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x125.

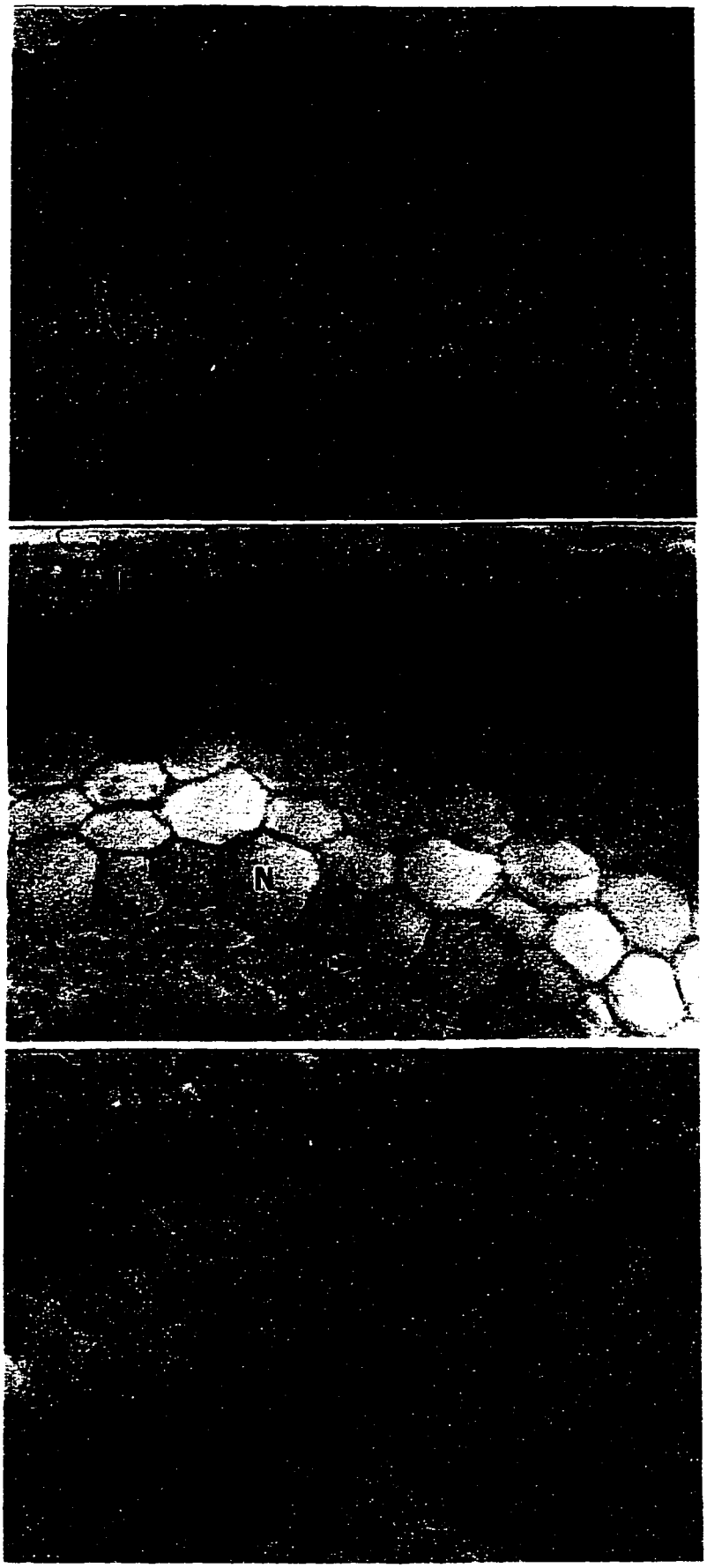

A
B
N
C

Plate 3.6. Sudan stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown for 10 days under LDW conditions. E, epidermis; C, cortex; N, endodermis; B, Casparian bands; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x125.

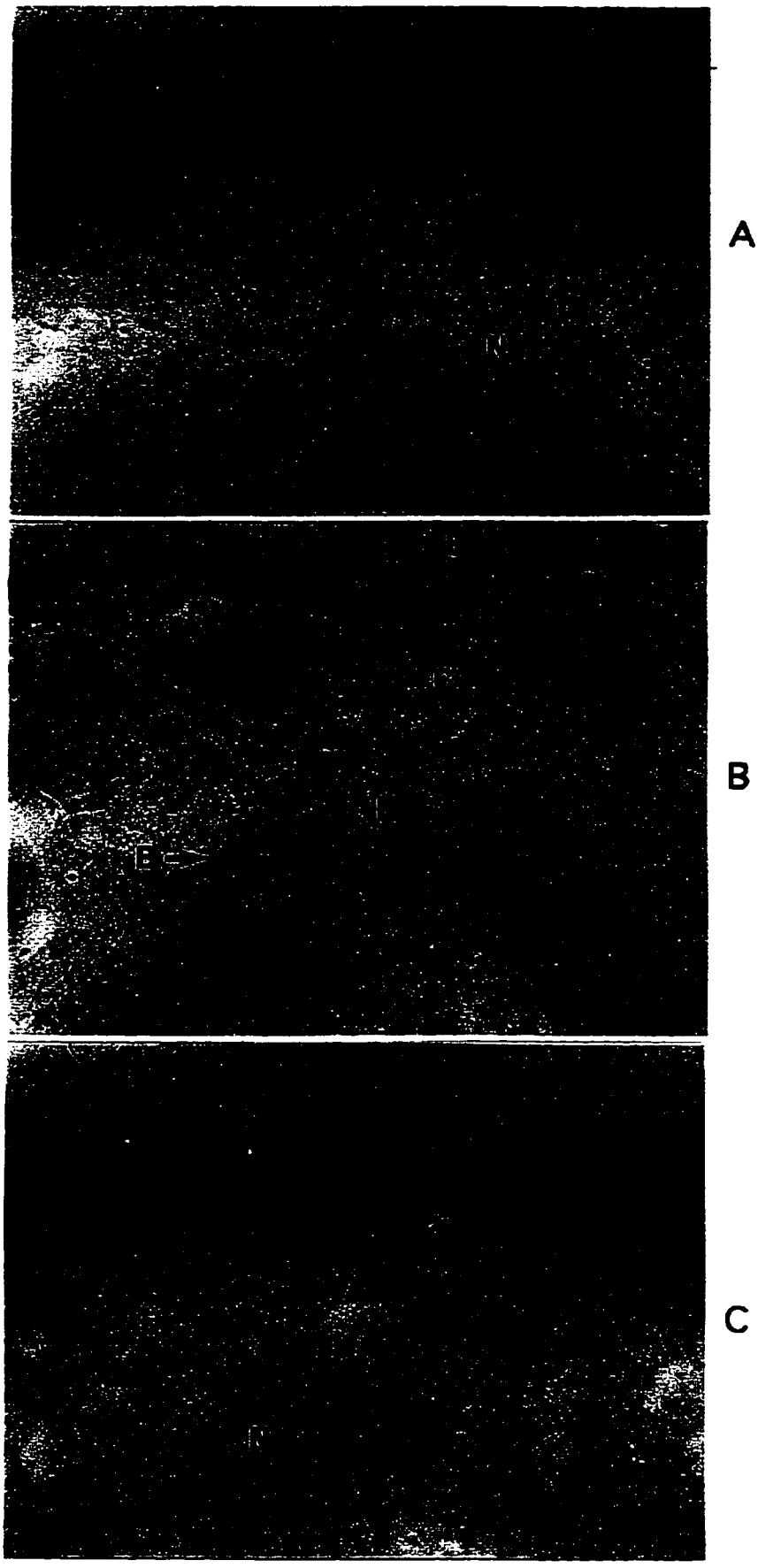

A
B
C

Plate 3.7. Sudan stain of stem sections from the second internode below the shoot meristem of *Stellaria longipes* plants grown for 23 days under LDW conditions. E, epidermis; C, cortex; N, endodermis; →, suberin lamellae. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode.

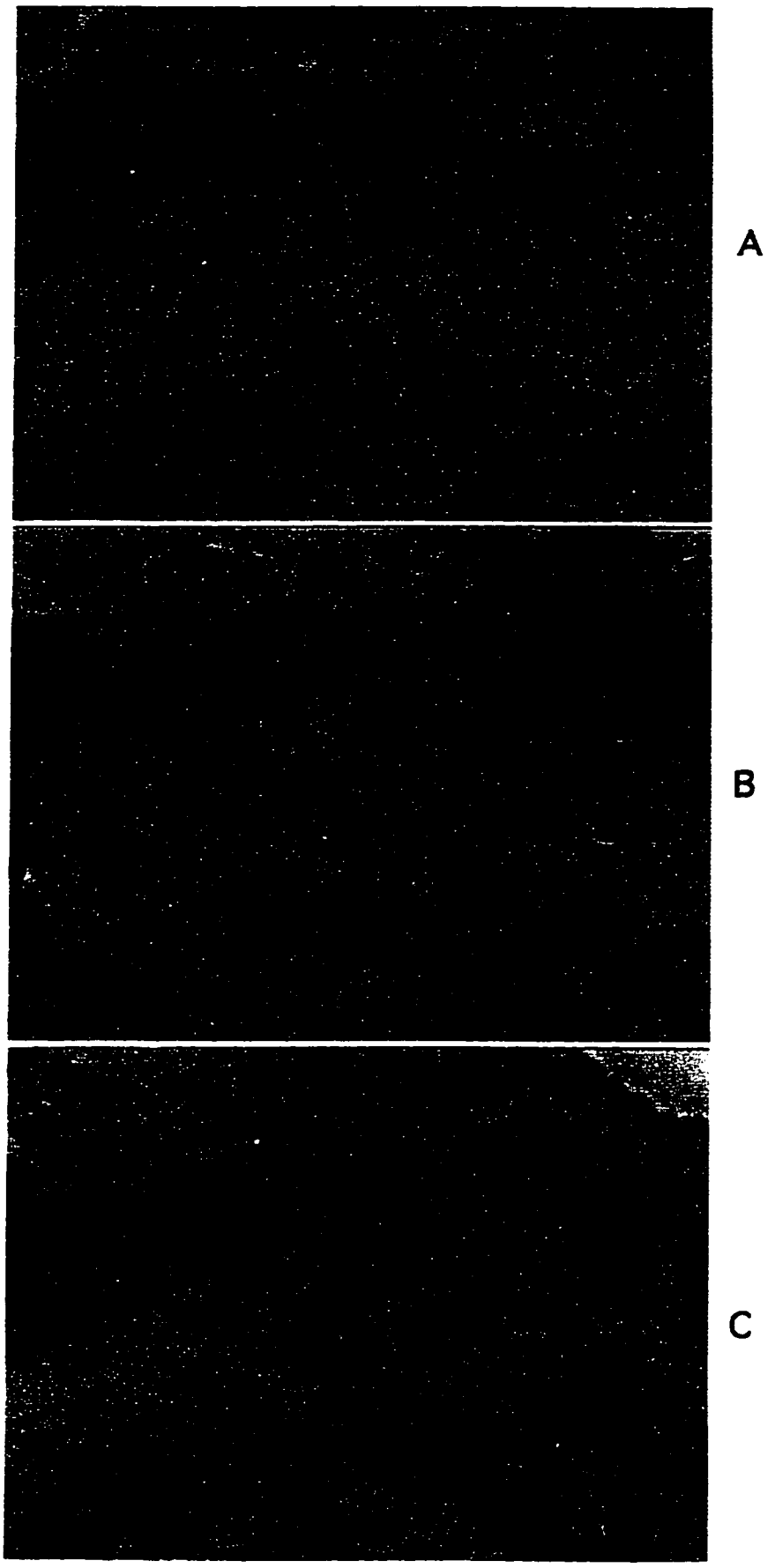
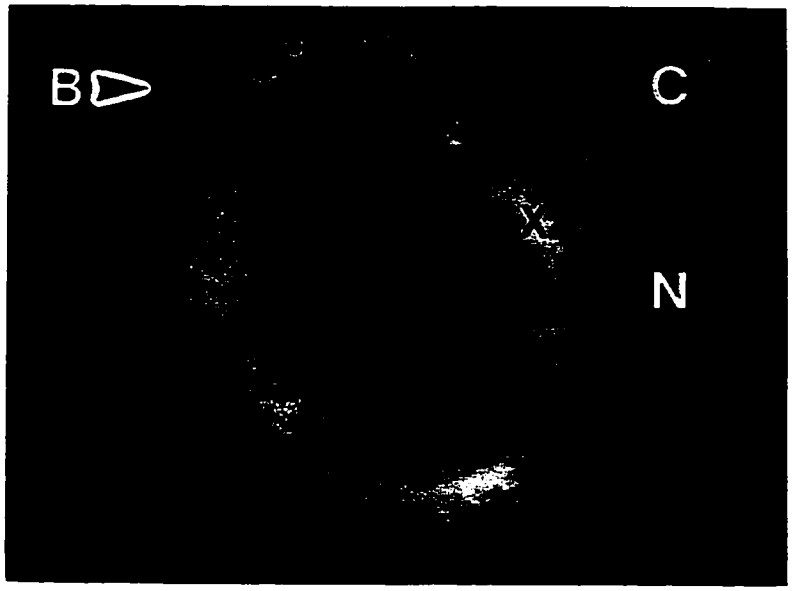
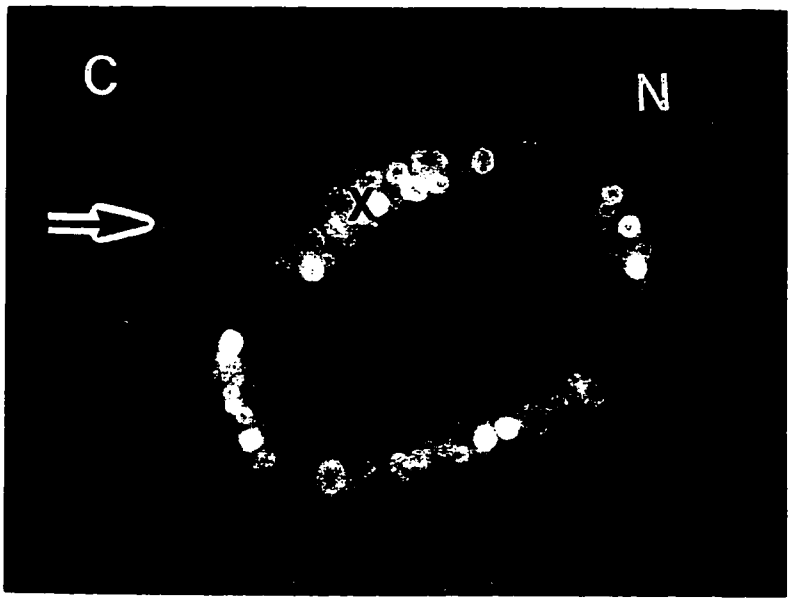

A
B
C

Plate 3.8. Photomicrographs of NaOH-treated stem sections from the second internode from the shoot meristem of *Stellaria longipes* plants grown under SDC conditions. C, cortex; N, endodermis; X, xylem; →, suberized/lignified thickening. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.



A



B

C

Plate 3.9. Photomicrographs of stem NaOH-treated sections from the second internode from the shoot meristem of *Stellaria longipes* plants grown for 10 days under LDW conditions. E, epidermis; C, cortex; N, endodermis; X, xylem; →, suberized/lignified thickening. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.

