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Regulation of Ethylene Biosynthesis and its Possible Role in Phenotypic Plasticity in *Stellaria longipes*

by

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Abstract

This study investigated the regulation of 1-aminocyclopropane-1-carboxylate (ACC) synthase and ACC oxidase, the two critical enzymes for ethylene biosynthesis in the context of stem elongation plasticity in alpine and prairie ecotypes of *Stellaria longipes*. The biochemical characteristics of ACC oxidase do not show any substantial difference between the two ecotypes. Full-length ACC oxidase cDNA clones were isolated from alpine and prairie cDNA libraries. Southern analysis suggests that ACC oxidase may be encoded by a single gene in both ecotypes. ACC oxidase exhibits circadian rhythmicity for its activity and mRNA abundance in both ecotypes. The steady-state mRNA and enzymatic activity levels reached their maxima by the middle of the light phase and minima by the middle of the dark phase. A 15 min red light pulse could reset rhythm in continuous darkness, suggesting the involvement of red light signal transduction pathway in the circadian regulation of ACC oxidase.

A genomic fragment encoding ACC synthase was amplified from alpine ecotype. Southern analysis indicates the presence of ACC synthase gene family with approximately 4 members in both ecotypes. Four unique ACC synthase cDNA clones that are expressed under different growth conditions were isolated from the two ecotypes. Northern analyses suggest that members are differentially regulated by photoperiod and temperature. It is proposed that such differential regulation of family members might represent one of the several molecular mechanisms of phenotypic plasticity.

The present investigation has identified γ-aminobutyric acid (GABA)

as a novel stimulus for ethylene production. Although GABA is structurally similar to ACC, it does not serve as an alternate substrate for ACC oxidase. Lower concentrations (upto 500 μM) of GABA promoted stem elongation. Higher concentrations (above 750 μM) of GABA significantly however inhibited stem elongation. Such inhibition was partly relieved by the presence of ethylene synthesis or action inhibitors, indicating that ethylene partly mediates the inhibitory effect of GABA. The dynamic changes in GABA levels associated with stem elongation and the ability of GABA to influence stem elongation suggest that GABA is involved in a general plastic response in *S. longipes.*

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....dedicated to my parents

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CHAPTER ONE
General Introduction

Through years of evolution, some plant species have gained the ability to control their growth and developmental processes in an opportunistic manner. Such an ability, often referred to as 'phenotypic plasticity' (Bradshaw, 1965), punctuates the genetic program under hostile environments but allows maximal growth under favorable conditions. In general, the term phenotypic plasticity is used to describe the manifestations of a single genotype resulting in distinguishable phenotypes under different environmental conditions. Given the sessile life style of plants, phenotypic plasticity is important for survival in habitats where environmental changes are unprecedented. Many studies suggest that plasticity of a trait is specific in relation to a particular environment and is under genetic control (Bradshaw, 1965; Schlichting, 1986; Stearns, 1989; Smith, 1990; Eiguchi, 1993). The type and degree of phenotypic plasticity may vary from subtle cellular level modifications to obvious morphological changes (Bradshaw, 1965). Because cellular modifications are less easy to observe than morphological changes, most of the evidence in the literature on phenotypic plasticity in higher plants is concerned with morphological or developmental changes.

Phenotypic plasticity is generally believed to have adaptive significance (Stearns, 1989), although all types of environmentally induced phenotypic variation are not necessarily always beneficial to a plant. Therefore evolution of phenotypic plasticity might rely on the ability to provide useful variation and adaptation to variable environments. A large