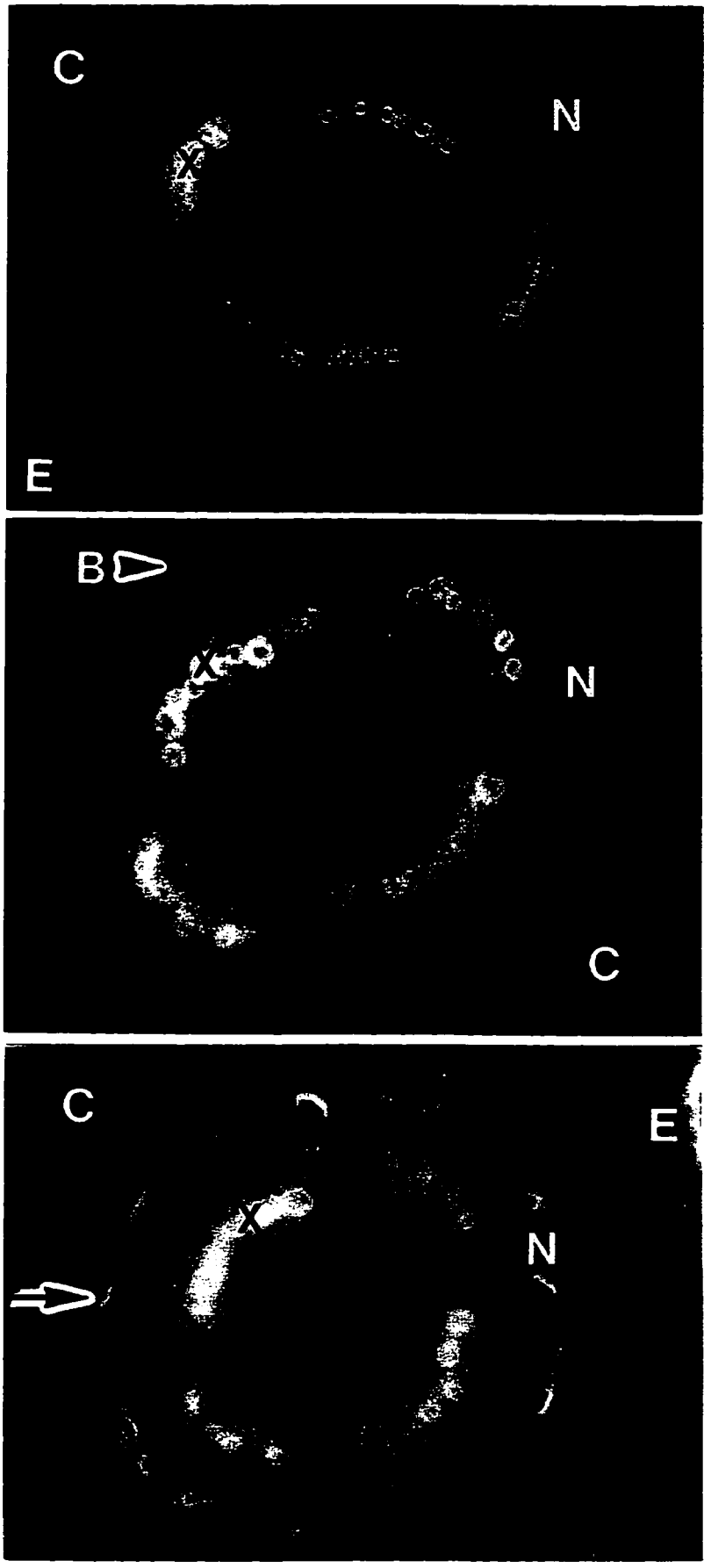
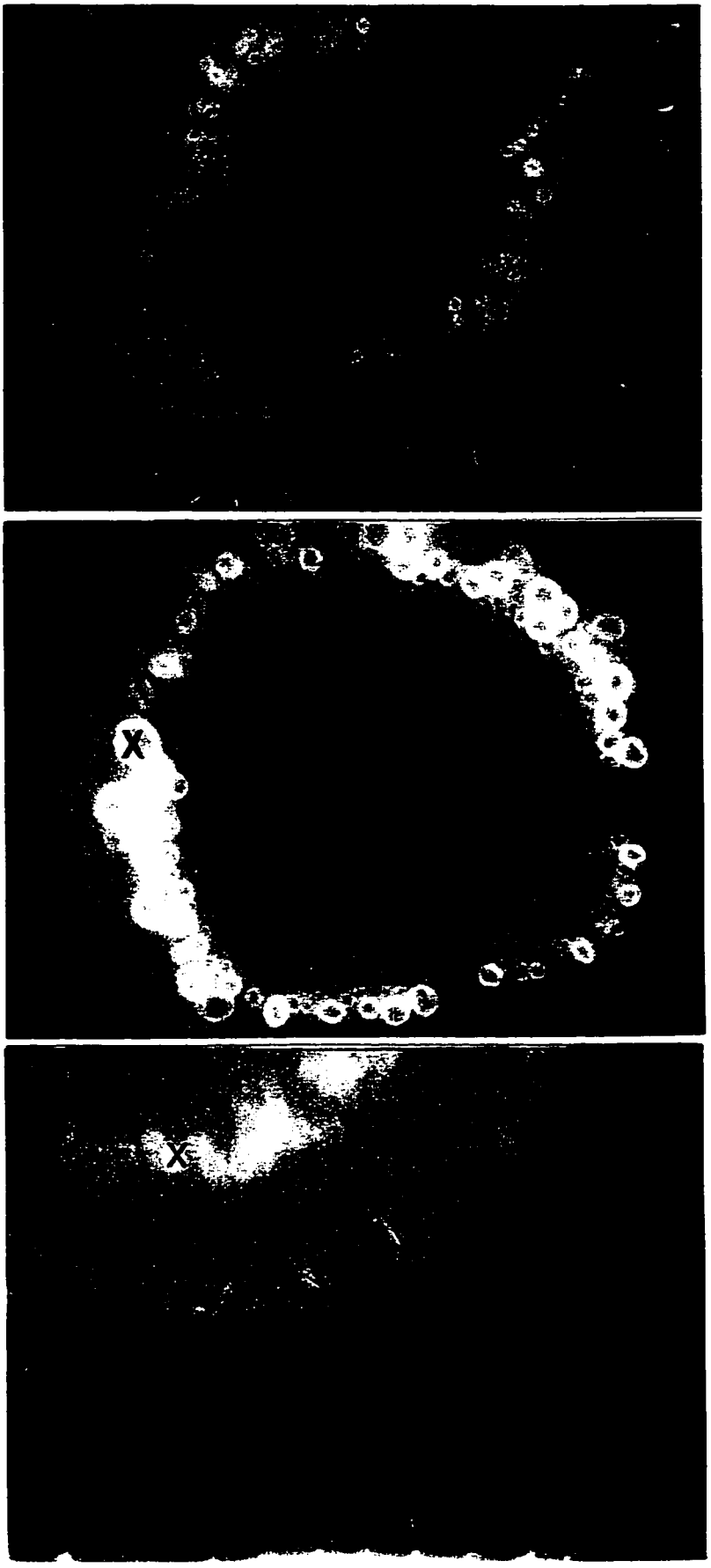

C
N
E
A
B
B
N
C
B
C
E
N
C

Plate 3.10. Photomicrographs of stem NaOH-treated sections from the second internode from the shoot meristem of *Stellaria longipes* plants grown for 23 days under LDW conditions. E, epidermis; C, cortex; N, endodermis; X, xylem; →, suberized/lignified thickening. A) stem section from the top region of the internode, B) stem section from the central region of the internode, and C) stem section from the basal region of the internode. x80.


A
B
X
C
X

Discussion

The observations made here indicate changes occurring in the wall chemistry as the cells elongate. The growth pattern of *Stellaria longipes* internode began at the basal region of each internode and proceeded upwards. The concomittant appearance of phenolic compounds in the walls of epidermal and endodermal cells of this region as the cells matured strongly suggests that phenolic polymers may have cross-linked with other matrix components and terminated cell elongation (Plate 3.4). Thus inhibition of cell elongation may have been brought about by a mechanical stiffening of the growth-limiting epidermal and endodermal cell walls which in turn restrain stem elongation. It has been suggested that wall-bound peroxidase play a major role in cross-linking phenolic residues (Schopfer, 1996). Preliminary histochemical results showed a higher level of peroxidase in the walls of epidermal cells from SDC or mature internode than those of the rapidly elongation internode. These results indicate that suppression of stem elongation is largely due to the hardening of epidermal and endodermal cell walls.

During growth and development, changes in the composition of the wall is inevitable. Therefore, it is reasonable to assume that cross-links within the wall matrix and/or at the cellulose microfibril-matrix interphase are weakened to bring about wall extensibility. Mechanical weakening of the walls is possibly caused by the action of wall-loosening enzymes such as xyloglucan endotransglycosylase (XET), glycanases and expansins (Brett and Waldron, 1996). However, as a wall matures or is exposed to stressful environmental factors, changes in wall extensibility must occur leading to an interruption of cell elongation. This is possibly due to both a decrease in wall-loosening enzyme activity and an increase in interpolymeric cross-links.

Many studies have focused on the relationship of biochemical events with the decrease in wall extensibility (Cosgrove, 1993, McQueen-Mason et al., 1993). It is possible that changes in phenolic cross-links between polymers are involved in the control of wall stiffening, which is in turn likely to be under the control of wall bound peroxidases.

Environmental stimulus such as low temperature not only caused phenolics accumulation in the epidermis but also induced precocious development of endodermis in stems of *S. longipes*. The endodermis, cells that separate the rest of the cortex from the vascular tissues, is normally found in many subaerial stems and roots (Lersten, 1997; Van Fleet, 1961). However, the endodermis is also detected in stems of some plant species (Lersten, 1997). These include the stems of several members of the *Commelinaceae* (Brundrett et al., 1988), etiolated stems of *Sorghum* (Espelie and Kolattukudy, 1979), etiolated pea epicotyls (Sack, 1986; Karakara and Shibaoka, 1994) and stem of *Senecia vulgaris* (Warden, 1937). Endodermal cells are distinguished from other cortical cells in that the radial and transverse walls contain Casparian bands which consist of suberin, lignin or both (Peterson and Enstone, 1996). In plants, the endodermis is generally developed in response to environmental fluctuations in habitat. The functions of the endodermis include fat metabolism, selection of ions, water accumulation, gas exchange barrier, food storage and exchange, and pathogenic barrier (Van Fleet, 1961).

The development of endodermal cells follow three general stages: a) a primary stage which involves the deposition of fatty and other substances in the radial and transverse walls, commonly known as the Casparian strips; b) a secondary phase which includes the oxidation and deposition of fatty and other substances in a continuous suberin lamella on the surface of the

protoplast next to the cell wall; and c) a tertiary phase which comprises of the deposition of cellulose on all the walls except the outer tangential wall to give the cell a U-shape appearance in transverse view (Van Fleet, 1961). So far most studies have shown the development of endodermis mostly in roots and subaerial stems with very few cases involving aerial stems.

This study confirms the development of Casparian bands in the endodermis of *S. longipes* aerial stems. Under low temperature treatment, the Casparian bands could be detected near the top of the growing internode by UV microscopy (Plate 3.2 A and Plate 3.8 A). This structure can also be observed in the innermost cortical cells of the central region of the internode where cell elongation has just stopped during normal developmental program (Plate 3.3 B). Casparian bands can be detected more easily when the sections were cleared in hot aqueous alkaline (Plate 3.8-Plate 3.10). The patterns observed here are in agreement with the results in Chapter 2 which indicated that the internode developed in an acropetal pattern. The ontogeny of the endodermis in *S. longipes* stems seems to follow the general patterns as described for all endodermis (Van Fleet, 1961). The endodermal cells begin the initial stage of development with the deposition of the Casparian bands in the radial and transverse walls (Plate 3.8 A and Plate 3.9 B). These cells further modified their development by forming suberin lamellae on the inner tangential walls of the cells (Plate 3.4 B,C, Plate 3.5 C and Plate 3.6 A-C). In the final stage of development, a thick, cellulosic secondary wall which may be lignified or suberized is deposited internal to the suberin lamella (Plate 3.9 C). This is predictable because secondary cell walls do not expand and grow but they can provide a wide variety of important functions such as defense, structural support and storage (Fry, 1988). However, it is unclear in this study whether it was the deposition of the secondary walls that stopped

growth or that the primary walls underwent some modifications to resist further expansion.

Although the stages of the endodermis formation were similar between alpine and prairie ecotypes of *S. longipes*, the onset in which this structure developed differed in the two ecotypes. This is not surprising since the stem growth pattern between the two ecotypes was different (Chapter 2). Precocious development of the endodermis could also be induced when plants were exposed to low temperature. Such development is consistent with its functions which serve to protect the plants in response to environmental changes. In *S. longipes*, the emergence of the endodermis not only provided structural support but also functioned as a barrier to the apoplastic movement of ions, nutrients and water between tissues under stressful conditions. Low temperatures can cause ice crystals to form in the spaces in the primary walls which are lethal to the plants. Therefore, endodermal cells have Casparian bands which infiltrate the apoplastic spaces thus preventing the formation of ice crystals and imparting a tight control of cell-to-cell transport. Regardless of the ecotypic differences, differentiation of the endodermis was linearly related to the growth rate or maturation process of the stem. In growing internodes the Casparian bands developed away from the rapidly growing region (Plate 3.9B). Alternatively, in low temperature treated plants, the Casparian bands were formed in the growing region of the internode (Plate 3.8A). A non growing or slowly growing internode was characterized by the production of the well developed Casparian bands or an endodermis near the top of the internode (Plate 3.10A).

In *S. longipes*, the presence of an endodermal cell layer in the aerial stem is most likely an adaptive trait acquired by the organism during evolution in response to the environmental changes in their habitats. One of

the unique features in *S. longipes* is the ability of these plants to propagate vegetatively with the underground stem acting as running stolon to give rise to new roots and vegetative clones upon subaerial exposure. This strategy could be assumed as one of the survival mechanisms demonstrated by *S. longipes* in a competitive environment. Because of their fragile stem structure, it would be unfavorable for these plants to maintain their upright position, thus creeping on the habitat floor could be their only chance of survival during the winter months. It can also be concluded that environmental changes induced the formation of this endodermis in plants under the exposure to low temperature. In *S. longipes,* the endodermis not only functioned to provide protection and tight regulation of ions and nutrient exchanges but also served as a support structure because these plants do not have secondary growth. The development of the endodermis is probably associated with termination of stem elongation.

The results obtained from this study are in agreement with the results from previous studies on the localization of lignin and suberin in the endodermis of onion roots (Brundrett et al., 1988; Wilson and Peterson, 1983). The use of the berberine-aniline blue technique allows the unveiling of details of the endodermal cell walls structure. Berberine has a high affinity for walls containing phenolic compounds such as lignin and suberin while aniline blue has the ability to quench autofluorescence of plant tissues which contain chlorophyll and other secondary metabolites (Brundrett et al., 1988). Conventional histochemical techniques such as Sudan stain was also used to further confirm the presence of suberin in the form of Casparian bands or suberin lamellae in the endodermis. When cross sections of *S. longipes* were stained with Sudan stain, suberin was detected, confirming the deposition of polymeric material containing phenolic components in the walls of the

endodermis (Plate 3.5-Plate 3.8). The cuticle also stained positively suggesting the presence of waxes or lipidic substances. However, negative results were observed for the inner tangential walls of the epidermis. Preliminary results from Phloroglucinol test for lignin of these sections did not indicate the presence of lignin in the tangential walls of the epidermis. Thus, there appears to be another source of phenolics in the walls of the epidermis that neither react with suberin nor lignin stains. Furthermore, this observation provides direct evidence that the epidermal cell walls contains other alkaline soluble material in the tangential walls of the epidermis with the fluorescence properties of phenolic compounds.

Treatment of the sections with 1% NaOH for 12 hours at 60°C successfully removed the autofluorescence from the cell walls especially the inner tangential walls of the epidermis (Plate 3.8 A-C-Plate 3.10 A-C). This treatment is known to remove esterified phenolic acids from cell walls and leaves most of the lignin intact (Willemse and Emons, 1991). However, it is not clear why this autofluorescence was removed from most cell walls but not the radial walls of the endodermis. Perhaps the Casparian bands found in *S. longipes* is composed of lignin as well. Direct chemical evidence obtained from Casparian strips of *Clivia miniata* roots contain lignin as a major cell wall polymer and the molecular structure of this lignin polymer was different from that of the xylem vessels (Schreiber, 1996). Another explanation may be that the Casparian bands contain a large domain of hydrophobic wall which is impossible for the NaOH to penetrate (Peterson et al., 1982). It has been shown that the Casparian bands were more tolerable to strong acid and alkaline treatment than the suberin lamellae (Priestley and North, 1922).

In summary, the results from the present study indicate that cross-linking and deposition of phenolic compounds may be the processes that lead

to the termination of stem elongation of *S. longipes*. The endodermis found in the stems developed under the normal developmental program as the cells reach maturation. In rapidly growing stem under LDW conditions, precocious induction of the endodermis development by low temperature was also observed. However, based on these results, it cannot be concluded whether the onset of phenolics accumulation and endodermis development caused stem elongation to stop, or the termination of cell elongation signaled the deposition of phenolic compounds and formation of the endodermis. Further experiments should examine whether other environmental or mechanical stresses induce precocious development of the endodermis. Also, by analyzing the role of cell wall peroxidase, insights into the mechanisms of cell wall stiffening can be gained. Chemical quantitative analyses would help in identifying the phenolic acids that cross-linked to the epidermal and endodermal cell walls of *S. longipes* during growth and stress. A detailed knowledge of the exact chemical composition of the endodermis will enhance our understanding of the function of the Casparian strips in plants.

CHAPTER FOUR

Differential Regulation of Phenylalanine ammonia-lyase in *Stellaria longipes* under different growth conditions

Summary

To elucidate the mechanism by which temperature and photoperiod control stem elongation in alpine and prairie ecotypes of *Stellaria longipes* grown under four different growth conditions, long day and warm (LDW), short day and cold (SDC), dark and warm (DW) and dark and cold (DC), the effects on phenylalanine ammonia-lyase (PAL) activities were examined. An increase in PAL activity was strongly correlated with phenolic products accumulation and the decrease in stem elongation rate under SDC treatment. Using a complementary DNA clone for PAL, plants under SDC treatment were shown to accumulate PAL mRNA transcripts and this accumulation paralleled the *in vivo* enzyme activity. Time-course studies show that PAL transcripts levels increased within the first 12 hours of cold stress and then declined to pretreatment levels within 2 days. The time and magnitude of the response to SDC treatment differred in alpine and prairie ecotypes suggesting that these plants possess different regulatory mechanisms in response to environmental stresses. The expression and activity of PAL upon exposure to low temperature was light dependent. However, our results support the view that PAL activity and phenolic compounds produced by plants are protective mechanisms adopted by the plants against stressful environments. These observations indicate that phenylpropanoid metabolism may play a crucial role in the development of plant tolerance to low temperature.

Introduction

Phenylpropanoid metabolism consists of many complicated pathways leading to the synthesis of various plant secondary products. These products provide structural and adaptive functions in plants. The biosynthesis of phenylpropanoid compounds is developmentally activated but can also be activated in response to environmental cues such as wounding, light, pathogen infection and ultraviolet irradiation (Hahlbrock and Scheel, 1989).

The role of plant secondary metabolites, including organic compounds derived from phenolic precursors, has long been recognized. In plants, the term phenolic compound is used to describe a large group of chemical compounds with an aromatic ring bearing one or more hydroxyl group together with a number of substituents (Wallace and Fry, 1994). Attempts have been made to classify plant phenolics according to the phenolic substituents. Ribereau-Gayon, (1972) classified the phenolic substituents into four groups: 1) the phenolic acids and coumarins, 2) the flavones, flavonols and related compounds, 3) the chalcones, dihydrochalcones and aurones and 4) the anthocyanins. Among the many enzymes involved in the phenylpropanoid pathways, phenylalanine ammonia-lyase (EC 4.3.1.5; PAL) is the most studied enzyme. One example of these important natural products includes lignin, the world's most abundant phenolic polymer which is a major component in plant cell walls, providing both mechanical and structural support. Stresses such as pathogen infection and wounding also induce lignin deposition to provide protection (Whetten and Sederoff, 1995). Other major plant secondary products, the flavonoids which include anthocyanins, are commonly found in petals, fruits and seeds (Holten and Cornish, 1995). Anthocyanins can also protect plants from damage by filtering UV irradiation. Other lipidic polymers such as cutin and suberin also serve

to protect plant cells from an unpredictable environment and against fungal and microbial attacks (Kolattukudy, 1984). Observations from chapter 3 suggest that the deposition of suberin, lignin and other phenolic compounds in the walls of epidermal and endodermal cells may be involved in the regulation of stem elongation of *Stellaria longipes*. Thus, examining the key enzymes in the phenylpropanoid pathway will shed light on the roles of phenolic compounds in plant growth and development.

Phenylalanine ammonia-lyase (PAL) is the key enzyme which provides the initial committed step for the phenylpropanoid biosynthetic pathway by eliminating ammonia from L-phenylalanine to *trans*-cinnamate, a precursor for the biosynthesis of many plant phenylpropanoid derivatives such as lignin, suberin, flavonoids, coumarins and numerous soluble and wall-bound esters and amides (Figure 1.1; Solecka and Kacperska, 1995). PAL is encoded by a small gene family in higher plants (Ohl et al., 1990) and that the transcripts of individual PAL genes show different patterns of accumulation during different stages of growth and development (Hahlbrock and Scheel, 1989). So far all studies in higher plants indicate that changes in PAL activity are regulated at the transcriptional level and that its subcellular localization is mainly cytoplasmic (Jones, 1984). An increase in PAL activity, however, has not always been correlated with the production of a specific phenylpropanoid compound (Camm and Towers, 1977). In most but not all cases, PAL has been shown to be the rate-limiting enzyme in phenylpropanoid biosynthesis and that it is sensitive to inhibition by its product, cinnamic acid (Bolwell et al., 1986). Thus the activation of PAL transcripts and enzyme must lead to phenolic polymer(s) biosynthesis.

Developmental and environmental controls of PAL levels have been investigated in many higher plants (Hahlbrock and Scheel, 1989). In plants,

PAL activity was found to vary under stresses such as wounding, nutrient deficiencies, pathogen attack, and UV irradiation (Camm and Towers, 1977, Lawton and Lamb, 1987, Liang et al., 1989). The effects of light and low temperature on PAL activity have also been widely studied in other systems (Zucker, 1965, 1971, 1972; Tong and Schopfer, 1976; Loschke et al., 1981 and Parvez et al., 1997).

As mentioned earlier, phenolic compounds may be involved in plant responses to mechanical and environmental stresses. PAL activity has been shown to increase in response to low temperature in tomatoes (Rhodes and Wooltorton, 1977), *Brassica napus* (Parra et al., 1990), sweet potato (Tanaka et al., 1989) and maize (Christie et al., 1994). Some studies correlated the increase in PAL activity with an accumulation of phenolic compounds, for example, the deposition of anthocyanins in maize seedlings and in stems and leaves of *Arabidopsis thaliana* under low temperature treatment (Christie et al., 1994; Leyva et al., 1995), and the deposition of suberin-like lipids and cell walls thickenings in cold-acclimated winter rye leaves (Griffith et al., 1985). White light also promoted PAL activity which correlated with increase in diferulic acid, ferulic acid and p-coumaric acid levels in maize coleoptiles (Parvez et al. 1997). Although many studies demonstrated a parallel correlation between PAL activity and environmental stresses, the underlying biological mechanisms are still poorly understood.

Stellaria longipes grown under low temperature and short photoperiod show stunted leaf and stem morphology primarily caused by the thickening of the epidermal cell walls and the development of an endodermal layer (Chapter 3). In addition, the accumulation of anthocyanins in leaves and stems was also observed (personnal observation). Anatomical and histochemical studies revealed that the deposition of suberin and lignin in

the epidermal and endodermal cells played a key role in the control of stem elongation plasticity. To understand the involvement of phenolic compounds accumulation in stem elongation and to further elucidate the role of plant phenolics during cold stress, the expression and activity of PAL under cold and warm conditions, in the presence and absence of light, were examined in both the alpine and prairie ecotypes of *S. longipes*.

Materials and Methods

Plant growth conditions

Plants from alpine and prairie ecotypes were collected and vegetative clones were made and transplanted into 6 cm pots. These pots were maintained in growth chambers (Conviron, Winnipeg, Canada) under short photoperiod (8 hours) and cold temperature (8°C day, 5°C night) (SDC conditions) for at least 120 days. This winter simulated treatment is required to elicit maximum growth in *S. longipes* (Macdonald et al., 1984). Randomly selected SDC-treated pots of each ecotype were then transferred to long photoperiod (16 hours) and warm temperature (22°C day, 18°C night) (LDW conditions) and allowed to grow for 9 days in long day warm (LDW) conditions (the time of rapid stem elongation as seen in Chapter 2). These plants were then placed into chambers of different conditions (LDW=long photoperiod and warm temperature, SDC=short photoperiod and cold temperature, DC=dark and cold temperature and DW= dark and warm temperature). These conditions were chosen in order to determine the factor responsible for PAL induction. A time-course study was performed where randomly selected tissues (0.3 g) were harvested, frozen in liquid nitrogen and stored at -80°C. Photosynthetically active radiation measured at plant height

with a LI-COR LI-185B quantum sensor was remained constant in all chambers (220 μmoles $m^{-2}s^{-1}$).

Genomic DNA extraction

Genomic DNA from stem and leaf tissues were extracted using the procedure described by Doyle and Doyle (1987) with slight modification. Fresh tissues were frozen in liquid nitrogen and ground to powder with a sterile mortar and pestle. 2 X CTAB buffer (2% (w/v) hexadecyltrimethylammonium bromide (CTAB), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, (pH 8.0) was added to the powderized tissues and incubated at 60°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 rpm at room temperature. The aqueous phase was removed and 2/3 volume of prechilled isopropanol was added to precipitate the nucleic acid. The DNA was spooled with a glass pasteur pipette and rinsed in the washing buffer containing 76% ethanol and 10 mM ammonium acetate. The DNA pellet was air dried and resuspended in sterile distilled water. The DNA was subsequently treated with RNase A to a final concentration of 10 ug/ml at 37°C for 30 minutes. The DNA was precipitated with equal volume of 5 M ammonium acetate and 2.5 volumes of absolute prechilled ethanol at -20°C for at least 1 hour and centrifuged at 15,000 rpm for 15 minutes. The pellet was washed with 70% ethanol, lyophilized and resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.4). DNA concentrations were determined by a spectrophotometer via standard methodology (Sambrook et al, 1987).

Southern hybridization

DNA samples (20 µg per lane) were digested by restriction endonuclease (Pharmacia, Uppsala, Sweden, 3 units/µg DNA) overnight at 37°C. The digested DNA was separated by electrophoresis and blotted onto a nylon membrane (Hybond-N+, Amersham, UK) in 20 X SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7). After blotting, the membrane was baked at 80°C for 2 hour and prehybridized in Rapid-hyb buffer (Amersham, UK) at 58°C for 2 hours. A 1.8 Kb insert of a cDNA clone encoding for PAL gene in *Phytolacca* (Joy et al., unpublished) was radiolabeled by random prime procedure (Sambrook et al., 1989) and used as probe. Hybridization was performed in the same buffer at 58°C for 3 hours. Membranes were washed once in 2 X SSPE plus 0.1% SDC for 10 minutes and twice in 1 X SSPE plus 0.1% SDS for 10 minutes at 50°C. The membranes were then exposed to Kodak XAR-5 film at -80°C overnight.

RNA extraction

Total RNA was isolated using the guanidinium thiocyanate method described by Strommer et al., (1993). The frozen tissues were ground to a powder in liquid nitrogen and homogenized in 5 ml of extraction buffer containing 4 M guanidinium thiocyanate and 50 mM mercaptoethanol pH 7.0 per gram of fresh weight tissues. After the addition of 1 ml of 2 M sodium acetate pH 4.0 the homogenate was vortexed for 30 seconds. Five ml of water saturated phenol was added and the slurry was vortexed for 30 seconds and then 2 ml of chloroform : isoamyl alcohol (24:1 v/v) was then added before another round of 30 seconds vortex was given. The slurry was centrifuged at 10,000 rpm for 15 minutes at room temperature. The supernatant was extracted twice with equal volume of phenol : chloroform (1:1 v/v) and once

with equal volume of chloroform. After centrifugation, the aqueous phase was removed and an equal volume of prechilled isopropanol was added. The mixture was kept at -20°C for at least one hour. RNA was collected by centrifugation at 15,000 rpm for 15 minutes at 4°C. The RNA pellet was resuspended in 500 µl of DEPC-treated water and the RNA was further precipitated with an equal volume of 4 M LiCl in an eppendorf tube on ice for at least 1 hour. After centrifugation at 14, 000 rpm for 15 minute at 4°C, the pellet was washed in 70% ethanol, lyophillized and dissolved in DEPC-treated water. Quantification of RNA was spectrophotometrically determined.

Northern hybridization

For Northern analysis, 10 µg of total RNA was denatured by 50% formamide and 6% formaldehyde at 65°C for 15 minutes and separated by 1.5% agarose gel electrophoresis over 6% formaldehyde and 1x MOPS buffer (40 mM 3-(N-morpholino) propanesulfonic acid, 10 mM sodium acetate, 1 mM EDTA, pH 7.0). The RNA was then blotted onto Hybond N+ nylon membranes with 20x SSC (Sambrook et al., 1989). The membranes were baked for 2 hours at 80°C and prehybidized in Rapid-hyb buffer (Amersham, UK) at 65°C. The same PAL probe was used and labeled as mentioned in the Southern hybridization section. Hybridization was performed in the same buffer at 65°C for 3 hours. The membranes were washed in 2x SSC plus 0.1 % SDS at room temperature for 10 minutes, once in 0.2x SSC plus 0.1 % SDS at 50°C for 10 minutes. The membranes were then exposed to Kodak XAR film at -80°C. The same membranes were stripped for reprobing by washing several times in 0.01x SSC and 0.01% SDS at 95°C and re-exposed to Kodak XAR film to confirm for complete probe removal.

Phenylalanine ammonia-lyase (PAL) extraction and assay

PAL extraction was performed as described by Khan and Vaidyanathan (1986). Briefly, stem tissues were ground in liquid nitrogen and extracted in 150 mM Tris-HCl (pH 8.8) and 12 mM β-mercaptoethanol and one half of a spatula-full of polyvinylpolypyrrolidone (PVPP) to remove phenolic compounds. The homogenate was centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was purified by solid ammonium sulphate fractionation. The precipitate was recovered by centrifugation at 14, 000 rpm at 4°C for 5 minutes and dissolved in 2 ml of 50 mM Tris-HCl (pH 8.8). The dissolved pellet was subjected to column chromatography on Biogel PD-10 (Sephadex G-25, Pharmacia) that was pre-equilibrated with 20 ml of elution buffer which contains 50 mM Tris-HCl (pH 8.8) and 12 mM β-mercaptoethanol. Once the 2 ml of dissolved precipitate was applied and completely absorbed in the column, 4 ml of elution buffer was used to elute the protein. The eluant was used for determination of enzyme activity and protein concentration. The reaction mixture contained 250 µl of 50 mM Tris-HCl (pH 8.8), 10 µl of 100 mM L-phenylalanine and 250 µl of enzyme extract. The mixture was incubated at 30°C for 30 minutes and stopped by the addition of 100 µl of 5 M HCl. The reaction was centrifuged at 10,000 rpm for 10 minutes to remove the precipitate that may influence the absorbance reading. PAL activity was determined spectrophotometrically by measuring the amount of *trans*-cinnamic acid formed at 290 nm. The specific activity of PAL was expressed as µmoles of *trans*-cinnamic acid (CA)/mg protein/hour. Protein concentration was determined with the Bradford dye-binding assay (Bradford, 1976).

Results

Organization of *S. longipes* PAL genes

Genomic DNA of alpine and prairie ecotypes was digested with EcoRI, BamHI, XbaI, EcoRV and HindIII restriction endonucleases. The digested DNA was then separated by 1% agarose gel electrophoresis, transferred to Nylon membrane and hybridized with *Phytolacca* cDNA insert encoding for PAL. As shown in Fig 4.1, this probe hybridized to several fragments in each reaction containing *S. longipes* genomic DNA digested with different restriction endonucleases ranged in size from 2.5 Kb to 18 Kb. The presence of multiple hybridizing bands suggests that there is a small PAL gene family in the *S. longipes* genome. Genomic DNA of alpine and prairie ecotypes showed some polymorphisms for the restriction enzymes used.