number of studies indicate that phenotypic plasticity can be selected for during the course of evolution (Schlichting, 1989). Simmonds (1981) suggested that modern cultivars with improved crop yield are a result of an unconscious selection of plasticity for more responsive genotypes to culture conditions. A good example of a plastic response in cultivated crops is deepwater rice. Deepwater rice is mostly grown in flood plains of Southeast Asia where flood waters rise up to several meters during the rainy season. A distinctive feature of deepwater rice plant is its ability to elongate its internode in response to rising water level as a means of preventing its foliage from being submerged. In contrast, air-grown deepwater rice or non deep water rice exhibit little or no elongation (Kihara *et al.*, 1962). In fact most of the recent crop improvement programs in deepwater rice is based on selection for plastic responses in internodal elongation (Eiguchi, 1993). Thus the phenotypic plasticity is a trait by itself and could have contributed to the fitness and success of the individuals or species during the course of evolution. It is often thought that polyploidy is an important force in the evolution of phenotypic plasticity (Schlichtig, 1989). Duplication of functional genes might allow significant changes in the structure of genes and thereby enhance the adaptivity of individuals under unprecedented environments (Dover, 1986). Thus polyploidy is often considered as a source of many biological novelties in higher plants. However, convincing evidence on the role of polyploidisation on phenotypic plasticity in plants is still lacking. It is certain that understanding the evolutionary framework in which phenotypic plasticity might have come about can help in studying the interaction between environment and physiological processes.

In any ecosystem, the amount and direction of plasticity may vary for different traits in different plants (Schlichting, 1986). Consequently the competing ability of different individuals or species for environmental variables such as light, water, minerals, etc. will vary in a community. For example, in a densely populated habitat, there is intense competition for light. Under such circumstances, stem elongation is crucial for a late emerging seedling to establish in a world already occupied by other green plants. This phenomenon, known as 'shade-avoidance response' (Smith, 1982), also enables other shaded plants to reach the top of the canopy to receive maximum light for photosynthesis. Dwarfing of most arctic and alpine plant species in response to severe wind stress and drought in winter is another example of the ecological significance of phenotypic plasticity. Arctic shrubs such as *Betula nana* and *Salix glauca* are rarely taller than 15 cm, but they surpass this height when grown against boulders or other taller shrubs and trees (Fitter and Hay, 1989). Thus phenotypic plasticity is an adaptive behavior of plants and is exhibited in response to environmental cues.

Although the evolutionary and ecological significance of phenotypic plasticity is widely appreciated, much less is known about the biological mechanisms by which plants exhibit phenotypic plasticity. Progress in our understanding of phenotypic plasticity has long been hindered by the lack of interdisciplinary approaches in dissecting its underlying biological processes. From a taxonomist's perspective, phenotypic plasticity is an unfavorable feature since varying phenotypes of a plant make it difficult to classify the species based on its appearance. To an ecologist, it exemplifies the adaptive strategies that a plant can offer to face the extremities in the

environment. For a plant physiologist, phenotypic plasticity can be a good phenomenon to understand how environmental cues influence growth and developmental patterns. To a plant molecular biologist, it offers an excellent opportunity to learn more about different repertoires of a genome that can be orchestrated by environmental signals. In this study an attempt has been made to integrate the different approaches to understand phenotypic plasticity using *Stellaria longipes* as a model system.

S. longipes is a weedy caryophyllaceous plant that grows in a wide range of habitat such as sand dunes, alpine, and prairie habitats (Macdonald and Chinnappa, 1989). It has several features of a good model system to study phenotypic plasticity. Throughout its life cycle, it exhibits discernible phenotypic plasticity for various traits (Chinnappa and Morton, 1984). For instance, the stem and leaves elongate under long day (16 h photoperiod) and warm (22° C day/18° C night) conditions (LDW) but not under short day (8 h photoperiod) and cold (8° C day/5° C night) conditions (SDC; Chinnappa and Morton, 1984). It is now understood that phenotypic plasticity is one of the vital means by which this plant species is able to thrive in contrasting habitats (Macdonald and Chinnappa, 1989).

S. longipes exists as a polyploid complex (2X, 4X, 6X and 8X; 2n=26; Chinnappa and Morton, 1974). Despite the variability and gene duplication that have been reported for several isozymes within the polyploid complex (Cai and Chinnappa, 1989; Cai *et al.*, 1990), there is no strict positive correlation between the ploidy levels and degree of plasticity among members of this polyploid species (Macdonald and Chinnappa, 1988). Yet natural selection has generated a considerable amount of genetic

variability and allowed population differentiation in *S. longipes* complex (Emery *et al.*, 1994b). It appears that over the years, such forces have allowed divergent plant strategies resulting in morphological differences between populations that can be revealed on a fine-scale differentiation (Macdonald and Chinnappa, 1989; Emery *et al.*, 1994b).