Differential expression pattern under different growth conditions

The differential regulation of PAL mRNA in alpine and prairie ecotypes was examined under different photoperiod and temperature conditions. Total RNA extracted from SDC, LDW and LDW-transferred back to SDC, DC and DW conditions plants was probed with a PAL cDNA clone of *Phytolacca* (Dr. R. Joy et al., unpublished). Under the LDW conditions, both the prairie and alpine ecotypes show stable levels of both PAL transcripts and enzyme activity (Fig 4.2 A and Fig 4.3 A). Upon transfer to SDC conditions there is an induction of both PAL transcripts and enzyme activity in both ecotypes (Fig 4.4 A and Fig 4.5 A). The accumulation of PAL transcripts of alpine and prairie ecotypes shows differences in their temporal regulation. PAL transcript levels in prairie ecotype increased upon transfer to SDC conditions to a maximum at 3 hour; PAL enzyme activity increased

transiently and peaked at 12 hour after transfer to SDC conditions (Fig 4.4). Conversely, the alpine ecotype showed maximal PAL expression 12 hours after transfer and showed an increase in activity only after 1 day in SDC (Fig 4.5). While the PAL transcript of prairie ecotype diminished after about 3 hours (Fig. 4.4 A), accumulation of PAL mRNA of alpine ecotype was briefly induced and disappeared after 12 hours (Fig. 4.5 A). This indicates that PAL gene expression appears to be regulated differently in these two ecotypes.

Figure 4.4 A shows that the steady-state levels of prairie plants PAL mRNA started to increase after 30 minutes of low-temperature exposure, reaching a maximum level after 3 hours and declining after 6 hours of exposure. These results indicated that PAL mRNAs accumulate in response to low temperature. Since PAL expression has been shown to be regulated by light (Zucker, 1965; Ohl et al., 1990; Lyeva et al., 1995), an investigation was carried out to determine whether its accumulation in response to SDC conditions was light dependent. To do this, rapidly elongating plants (9 days) of both ecotypes in LDW conditions were transferred to DC or DW chambers, total RNA from these plants at different time intervals were prepared. Northern analyses showed that transcripts that accumulated under SDC did not appear under DC and DW conditions (Fig 4.6 A-Fig 4.9 A). However, a moderate hybridization signal was observed in DC-treated prairie plants (Fig 4.6 A). These results suggest that the accumulation of PAL transcripts by low temperature in SDC in alpine and prairie plants is actually light dependent.

PAL enzyme activity under different growth conditions

Macdonald *et al.*, (1984) showed that photoperiod and temperature are two of the most important factors involved in the regulation of stem elongation in *S. longipes*. The separate effect of photoperiod and temperature

on PAL activity was examined. Plants of alpine and prairie ecotypes were grown in LDW chambers until the period of rapid stem elongation (9 days), then transferred to SDC, DC and DW chambers for a treatment period of 21 days. PAL activity for plants that were left in LDW chamber was used as a control to show that the increase in activity was caused by the treatment and independent of daily fluctuation.

Basal PAL activities were observed in both ecotypes under LDW conditions (Fig 4.2 C and Fig 4.3 C). Rapidly elongating prairie plants in LDW conditions placed in the SDC chamber showed a marked increase in PAL activity after 2 hours and peaked at 12 hour after transfer. Thereafter the specific activity of PAL declined steadily within the next 7 days to the level observed before treatment (Fig 4.4 C). LDW grown alpine plants also showed a moderate increase in PAL activity when subjected to SDC conditions treatment but the magnitude of this was not as dramatic as that shown by the prairie plants, and the response time recorded was delayed by 12 hours (Fig 4.5 C). PAL activity of DC-treated prairie plants increased slightly initially and then drop back to the basal level. Prolonged dark exposure in cold temperature decreased the enzyme activity to a level below that of the basal activity (Fig 4.6 C). DC-treated alpine plants on the other hand did not show any changes in their PAL activity (Fig 4.7 C). DW-treated plants showed no increase in PAL activities for both ecotypes. In fact, after two hours the PAL activities decreased below the levels recorded for the LDW plants (Fig 4.8 C and Fig 4.9 C).

Figure 4.1 (A-B). Southern analysis of genomic DNA from alpine (A) and prairie (B) ecotypes of *Stellaria longipes*. 10 μg of DNA was digested with BamHI (a), EcoRV (b), HindIII (c), XbaI (d), and EcoRI (e), blotted onto a nylon membrane and hybridized with *Phytollaca* PAL cDNA fragment. The numbers represent the size standards in Kbp from Hind III digested λ DNA.

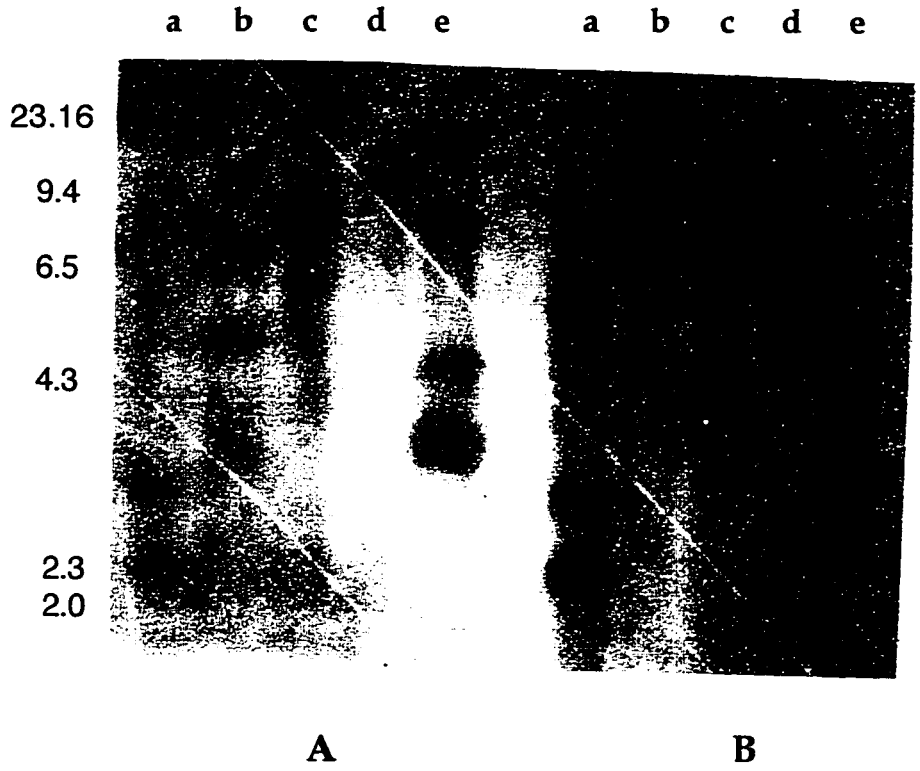

a b c d e
a b c d e
23.16
9.4
6.5
4.3
2.3
2.0
A
B

Figure 4.2. Effect of LDW conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the prairie ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from prairie plants grown under SDC conditions and from different time intervals after their transfer to LDW conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

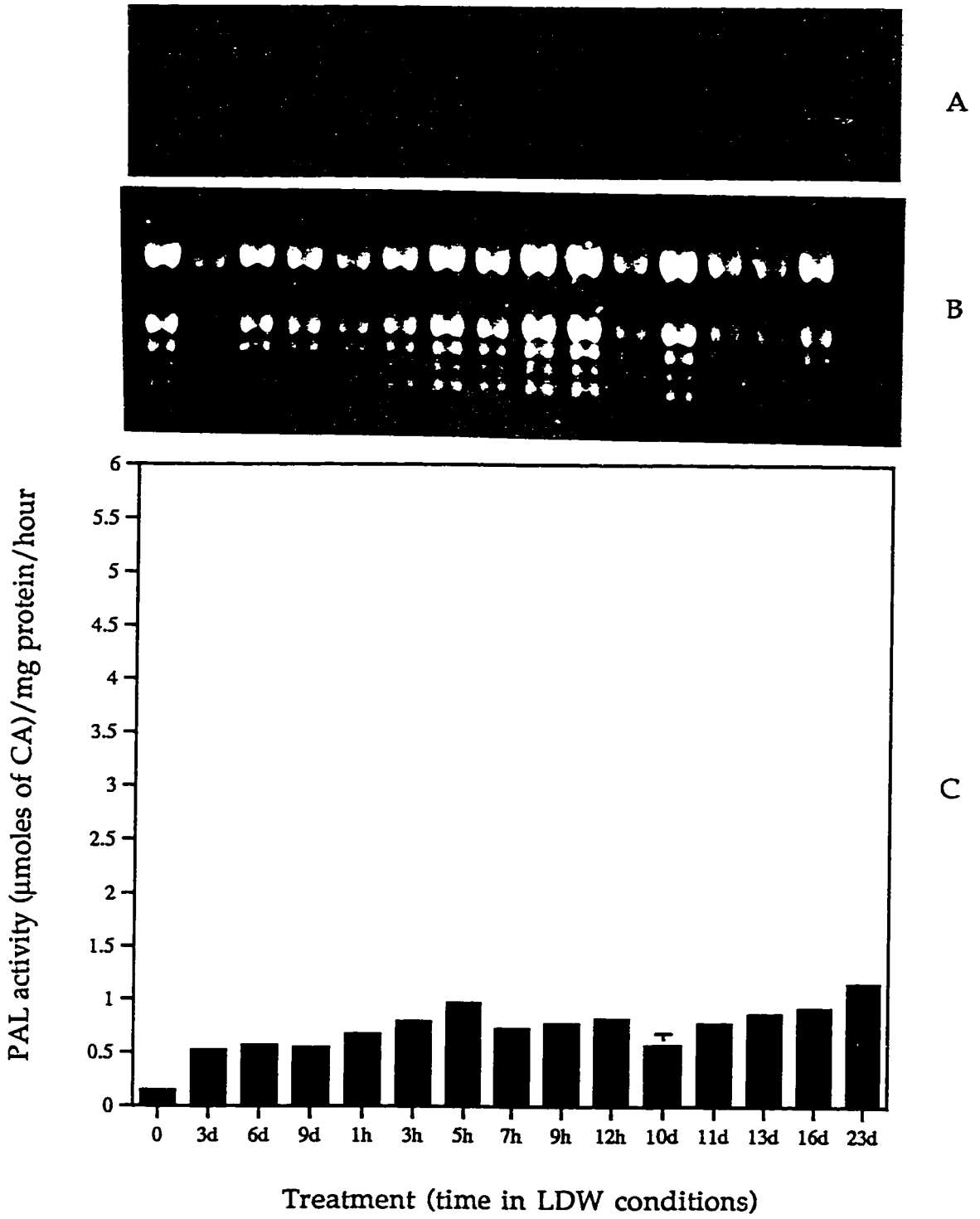

A
B
C
PAL activity (µmoles of CA)/mg protein/hour
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
0
3d
6d
9d
1h
3h
5h
7h
9h
12h
10d
11d
13d
16d
23d
Treatment (time in LDW conditions)

Figure 4.3. Effect of LDW conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the alpine ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from alpine plants grown under SDC conditions and from different time intervals after their transfer to LDW conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

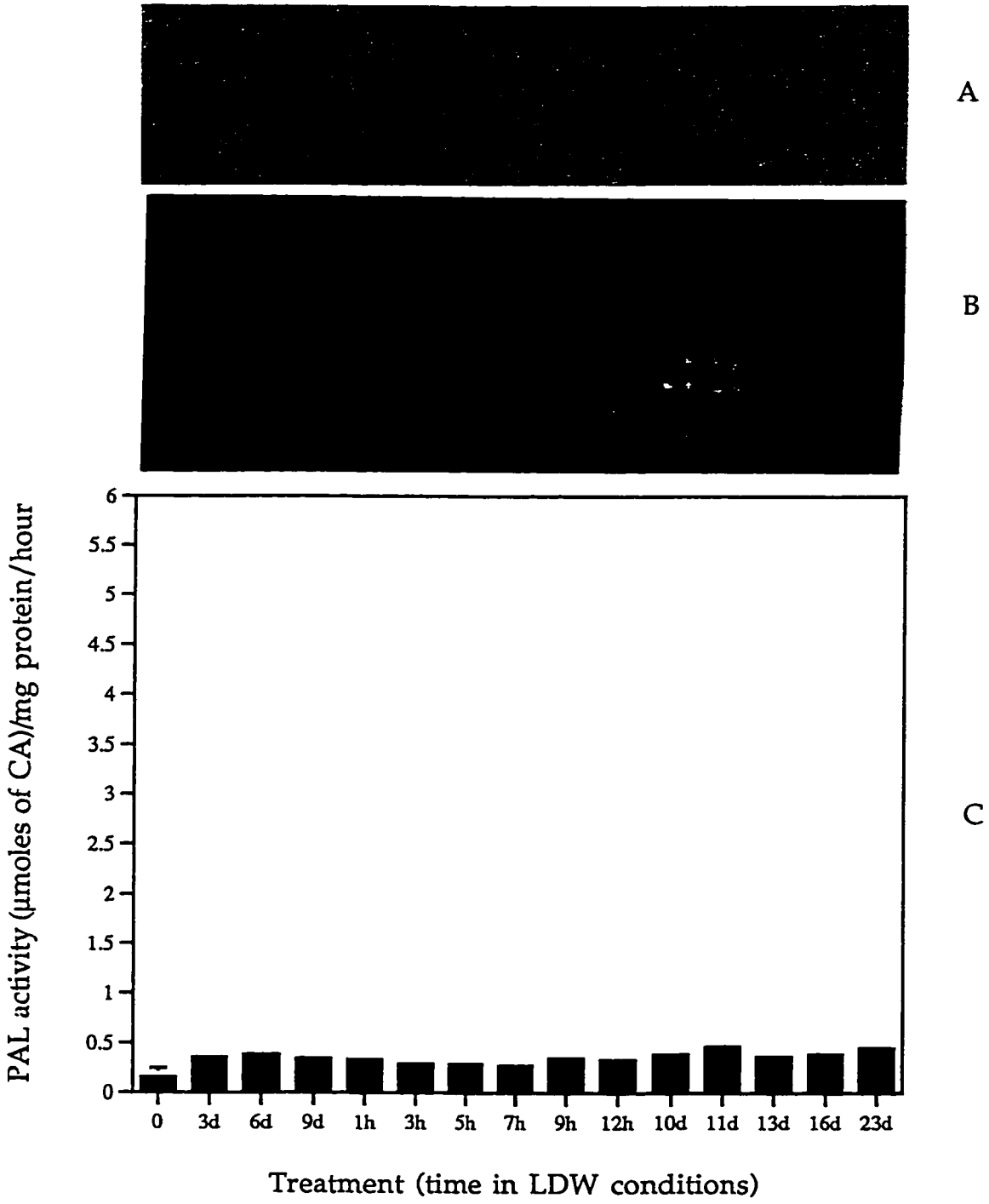

A
B
C
PAL activity (µmoles of CA)/mg protein/hour
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
5.5
6
0
3d
6d
9d
1h
3h
5h
7h
9h
12h
10d
11d
13d
16d
23d
Treatment (time in LDW conditions)

Figure 4.4. Effect of SDC conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the prairie ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from prairie plants grown under LDW conditions for 9 days and from different time intervals after their transfer to SDC conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

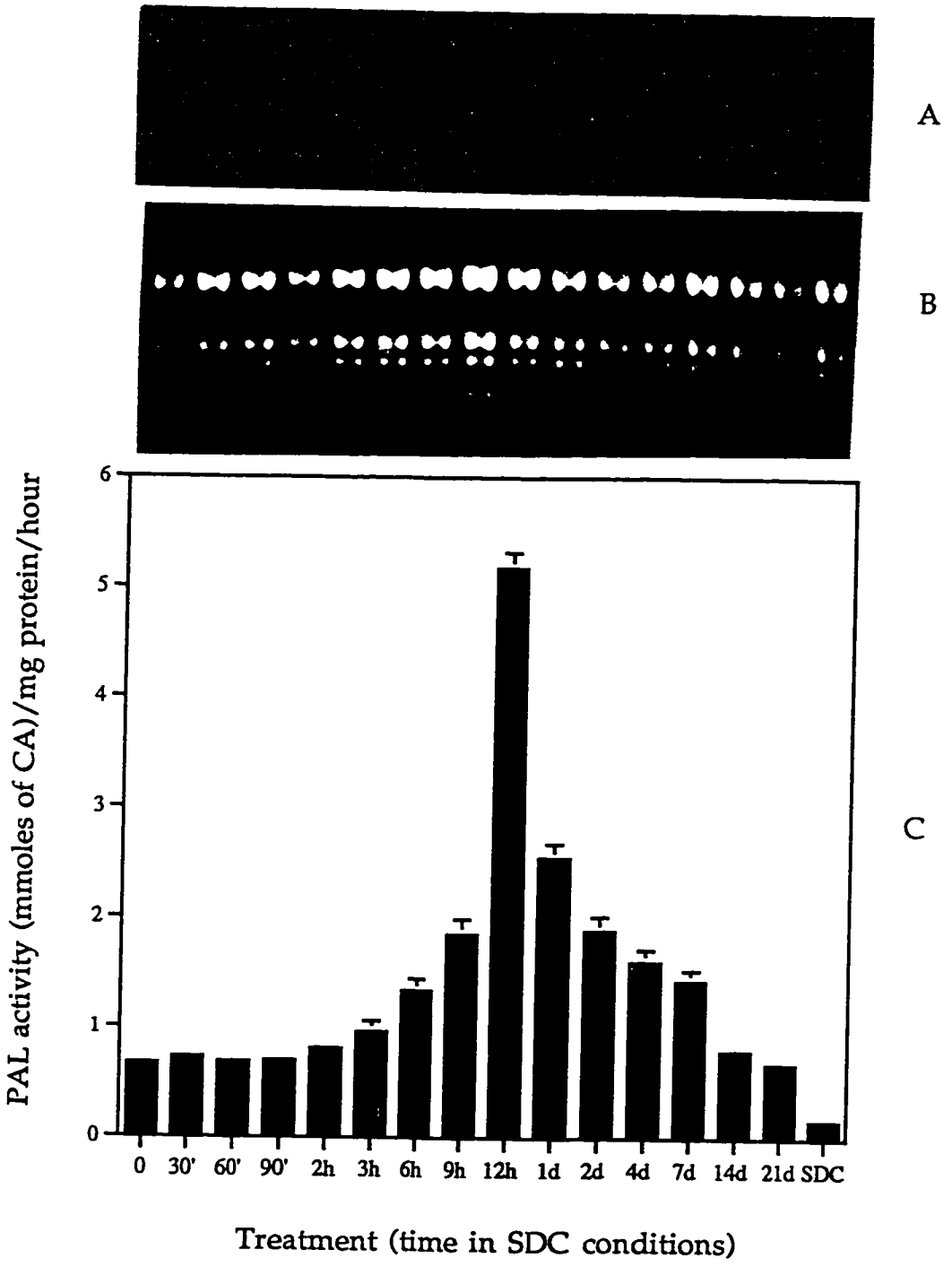

A
B
C
PAL activity (mmoles of CA)/mg protein/hour
0
1
2
3
4
5
6
0 30' 60' 90' 2h 3h 6h 9h 12h 1d 2d 4d 7d 14d 21d SDC
Treatment (time in SDC conditions)

Figure 4.5. Effect of SDC conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the alpine ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from alpine plants grown under LDW conditions for 9 days and from different time intervals after their transfer to SDC conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

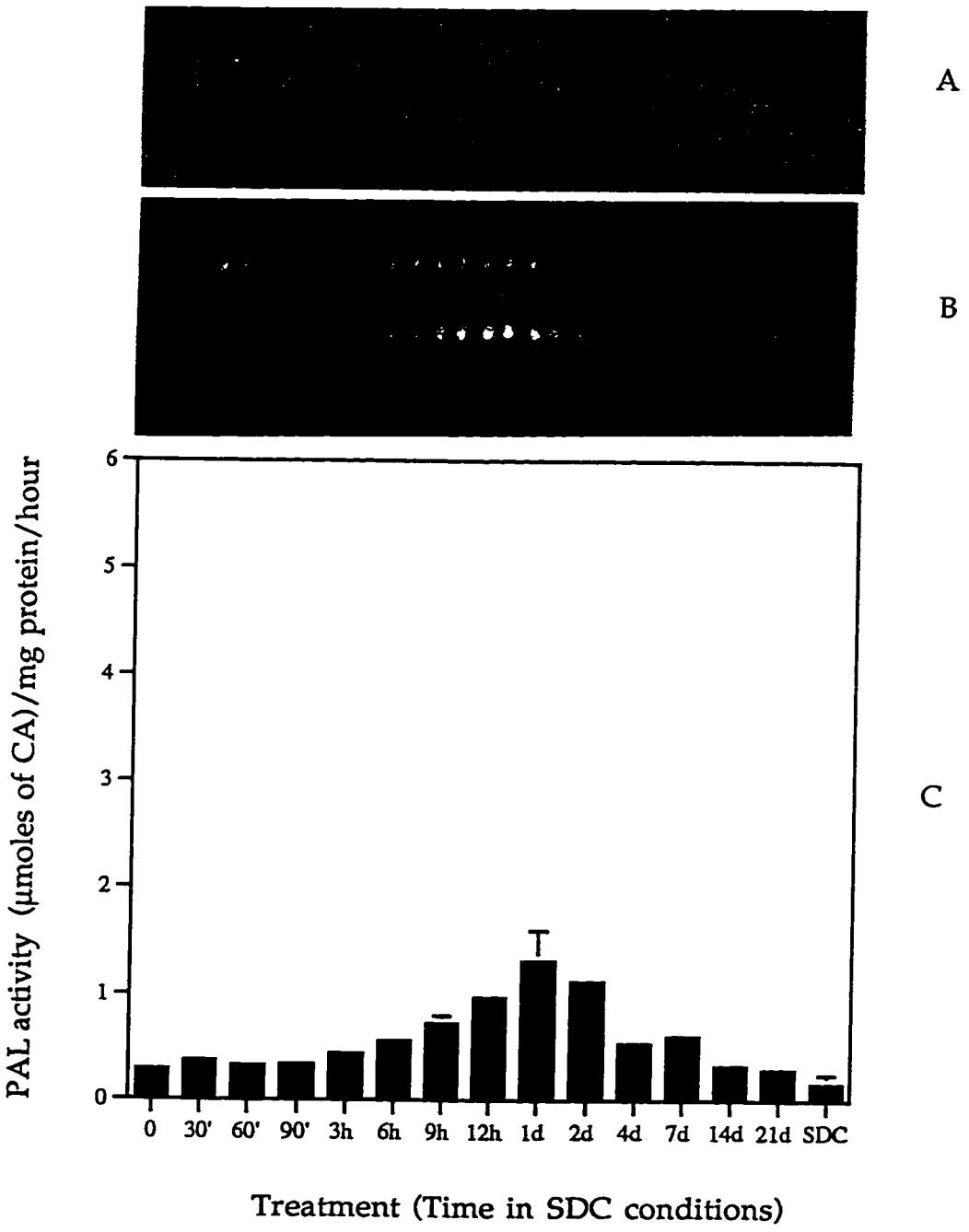

A
B
C
PAL activity (µmoles of CA)/mg protein/hour
0
1
2
3
4
5
6
0
30'
60'
90'
3h
6h
9h
12h
1d
2d
4d
7d
14d
21d
SDC
Treatment (Time in SDC conditions)

Figure 4.6. Effect of DC conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the prairie ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from prairie plants grown under LDW conditions for 9 days and from different time intervals after their transfer to DC conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

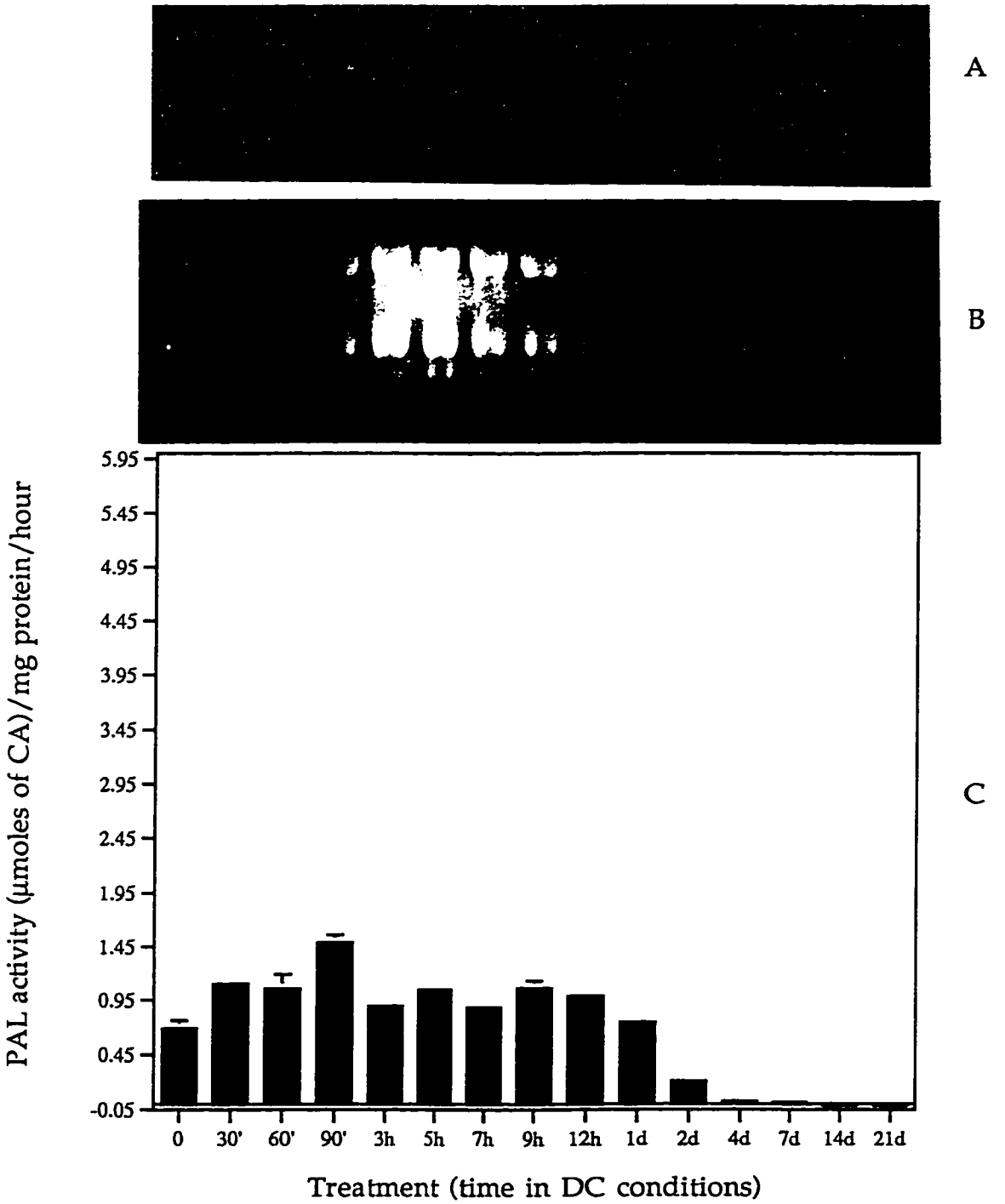

A
B
C
PAL activity (µmoles of CA)/mg protein/hour
5.95
5.45
4.95
4.45
3.95
3.45
2.95
2.45
1.95
1.45
0.95
0.45
-0.05
0
30'
60'
90'
3h
5h
7h
9h
12h
1d
2d
4d
7d
14d
21d
Treatment (time in DC conditions)

Figure 4.7. Effect of DC conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the alpine ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from alpine plants grown under LDW conditions for 9 days and from different time intervals after their transfer to DC conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

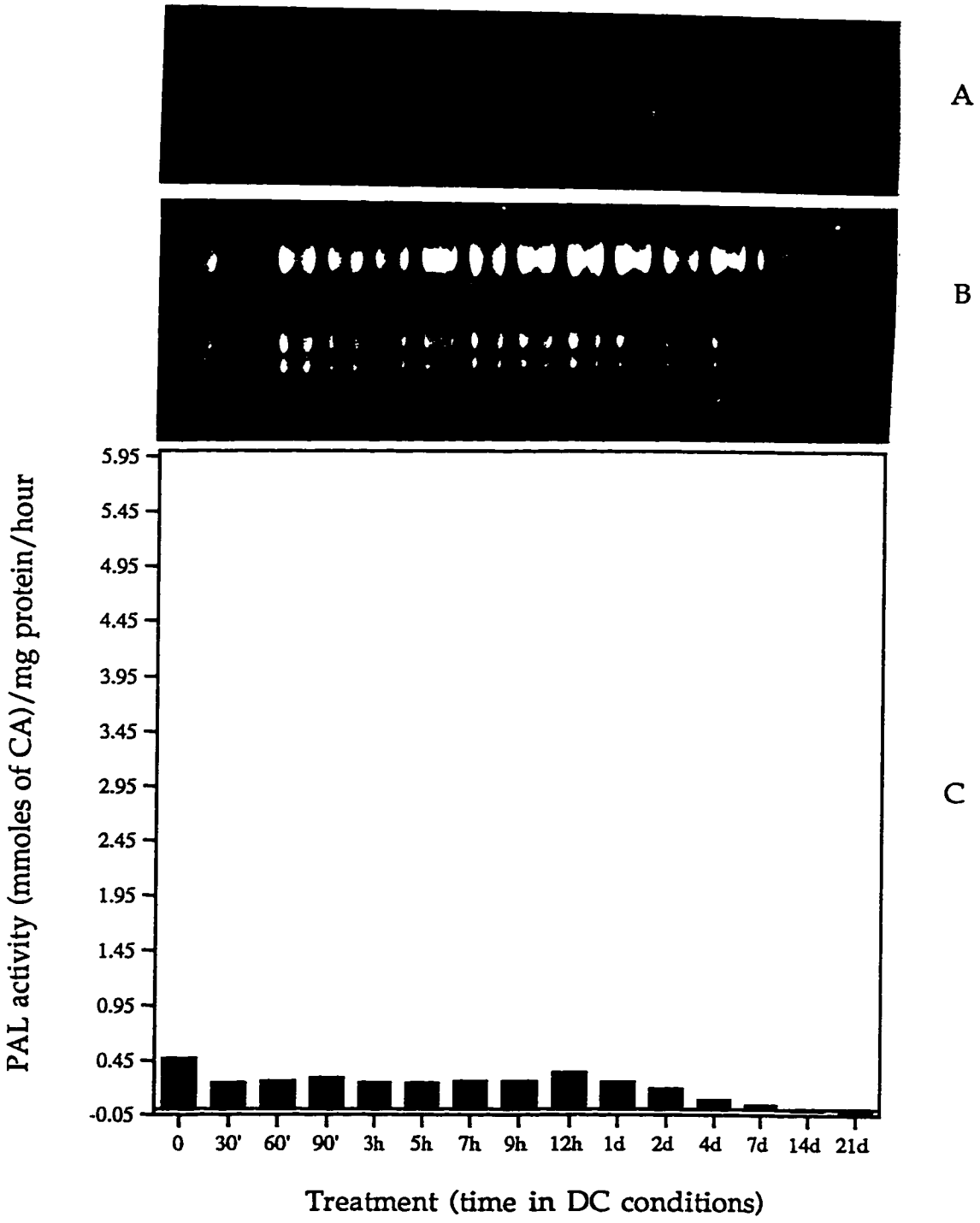

A
B
C
PAL activity (mmoles of CA)/mg protein/hour
5.95
5.45
4.95
4.45
3.95
3.45
2.95
2.45
1.95
1.45
0.95
0.45
-0.05
0
30'
60'
90'
3h
5h
7h
9h
12h
1d
2d
4d
7d
14d
21d
Treatment (time in DC conditions)

Figure 4.8. Effect of DW conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the prairie ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from prairie plants grown under LDW conditions for 9 days and from different time intervals after their transfer to DW conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).

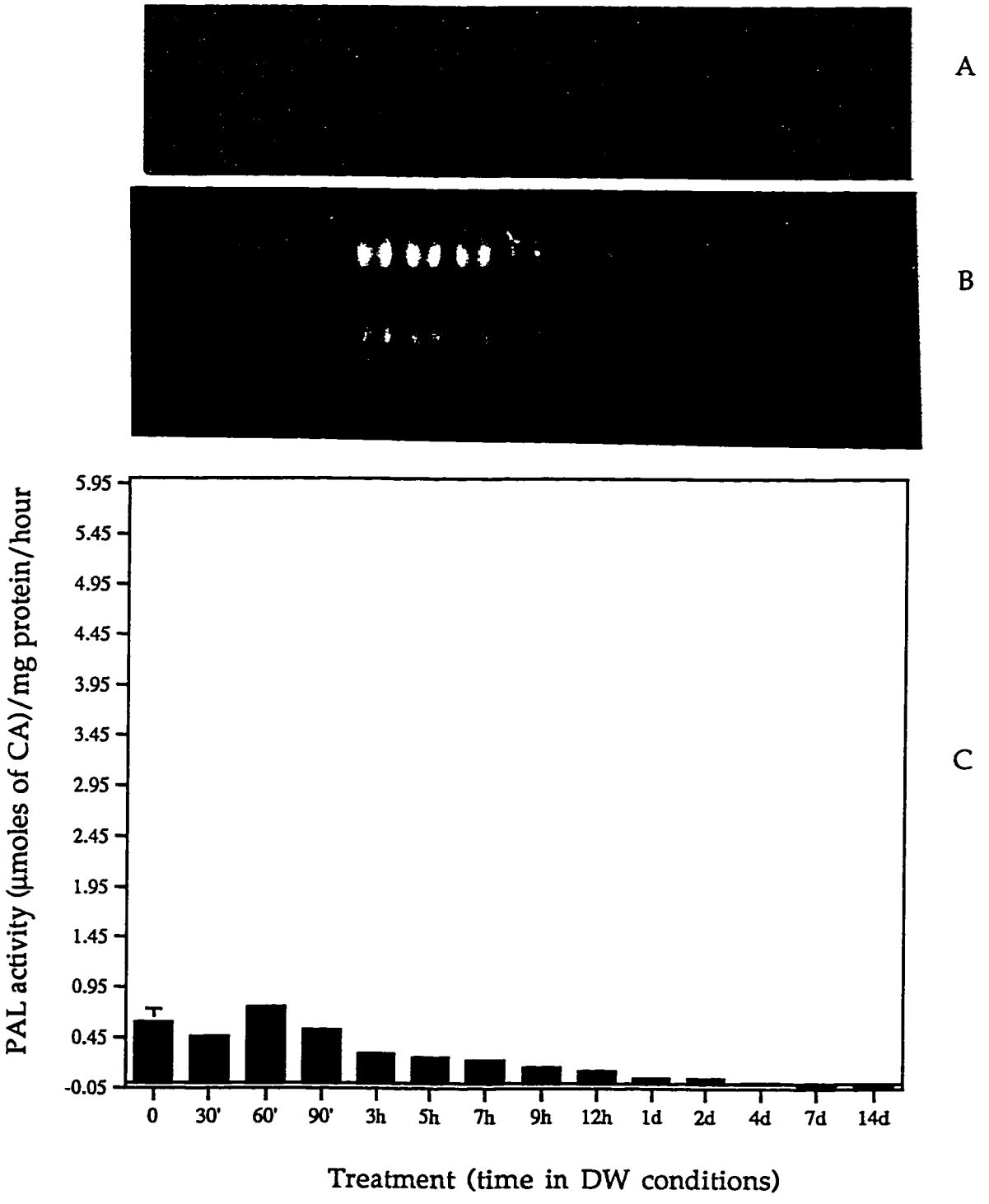
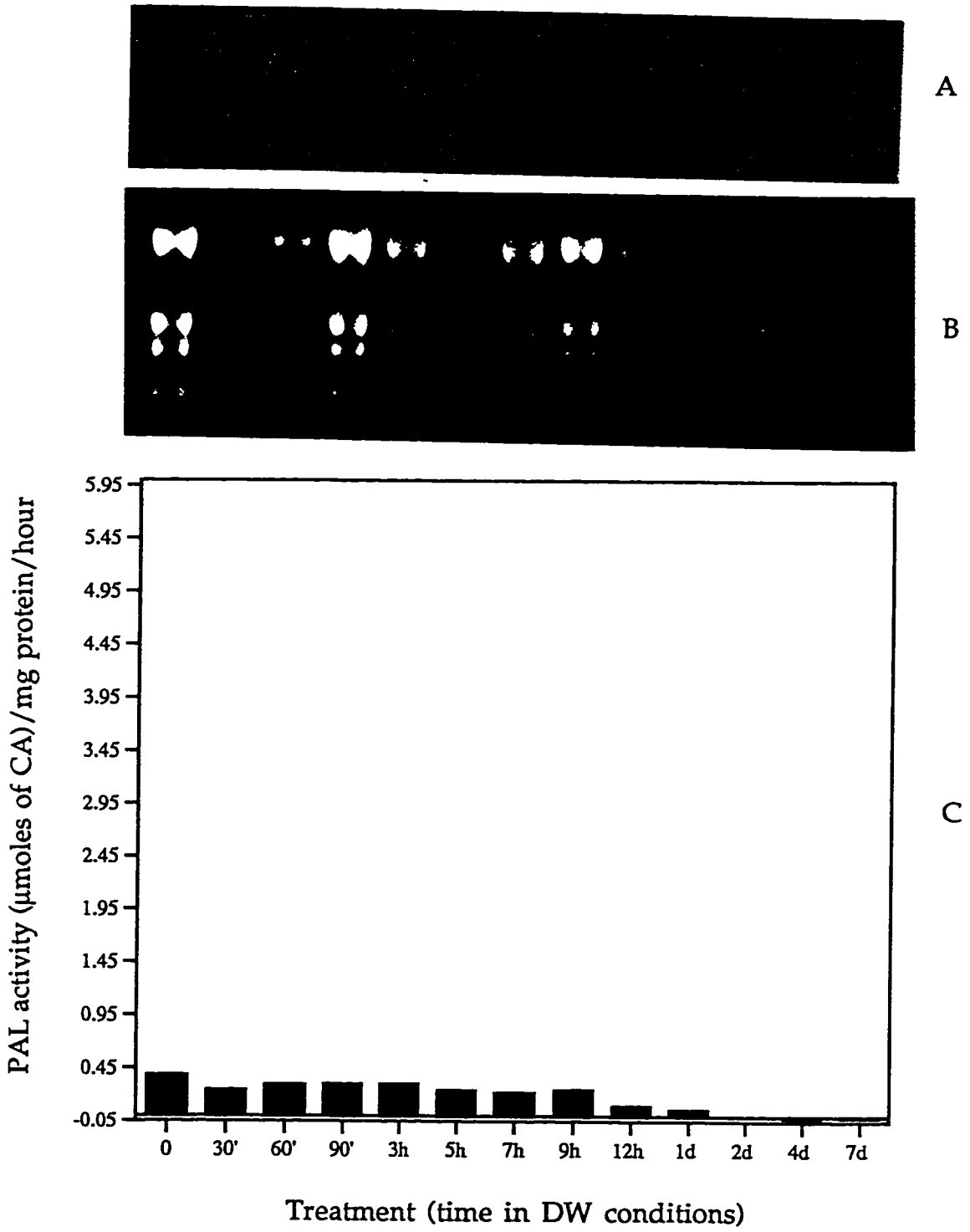

A
B
C
PAL activity (µmoles of CA)/mg protein/hour
5.95
5.45
4.95
4.45
3.95
3.45
2.95
2.45
1.95
1.45
0.95
0.45
-0.05
0
30'
60'
90'
3h
5h
7h
9h
12h
1d
2d
4d
7d
14d
Treatment (time in DW conditions)

Figure 4.9. Effect of DW conditions on expression of PAL gene (A) and PAL enzyme activity (C) in the alpine ecotype of *Stellaria longipes*. Stem and leaf tissues were collected from alpine plants grown under LDW conditions for 9 days and from different time intervals after their transfer to DW conditions. Each lane in the Northern blot contains 10 μg of total RNA. The size of the PAL transcript in A is 2.5 Kb. B shows the ethidium bromide stained ribosomal bands of the RNA gel prior to nylon membrane blotting. PAL enzyme activity of tissues from the same treatment is shown in C. Vertical bars represent ± standard error (SE).


A
B
C
PAL activity (µmoles of CA)/mg protein/hour
5.95
5.45
4.95
4.45
3.95
3.45
2.95
2.45
1.95
1.45
0.95
0.45
-0.05
0
30'
60'
90'
3h
5h
7h
9h
12h
1d
2d
4d
7d
Treatment (time in DW conditions)

Discussion

The general response of plants to environmental changes has been reported to result in fluctuations in PAL expression and subsequently accumulation of phenolic compounds. Since PAL is the primary enzyme that commits phenylalanine to phenylpropanoid metabolism, it is important in the channeling of carbon flow into the production of secondary metabolites. Phenolic compounds such as anthocyanins have been proposed to function as photoprotective pigment and have been shown to accumulate in plants in response to low temperature treatment in a light-dependent manner (Christie et al., 1994; Leyva et al., 1995). Since photosynthesis is the first process affected by low temperature, it is possible that the photosynthetic ability of cells is reduced under low temperature leading to a build up of photon flux which in turn could produce a light stress causing an additional induction of the phenylpropanoid metabolism (Somersalo and Krause, 1989). In agreement with these observations, and in addition to the increase in PAL activity under low temperature in the presence of light, the accumulation of anthocyanins in stems and leaves of prairie plants was observed (data not shown). Under cold temperature, the accumulation of anthocyanins may serve to filter out the excess photon that can cause damage to the photochemical reactions. Furthermore, a precocious development of an endodermis and heavy phenolics accumulation in epidermal cells were also observed in low temperature treated stems (Chapter 3). This is further evidence of the production of phenolic products in response to stress conditions. These observations coincide with the decline in stem elongation rate suggesting that the activation of phenylpropanoid metabolism by low temperature is probably associated with the accumulation of phenolic compounds in the cell walls.

Previous results demonstrated that temperature and photoperiod are two important environmental signals that regulate the expression of PAL genes (Christie et al.,1994). Using a time-course approach, the effects of these two signals on the differential expression of PAL gene in *S. longipes* were

examined. A basal PAL level is present in tissues of both ecotypes under LDW conditions suggesting the existence of secondary metabolism during the normal course of these plants' develompment (Fig 4.2 and Fig 4.3). The transfer of LDW grown plants to SDC conditions resulted in the accumulation of PAL transcripts, suggesting that the cold temperature upregulates the abundance of the PAL mRNA that normally exists under LDW conditions (Fig 4.4 and Fig 4.5). However, in the absence of light under both cold and warm temperature, the transcript levels that normally accumulated under LDW were reduced (Fig 4.6-Fig 4.9). This strongly confirms the inductive action of the PAL gene by cold temperature is light dependent. The increase of PAL transcripts coincides with the increase in PAL enzyme activity with a lag phase in between (Fig 4.4, Fig 4.5). The synergistic regulation of PAL transcripts by cold temperature and light has been previously reported in *Arabidopsis thaliana* by Christie et al., (1994). It is thus not uncommon that light appears to be a requirement for the induction of this defense gene. In addition, PAL in *Arabidopsis thaliana* (Leyva et al., 1995) has been shown to be light dependent. Other genes involved in a plant's defense response include a dessication-inducible gene of *Craterostigma plantagineum* (Bartels et al., 1992), and chalcone synthase. Using *S. longipes* cDNA encoding for CCoAMT (Zhang, 1994) as probe, preliminary results from Northern blot analyses also showed an induction of CCoAMT transcripts by low temperature in both ecotypes. It is possible that the molecular mechanisms regulating defense response in plants and the cold-induction of phenylpropanoid metabolism share some common steps.

The regulation of PAL activity in response to various environmental and mechanical stresses has been studied in several plants and three mechanisms have been proposed. The first two mechanisms suggested that the increase in PAL activity is caused by an increased rate of activation of pre-existing zymogen 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 on receipt of the stimulus (Tong and Schopfer, 1976; Loschke et

al., 1981; Jones, 1984 and Leyva et al., 1995). The observations obtained here are likely to support the first two mechanisms which proposed the activation of pre-existing zymogen and the decrease in the degradation of active enzyme. The third mechanism which suggested the *de novo* synthesis is less likely to be the case because of the extended time lag between the appearance of the transcripts and the enzyme activity. However, these results do not ruled out other possible mechanisms. Another possibility is that stored PAL-mRNA is released from a ribonucleoprotein particle on receipt of the stimulus followed by the usual ribosomal initiation (Jones, 1984). The time lag of PAL activity can be explained by the fact that the production of active enzyme requires transcription and translation of the PAL gene with the subsequent post-transcriptional and post-translational processing. The decline in PAL activity after its peak is most likely due the termination of enzyme synthesis or due to the inactivation or degradation of the active enzyme.

It has been reported that potato contains 40 to 50 PAL genes (Joos and Hahlbrock, 1992) whereas loblolly pine only contains a single PAL gene (Whetten and Sederoff, 1992). Several other plants such as parsley, bean and *Arabidopsis thaliana* have been shown to contain three to four PAL genes which are expressed differentially during development and in response to environmental stimuli such as light, UV irradiation, wounding, fungal elicitor (Zucker, 1965; Lawton and Lamb, 1987; Hahlbrock and Scheel, 1989; Liang et al., 1989; Lois et al., 1989; Ohl et al., 1990). In this study, the Southern analysis suggests the possibility that PAL encoded a small gene family in *S. longipes*. Some polymorphism was observed between alpine and prairie ecotypes with the restriction endonucleases used (Fig 4.1). However, minimal variations in both the size and number of hybridizing bands suggested the conservative nature of PAL genes in the genome of these ecotypes.

The above results show strong correlations in the increase in PAL expression and activity, the accumulation of phenolic substances and the termination of stem elongation in both the alpine and prairie ecotypes of *S. longipes*. However, the prairie ecotype was more responsive to low

temperature treatment than the alpine ecotype suggesting an adaptive strategy acquired during evolution by these plants to improve their fitness of survival in their habitats. Prairie plants come from a very competitive yet more stable environment while alpine plants are from a less competitive habitat but are subjected to constant and abrupt environmental changes. Obviously, the differences in PAL expression and enzyme activity suggest that alpine plants are well adapted to cooler environments than the prairie plants. These results indicate that alpine and prairie ecotypes possess different regulatory mechanisms in response to environmental stimuli.

In conclusion, the present studies show that the expression of PAL gene is regulated by low temperature in a light-dependent manner. These results also suggest that regulation of PAL gene provides a key entry point for the biosynthesis of phenolic compounds which in turn control stem elongation in *S. longipes* perhaps by altering the mechanical properties of cell walls. Thus differential regulation of PAL could be an important aspect of phenotypic plasticity in *S. longipes*. It can also be speculated that phenolic compounds accumulation under cold condition in the presence of light could play an important role in the acquisition of stress tolerance in plants. Further experiments are required to elucidate the molecular mechanisms of the light-dependent transcriptional control of PAL. Also, the use of *in situ* hybribridization technique will provide a better understanding of the tissue-specific and the intracellular expression of PAL. Findings from such studies could provide a better understanding of plants interaction with the environments.

CHAPTER FIVE

General Discussion and Future Experiments

Stellaria longipes is a good model system to study phenotypic plasticity. Two populations with contrasting levels of plasticity were used to examine the adaptive mechanism that underly phenotypic plasticity in *S. longipes*. My study was designed to determine the anatomical and biochemical changes in the cell wall components that lead to the different stem elongation responses of alpine and prairie ecotypes under different environmental cues. The results obtained in this study provided useful information not only on phenotypic plasticity but also on biochemical events that lead to morphological and anatomical changes related to stem elongation.

Stem elongation is a complicated process that involves the coordination of cell division and irreversible cell expansion. Cell expansion is control by the flexibility of the cell wall, a structural layer comprised of polysaccharides, glycoproteins and phenolic compounds (Fry, 1988). Cell expansion is under the regulation of internal factors such as cell wall extensibility, turgor pressure and biochemical processes that bring about wall extension and relaxation (Cosgrove, 1993; Iiyama et al., 1994). It is generally hypothesized that wall loosening enzymes are responsible for the modification of the wall to allow turgor-driven extension. Glucanases, XET and expansins have been suggested as the wall loosening enzymes. Expansins were the first endogenous wall proteins to induce extension of isolated walls. McQueen-Mason et al., (1992) were the first to show that under low pH, crude protein extract from the walls of growing cucumber hypocotyl could induce extension in heat-inactivated cucumber walls as well as walls of various dicots and monocots. These biochemical evidence suggest that expansins are

responsible for the acid-induced extension *in vitro* (in isolated walls) and possibly *in vivo* (of intact tissues) (McQueen-Mason et al., 1992, 1993). However, the mode of action of expansins is still unclear. In support of the notion that glucanases are wall-loosening enzymes, Inouhe and Nevins (1991) and Hoson and Masuda (1991) used antibodies against cell wall glucanases to interfere with auxin-induced growth which requires hydrolytic breakdown of matrix polysaccharides that bind to cellulose microfibrils. The evidence linking XET to wall extension however remains speculative because application of crude protein extract containing high XET activity to isolated cucumber walls under tension failed to cause wall extension (McQueen-Mason et al., 1993). This however does not ruled out XET's role in wall extension because its function *in vivo* serve to anchor newly deposited xyloglucan into the wall has been shown (Edelmann and Fry, 1992).

The results of this study showed that stem elongation involved both cortical cell division and cell elongation (Chapter 2). Specifically, elongation of the epidermis was the factor that controlled the rate of stem elongation in *S. longipes*. Similar observations were reported by Matsuda and Yamamoto (1972), Kutschera et al., (1987), Kutschera, (1987, 1992) and Kutschera and Briggs, (1988). These studies showed the direct involvement of the epidermis in auxin-mediated plant organ growth. Ultrastructural examination of the architecture of the rigid epidermal walls may provide insights into the mechanism of cell elongation. However, the physiological mechanisms of cell expansion remains to be investigated. How do phytohormones such as auxins, ethylene, and gibberellins affect stem elongation plasticity of *S. longipes*? Further research is needed to determine the synergistic or indirect role(s) of these hormones in stem elongation.

Fluorescence microscopy has provided invaluable observations on the structural changes occurring in the cell walls of *S. longipes*. Chapter 3 examined the changes in stem cell walls at various developmental stages and under different environmental treatments. The data obtained showed accumulation of suberin, lignin and other phenolic compounds in the walls of epidermal and endodermal cell in mature and cold-treated stems. Furthermore, histochemical studies indicated that the innermost cortical cells showed the similar characteristics and followed the pattern of development of an endodermis as described by Van Fleet (1961). The deposition of phenolic substances in these cell walls comprised mainly of suberin and lignin or both. In *S. longipes*, the endodermis developed under normal maturation of the stems or it can be precociously induced by low temperature treatment. Further work including the used of an apoplastic dye tracer is needed to determine the functions of the endodermis in aerial stems. Other histochemical techniques can be used to identify the phenolic compounds in the tangential walls of the epidermis.

Based on the observations from the histochemical and structural evidence suggesting the involvement phenolic compounds in stem elongation, it is essential to investigate the phenylpropanoid pathways which are responsible for the production of the products. Phenylpropanoid metabolism is activated in response to a wide range of developmental and environmental stimuli (Jones, 1984, Liang et al., 1989) and consequently, the deposition of phenolic polymers such as lignin, suberin, and anthocyanins ensued to provide plants with a new mechanical and protective barrier. So far no studies have looked into changes in PAL activity and correlated it with production and accumulation of a specific phenylpropanoid product. PAL, the enzyme that catalyzes the first step of the phenylpropanoid metabolism to

supply the precursors for many phenolic products, has been shown to be under developmental and environmental control (Hahlbrock and Scheel, 1989). An increase in PAL mRNA has been shown to correlate to an increase in PAL activity (Orr et al., 1993, Lawton and Lamb, 1987). In *S. longipes*, PAL mRNA induction preceded the increase in PAL activity. Similar investigations on the relationship of PAL and other enzymes of a specific phenylpropanoid product might help us understand the role of phenolic compounds in stem elongation plasticity more thoroughly.

In both ecotypes, PAL expression and activity were induced by stress such as low temperature treatment in a light-dependent fashion, suggesting the involvement of phytochromes. Phytochromes are known to be involved in all stages of plant growth and development.

Southern analysis suggests that PAL may be encoded by a small gene family in *S. longipes* and there are some polymorphisms in the alpine and prairie ecotypes. It is interesting, however, to note that the magnitude and timing of response were different in these ecotypes. It is possible to speculate that the alpine and prairie ecotypes differ in their sensing mechanism for environmental signals such as light and temperature. Such differences in responses could be due to the mere acquisition of regulatory controls and modification of existing responsive genes inherited by these plants to enhance their survival and adaptability in different habitats. Different plants species may have evolved different regulatory mechanisms for controlling PAL and it is possible that one or more mechanisms might be activating simultaneously in a tissue. However, these results could not distinguish whether the regulation of PAL is due to *de novo* synthesis of transcripts, activation of pre-existing inactive enzymes or the degradation of active enzymes. Immunochemical techniques would therefore determine whether

one or all of these mechanisms are at play in this instance. Also, analysis and comparison of PAL and other product specific genes (chalcone synthase, CCoAMT) of the phenylpropanoid metabolism between alpine and prairie ecotypes of *S. longipes* may provide more clues to the plants' response to different environmental conditions.

This work demonstrates that anatomical and biochemical changes are essential to bring about the morphological phenotypic plasticity expressed in *S. longipes*. Although the results could not fully explain the biological mechanisms of phenotypic plasticity, the results do show interesting evidence that could serve as the basis for further biochemical, physiological and molecular investigations in identifying and characterizing the regulatory mechanisms that initiate the array of plastic responses that provide the plants overall increased environmental fitness.

References

Bartels D., Hanke C., Schneider K., Michel D., and F. Salamini. 1992. A dessication-related Elip-gene from the resurrection plant *Craterostigma plantagineum* is regulated by light and ABA. **EMBO J** 11: 2771-2778.

Bolwell G.P., Cramer C.L., Lamb C.J., Schuch W., and R.A. Dixon. 1986. L-phenylalanine ammonia-lyase from *Phaseolus vulgaris*: Modulation of the levels of active enzyme by *trans*-cinnamic acid. **Planta** 169: 97-107.

Bradford M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. **Anal Biochem** 72: 248-254.

Bradshaw A. D. (1965). Evolutionary significance of phenotypic plasticity in plants. **Adv Genet** 13: 115-155.

Brett C.T. and K.W. Waldron. 1996. *Physiology and biochemistry of plant cell walls*. Second edition. Chapman and Hall. New York, U.S.A.

Brown C.L., Sommer H.E. and L.V. Pienaar. 1995. The predominant role of the pith in the growth and development of internodes in *Liquidambar styraciflua* (Hamamelidaceae). I. Histological basis of compressive and tensile stresses in developing primary tissues. **Am J Bot** 82: 769-776.

Brundrett M.C., Enstone D.E., and C.A. Peterson. 1988. A berberine-aniline blue fluorescent staining procedure for suberin, lignin and callose in plant tissues. **Protoplasma** 146: 133-142.

Camm E.L. and G.H.N. Towers. 1977. Phenylalanine ammonia-lyase. **Phytochemistry** 4: 169-181.

Carpita N.C. and D.M. Gibeaut. 1993. Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the walls during growth. **Plant J** 3: 1-30.

Casal J.J., Barlare C.L. and R.A. Sanchez. 1994. Anatomy, growth and survival of a long-hypocotyl mutant of *Cucumis sativus* deficient in phytochrome B. **Ann Bot** 73: 569-575.

Chinnappa C.C. and J. K. Morton. 1976. Studies on the *Stellaria longipes* Goldie complex- Variation in wild populations. **Rhodora** 78:488-512.

Chinnappa C.C. and J. K. Morton (1984). Studies on the *Stellaria longipes* complex (Caryophyllaceae)- Biosystematics. **Syst Bot** 9: 60-73.

Chory J. 1997. Light modulation of vegetative development. **Plant Cell** 9: 1225-1234.

Christie P.J., Alfenito M.R., and V. Walbot. 1994. Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedllings. **Planta** 194: 541-549.

Cosgrove D.J. 1993. How do plant cell walls extend. **Plant Physiol** 102: 1-6.

Cosgrove D.J. 1997. Relaxation in a high-stress environment: the molecular bases of extensible cell walls and cell enlargement. **Plant Cell** 9: 1031-1041.

Dixon R.A., Dey P.M., Lawton M.A., and C.J. Lamb. 1983. Phytoalexin induction in French bean. Intracellular transmission of eliciation in cell suspension cultures and hypocotyl sections of *Phaseolus vulgaris*. **Plant Physiol** 71: 251-256.

Doyle J.J. and J.L. Doyle. 1987. Isolation of plant DNA from fresh tissues. **Focus** 12: 13-15.

Dudley S.A. and J. Schmitt. 1996. Testing the adaptive hypothesis: Density-dependent selection on manipulated stem length in *Impatiens capensis*. **American Naturalist** 147: 445-465.

Edelmann H.G. and S.C. Fry. (1992). Effect of cellulose synthesis inhibition on growth and the integration of xyloglucan into pea internode cell walls. **Plant Physiol** 100: 993-997.

Eiguchi M., Sano R., Hirano H.Y. and Y. Sano. 1993. Genetic and developmental bases of phenotypic plasticity in deepwater rice. **J Heredidy** 84: 201-205.

Emery R.J.N. 1994. Morphological and physiological phenotypic plasticity in alpine and prairie ecotypes of *Stellaria longipes*. Ph.D dissertation. The University of Calgary, Calgary, Alberta. Canada.

Emery R.J.N., Reid D.M., and C.C. Chinnappa. 1994. Phenotypic plasticity of stem elongation in two ecotypes of *Stellaria longipes*: the role of ethylene and response to wind. **Plant Cell and Environ** 17: 691-700.

Erwin J.E., R.D. Heins, and M.G. Karlsson. 1989. Thermomorphogenesis in *Lilium longiflorum* Thunb. **Amer J Bot** 76: 47-52.

Erwin J.E., R.D. Heins, R. Moe, W.H. Carlson and S. Newport. 1992. Environmental and mechanical manipulation of stem elongation. A mini review. **PGRSA** 20: 1-17.

Erwin J.E., P. Velguth and R. Heins. 1994. Day/night temperature environment affects cell elongation but not division in *Lilium longiflorum* Thunb. **J Exp Bot** 45: 1019-1025.

Espelie E.K. and P.E. Kolattukudy. 1979. Composition of the aliphatic components of suberin of the endodermal fraction from the first internode of etiolated *Sorghum* seedlings. **Plant Physiol** 63: 433-435.

Firn R. and J. Digby. 1977. The role of the peripheral cell layers in the geotropic curvature of sunflower hypocotyls: a new model of shoot geotropism. **Aust J Plant Physiol** 4: 337-347.

Fry S.C. 1986. Cross-linking of the matrix polymers in the growing cell walls of angiosperms. **Ann Rev Plant Physiol** 37: 165-186.

Fry S.C. 1988. *The growing plant cell wall: chemical and metabolic analysis.* John Wiley and Sons, Inc., New York, USA.

Goldberg R.M., Liberman C., Mathieu M.P. and A.M. Catesson. 1987. Development of epidermal cell wall peroxidases along the mungbean hypocotyl - possible involvement in the cell wall stiffening process. **J Exp Bot** 38: 1378-1359.

Gottlieb L.D. 1977. Phenotypic variation in *Stephanomeria exigua* ssp. *coronaria* (Compositae) and its recent derivative species "Malheurensis". **Amer J Bot** 64: 873-880.

Graham D. and B.D. Patterson. 1982. Responses of plants to low, nonfreezing temperatures: proteins, metabolism and acclimation . **Annu Rev Plant Physiol** 33: 347-372.

Griffith M., Huner N.P.A., Esspelie K.E. and P.E. Kolattukudy. 1985. Lipid polymers accumulate in the epidermis and mestome sheat cell walls during low temperature development of winter rye leaves. **Protoplasma** 125: 53-59.

Hahlbrock K. and D. Scheel. 1989. Physiology and molecular biology of phenylpropanoid metabolism. **Annu Rev Plant Physiol Plant Mol Biol** 40: 347-369.

Heupel T. and U. Kutschera. 1997. Cell number and organ size in developing sunflower hypocotyls. **J Plant Physiol** 151: 379-381.

Hodick D. and U. Kutschera. 1992. Light-induced inhibition of elonation growth in sunflower hypocotyls. Biophysical and ultrastructural investigations. **Protoplasma** 168: 7-13.

Holton T.A. and E.C. Cornish. 1995. Genetics and biochemistry of anthocyanins biosynthesis. **Plant Cell** 7: 1071-1083.

Hoson T. and Y. Masuda. 1991. Inhibition of auxin-induced elongation and xyloglycan breakdonw in azyki bean epicotyl segments by fucose-binding lectins. **Physiol Plant** 82: 41-47.

Hoson T. and Y. Masuda. 1992. Inhibition of auxin-induced elongation and xyloglucan breakdown in azuki bean epicotyl segments by fucose-binding lectins. **Physiol Plant** 82: 41-47.

Hughes J.E., and E. Wagner. 1987. Photomorphogenesis in *Chenopodium album*. Effects of supplementary far-red light on the kenetics of stem elongation. **Planta** 172: 131-138.

Iiyama K., T.B.T. Lam and B.A. Stone. 1994. Covalent cross-link in the cell wall. **Plant Physiol** 104: 315-320.

Inouhe M. and D.J. Nevins. 1991. Inhibition of auxin-induced cell elongation of maize coleoptiles by antibodies specific for cell wall glucanases. **Plant Physiol** 96: 426-431.

Jensen W.A. 1962. *Botanical histochemistry:Principle and Practice.* W.H. Freeman and Company. San Francisco. U.S.A.

Jones D.H. 1984. Phenylalanine ammonia-lyase: regulation of its induction and its role in plant development. **Phytochemistry** 23:1349-1359.

Joos H.J. and K. Hahlbrock. 1992. Phenylalanine ammonia-lyase in potato (*Solanum tuberosum* L)-genomic complexity, structural comparison of two selected genes and modes of expression. **Eur J Biochem** 204: 621-629.

Karahara I. and H. Shibaoka. 1994. The Casparian strip of pea epicotyls: Effect of light on its development. **Planta** 192: 269-275.

Kathiresan A. 1997. Regulation of ethylene biosynthesis and its possible role in phenotypic plasticity in *Stellaria longipes.* Ph.D dissertation. The University of Calgary, Calgary, Alberta. Canada.

Khan N.U. and C.S. Vaidyanathan. 1986. A new simple spectrophotometric assay of phenylalanine ammonia-lyase. **Curr Sci** 55: 391-393.

Kolattukudy P.E. 1984. Biochemistry and function of cutin and suberin. **Can J Bot** 62: 2918-2933.

Kuhlemeier C., Green P.J., and N.H. Chua. 1987. Regulation of gene expression in higher plants. **Ann Rev Plant Physiol** 38: 221-257.

Kutschera U., R. Bergfeld and P. Schopfer. 1987. Cooperation of epidermis and inner tissues in auxin-mediated growth of maize coleoptiles. **Planta** 170: 168-180.

Kutschera U. 1987. Cooperation between outer and inner tissues in auxin-mediated plant organ growth. In: *Physiology of cell expansion during plant growth*, pp. 215-226, Cosgrove D.J. and P.D. Knievel, eds. The American Soc of Plant Physiologists. Rockville, MA. USA.

Kutschera U., and W.R. Briggs. 1987. Differential effect of auxin on in vivo extensibility of cortical cylinder and epidermis in pea internodes. **Plant Physiol** 84: 1361-1366.

Kutschera U., and W.R. Briggs. 1988. Interaction between cortical cylinder and epidermis during auxin-mediated growth in pea internodes. **Plant Sci** 54: 23-28.

Kutschera U. 1989. Tissue stresses in growing plant organs. **Physiol Plant** 77: 157-163.

Kutschera U. 1992. The role of the epidermis in the control of elongation growth in stems and coleoptiles. **Bot Acta** 105: 246-252.

Kutschera U. 1996. Cesation of cell elongation in rye coleoptiles is accompanied by a loss of cell-wall plasticity. **J Exp Bot** 47: 1387-1394.

Lawton M.A. and C.J. Lamb. 1987. Transcriptional activation of plant defense genes by fungal elicitor, wounding and infection. **Mol Cell Biol** 7: 335-341.

Lersten N.R. 1997. Occurrence of endodermis with a Casparian strip in stem and leaf. **Botanical Review** 63: 265-272.

Leyva A., J.A. Jarillo, J. Salinas and J.M. Martiner-Zapater. 1995. Low temperature induces the accumulation of phenylalanine ammonia-lyase and chalcone synthase mRNAs of *Arabidopsis thaliana* in a light-dependent manner. **Plant Physiol** 108: 39-46.

Liang X., Dron, M., Schmid J., Dixon R.A., and C.J. Lamb. 1989. Developmental and environmental regulation of a phenylalanine ammonia-lyase-β-glucuronidase gene fusion in transgenic tobacco. **Proc Natl Acad Sci USA** 86: 9284-9288.

Lois R., A. Dietrich, K. Hahlbrock, and W. Schulz. 1989. A phenylalanine ammonia-lyase gene from parsley: structure, regulation and identification of elicitor and light responsive *cis*-acting elements. **EMBO J** 8: 1899-1906.

Loschke D.C., Hadwiger L.A., Schroder J., and K. Hahlbrock. 1981. Effects of light and *Fusarium solani* on synthesis and activity of Phenylalanine ammonia-lyase in peas. **Plant Physiol** 68: 680-685.

Macdonald, S.E., Chinnappa C.C., and D.M. Reid. 1984. Studies on the *Stellaria longipes* complex: phenotypic plasticity. I. Response of stem elongation to temperature and photoperiod. **Can J Bot** 62: 414-419.

Macdonald, S.E. 1988. Ecological and physiological aspects of phenotypic plasticity in *Stellaria longipes* complex (Caryophyllaceae). Ph.D dissertation. The University of Calgary. Calgary, Alberta, Canada.

Macdonald, S.E., Chinnappa C.C., and D.M. Reid. 1988. Evolution of phenotypic plasticity in *Stellaria longipes* complex: comparisons among cytotypes and habitats. **Evolution** 42: 1036-1046.

Macdonald, S.E. and C.C. Chinnappa. 1989. Population differentiation for phenotypic plasticity in the *Stellaria longipes* complex. **Amer J Bot** 76(11): 1627-1637.

Masuda Y., and R. Yamamoto. 1972. Control of auxin-induced stem elongation by the epidermis. **Physiol Plant** 27: 109-115.

McCann M.C., and K. Roberts. 1994. Changes in cell architecture during cell elongation. **J Exp Bot** 45: 1683-1691.

McQueen-Mason S.J., D.M. Durachko and D.J. Cosgrove. 1992. endogenous proteins that indcue cell wall expansion in plants. **Plant Cell** 4: 1425-1433.

McQueen-Mason S.J., S.C. Fry, D.M, Durachko and D.J. Cosgrove. 1993. The relationship between xyloglucan endotransglycosylase and in vitro cell wall extension in cucumber hypocotyls. **Planta**

Moe R., R.D. Heins and J.E. Erwin. 1991. Effect of day and night temperature alterations, and plant growth regulators on stem elongation and flowering of the long-day plant *Campanula isphylla* Morettii. **Scientia Horticulturae** 48: 141-151.

Myster J., and R. Moe. 1995. Effect of diurnal temperature alterations on plant morphology in some greenhouse crops. A mini review. **Scientia Horticulturae** 62: 205-215.

Ohl, S., Hedrick, S., Chory, J. and C.J. Lamb. 1990. Functional properties of a phenylalanine ammonia-lyase promoter from *Arabidopsis*. **Plant Cell** 3:741-752.

Orr J.D., R. Edwards and R.A. Dixon. 1993. Stress response in alfalfa (*Medicago sativa* L.). XIV. Changes in the level of phenylpropanoid pathway intermediates in relation to regulation of L-phenylalanine ammonia-lyase in elicitor-treated cell-suspension cultures. **Plant Physiol** 101: 847-856.

Ozeki Y., Komamine A. and Y. Tanaka. 1990. Induction and repression of PAL and chalcone synthase enzyme proteins and mRNAs in carrot cell suspension cultures regulated by 2,4-D. **Physiol Plant** 78: 400-408.

Parra C., Saez J., Perez H., Alberdi M., Delsemy M., Hubert E., and L. Meza-Basso. 1990. Cold resistance in rapeseed (*Brassica napus*) seedlings. Searching biochemical markers of cold-tolerance. **Arch Biol Med Exp** 23: 187-194.

Parvez M.M., K. Wakabayashi, T. Hoson and S. Kamisaka. 1997. White light promotes the formation of diferulic acid in maize coleoptile cell walls by enhancing PAL activity. **Physiol Plant** 99: 39-48.

Peterson C.A., M.E. Emanuel and C. Wilson. 1982. Identification of a Casparian band in the hypodermis of onion and corn roots. **Can J Bot** 60: 1529-1535.

Peterson C.A. and D.E. Enstone. 1996. Functions of passage cells in the endodermis and exodermis of roots. **Physiol Plant** 97: 592-598.

Priestley J.H. and E.E. North. 1922. Physiological studies in plant anatomy. III. The structure of the endodermis in relation to its function. **New Phytol** 21: 113-139.

Quail P.H., M.T. Boylan, B.M. Parks, T.W. Short, Y. Xu and D. Wagner. 1995. Phytochromes: Photosensory perception and signal transduction. **Science** 268: 675-680.

Ray P.M. 1987. Principles of plant cell expansion. *in Physiology of cell expansion during plant growth.* eds D.J. Cosgrove and K.P. Knievel. The American Society of Plant Physiologists. PA. USA. p.p. 1-17.

Rayle D.L. and R.E. Clevand. 1992. The acid growth theory of auxin-induced cell elongation is alive and well. **Plant Physiol** 99: 1271-1274.

Ribereau-Gayon, P. 1972. *Plant phenolics.* Hafner Publishing Company. New York, USA.

Rhodes M.J.C., and L.S.C. Wooltorton. 1977. Changes in the activity of enzymes of phenylpropanoid metabolism in tomatoes stored at low temperatures. **Phytochemistry** 16: 655-659.

Sachs R.M. 1965. Stem elongation. **Ann Rev Plant Physiol** 16; 73-96.

Sack F.D. 1987. The structure of the stem endodermis in etiolated pea seedlings. **Can J Bot 65:** 1514-1519.

Sambrook J., Fritsch E.F., and T. Maniatus 1987. *Molecular cloning. A laboratory manual.* Cold Spring Harbor Laboratory, New York, USA.

Schlichting C.D. 1986. The evolution of phenotypic plasticity in plants. **Ann Rev Ecol Syst** 17: 667-693.

Schmitt J., McCormac A.C., and H. Smith. 1995. A test of the adaptive plasticity hypothesis using transgenic and mutant plants disabled in

phytochrome-mediated elongation responses to neighbors. **Am Nat** 146: 937-953.

Schmitt J., and S.A. Dudley. 1996. Testing the adaptive plasticity hypothesis for plant responses to neighbors. **Plant Sciences Biol** 11: 59-67.

Schopfer P. 1996. Hydrogen peroxide-mediated cell-wall stiffening in vitro in maize coleoptiles. **Planta** 199: 43-49.

Schreiber L. 1996. Chemical composition of Casparian strips isolated from *Clivia miniata* Reg. roots: Evidence for lignin. **Planta** 199: 596-601.

Smart M.G. and T.P. O'Brien. 1979. Observations on the scutellum. III: ferulic acid as a component of the cell wall in wheat and barley. **Aust J Plant Physiol** 6: 485-491.

Smith H. 1982. Light quality, photoreception and plant strategy. **Ann Rev Plant Physiol** 33: 481-518.

Smith, H. 1990. Signal perception, differential expression within multigene families and the molecular basis of phenotypic plasticity. **Plant, Cell and Environment** 13: 585-594.

Solecka, D. and A. Kacperska. 1995. Phenylalanine ammonia-lyase activity in leaves of winter oilseed rape plants as affected by acclimation of plants to low temperature. **Plant Physiol Biochem** 33:585-591.

Somersalo S. and G.H. Krause. 1989. Photoinbibition at chilling temperature. Fluorescence characteristics of unhardened and cold-acclimated spinach leaves. **Planta** 177: 409-416.

Stearns, S.C. 1989. The evolutionary significance of phenotypic plasticity. **BioScience** 39: 436-445.

Strommer, J., Gregerson R., and M. Vayda. 1993. *Isolation and characterization of plant mRNA. In Methods in plant molecular biology and biotechnology, eds.* B.R. Glick and J.E. Thompson, pp. 49-65, CRC Press Inc., Florida, USA.

Suge H., Nishizawa T., Takahashi H., and K. Takeda. 1997. Phenotypic plasticity of internode elongation stimulated by deep-seedling and ethylene in wheat seedlings. **Plant Cell and Environment** 147: 961-964.

Taiz L. 1984. Plant cell expansion: regulation of cell wall mechanical properties. **Ann Rev Plant Physiol** 35: 585-657.

Talbott L.D. and P.W. Ray. 1992. Molecualr size and separability features of pea cell wall polysaccharides. Implication for models of primary wall structure. **Plant Physiol** 98: 357-368.

Tanaka, Y., Matsuoka M., Yamamoto N., Ohashi Y., Kano-Murakami Y., and Y. Ozeki. 1989. Structure and characterization of a cDNA clone for phenylalanine ammonia-lyase from cut-injured roots of sweet potato. **Plant Physiol.** 90: 1403-1407.

Tong W. and P. Schopfer. 1976. Phytochrome-mediated de novo synthesis of phenylalanine ammonia-lyase. An approach using preinduced mustard seedlings. **Proc Natl Acad Sci USA** 73: 4017-4021.

Van Fleet D.S. 1961. Histochemical and function of the endodermis. **Botanical Review** 27: 165-220.

Varner J.E. and L.S. Lin. 1989. Plant cell wall architecture. **Cell** 56: 231-239.

Vladimirova S.V., McCommell D.B., Kane M.E., and R.W. Henley. 1997. Morphological plasticity of *Dracaena sanderana* 'Ribbon' in response to four light intensities. **HortScience** 32: 1049-1052.

Wallace, G. and S.C. Fry. 1994. Phenolic components of the plant cell wall. **Int Rev Cyto** 151: 229-270.

Waller, G.R. and E.K. Norwacki. 1978. *Alkaloid biology and metabolism in plants*. Pleum Press, New York, USA.

Warden W.M. 1937. On the structure, development and distribution of the endodermis and its associated ducts in Senecio vulgaris. **New Phyto** 24: 361-385.

Went F. 1952. The effect of temperature on plant growth. **Ann Rev Plant Physiol** 4: 347-362.

Whetten R.W. and R.R. Sederoff. 1992. Phenylalanine ammonia-lyase from loblolly pine. Purification of the enzyme and isolation of complementary DNA clones. **Plant Physiol** 96: 380-386.

Whetten R.W. and R.R. Sederoff. 1995. Lignin biosynthesis. **Plant Cell** 7: 1001-1013.

Willemse M.T.M and A.M.C. Emons. 1991. Autofluorescence and HPLC analyses of phenolics in *Zea mays* L. stem cell walls. **Acta Bot Neerl** 40: 115-124.

Wilson C.A. and C.A. Peterson. 1983. Chemical composition of the epidermal, hypodermal, endodermal and intervening cortical cell walls of various plants roots. **Ann Bot** 51: 759-769.

Yeung E.C. 1984. Histological and histochemical staining procedures. pp. 778-784 *in* I.K. Vassal, ed. *Cell culture and somatic cell genetics of plants. Vol 1. Laboratory Procedures and Their Application.* Academic Press, Orlando, Florida.

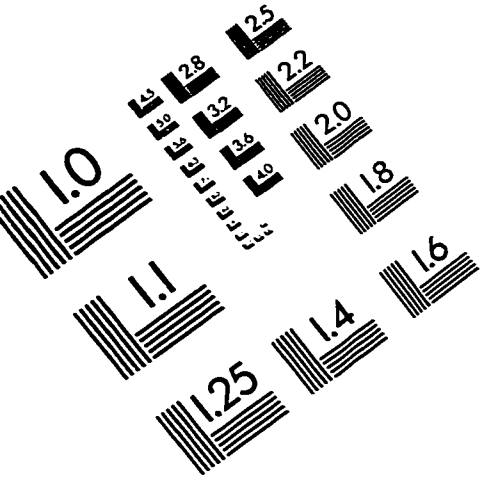
Zhang X.H. 1994. Characterization of protein-encoding cDNA clones from Stellaria longipes (Caryaphyllaceae): gene expression and evolution. PhD dissertation. The University of Calgary, Calgary, Alberta. Canada.

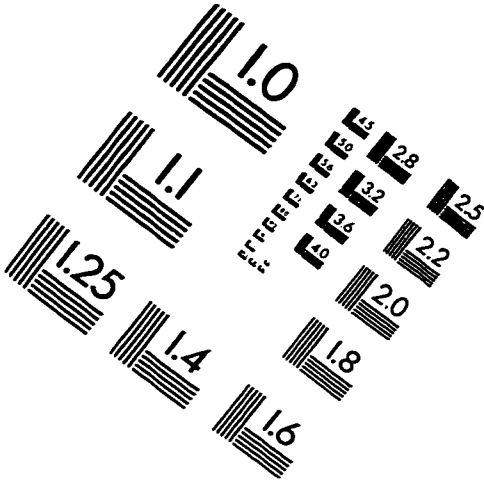
Zieslin N. and M.J. Tsujita. 1988. Regulation of stem elongation of lilies by temperature and the effect of gibberellin. **Scientia Hortic** 37: 165-169.

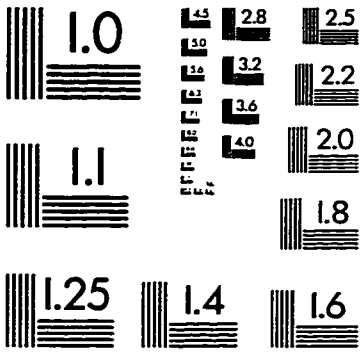
Zucker M. 1965. Induction of phenylalanine ammonia deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. **Plant Physiol** 40: 779-784.

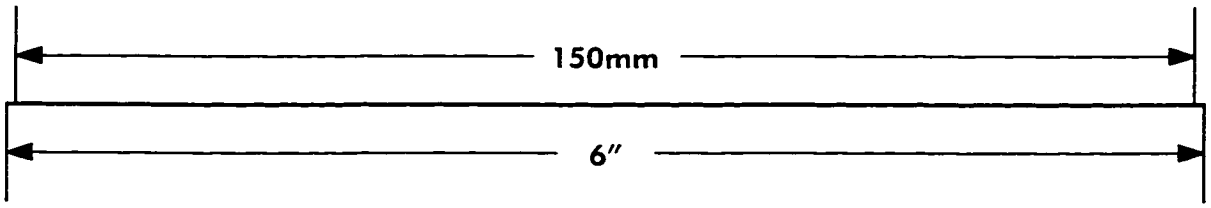
Zucker M. 1972. Light and enzymes. **Annu Rev Plant Physiol** 23: 133-156.

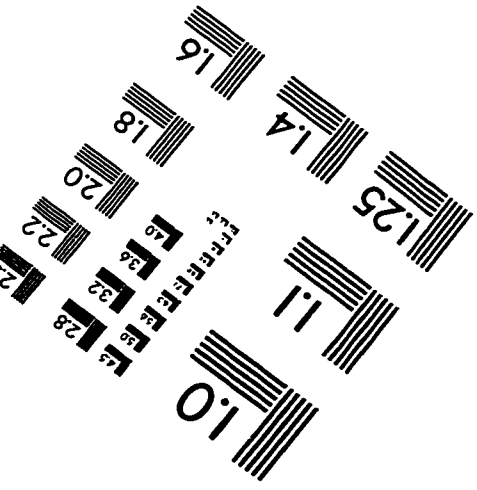
IMAGE EVALUATION TEST TARGET (QA-3)

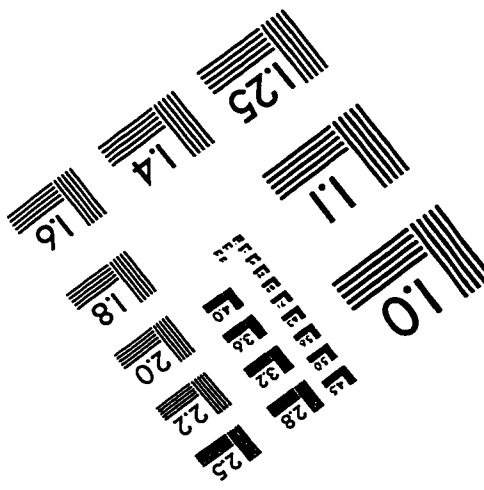












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989