Among several populations that have colonized divergent habitats, alpine and prairie ecotypes are widely used in comparative studies (Macdonald, 1984; Emery, 1994; Zhang, 1995). Even though both of these ecotypes are tetraploid (4X=52), the amount of plasticity for stem and leaf elongation traits is greater in prairie ecotype than in alpine ecotype (Macdonald and Chinnappa, 1989; Emery *et al.*, 1994a). Availability of such contrasting ecotypes and the reproducibility of their phenotypic variations under controlled growth chambers make it easy to study phenotypic plasticity at the ecophysiological levels. Since *S. longipes* can be propagated vegetatively (Chinnappa and Morton, 1984), the genetic identity of individual ecotypes can easily be maintained and multiplied in a green house. Despite the perennial nature of its life cycle and the large number of minuscule chromosomes per haploid genome (Chinnappa and Morton, 1974; Chinnappa, 1985), significant progress has been made in physiological (Macdonald, 1984; Emery, 1994), biochemical (Cai, 1987) and molecular analyses (Zhang, 1994). These studies conributed to our understanding on the genetic, ecophysiological or molecular bases of phenotypic plasticity in various populations of *S. longipes* complex.

Plasticity for stem elongation, hitherto referred as stem elongation plasticity, is an important trait for the survival of *S. longipes*. Alpine plants

have evolved dwarfism to cope with a high wind stress in mountain habitats and avoid damage to their photosynthetic tissues. Prairie plants on the other hand grow in a habitat where there is an intense competition for light from crowded neighboring plants. The adaptive significance of such contrasting stem elongation behavior was elegantly shown by Emery *et al.* (1994a) in a 'reciprocal transplantation' study. The poor performance and low fitness of survival of alpine plants in a prairie habitat and prairie plants in an alpine habitat indicate that stem elongation plasticity is an important trait in *S. longipes* (Emery *et al.*, 1994a).

Stem elongation is a complex trait controlled by various environmental cues such as photoperiod, quality of light, intensity of light, temperature, wind, etc. (Sachs, 1965). The stem elongation response to such environmental cues is usually mediated by endogenous factors. For example, phytochromes are well known for their ability to transduce the quality of light and photoperiod. Phytochromes are cytoplasmic proteins which act as 'photoreceptors' for red (R) and far red (FR) light, and regulate the expression of genes that are involved in growth and development of plants. In higher plants, phytochromes are encoded by a small gene family (Quail *et al.*, 1995). A large number of studies has shown that lesions in PHY genes severely alter the stem elongation pattern. In fact, stem elongation is an important photomorphogenic trait being used in screening for phytochrome mutants. For example, the *hy3* mutant in *A. thaliana* that lacks phytochrome B was originally selected for their long hypocotyls. Other examples include *aurea* (*au*) mutant in tomato (Koornneef *et al.*, 1985), *long hypocotyl* (*lh*) mutant in cucumber (Adamse *et al.*, 1987), *elongated internode* (*ein*) mutant in *Brassica rapa* (Devlin *et*

al., 1992), *lv* in pea (Weller and Reid, 1993), and *phy B* in sorghum (Pao and Morgan, 1986). Transgenic plants that overexpress phytochrome cDNAs in oat, tobacco, tomato, potato or *A. thaliana* also exhibit large scale modifications in their stem elongation pattern (Cherry and Vierstra, 1994). A common feature observed in almost all the known PHY mutants is that they show significant changes in synthesis and/or action of major hormones such as gibberellin, auxin and ethylene (Koornneef and Kendrick, 1994; Firn, 1994). It is now thought that many of the effects of phytochrome on stem elongation are mediated by plant hormones.

It is conceivable that sensing the environmental signal(s), and transforming the signal into a suitable phenotypic response are the two most important components of phenotypic plasticity. Depending upon the type of environmental signal, the type and degree of phenotypic responses may vary in plants. It is well known that a special group of organic compounds called hormones can act as 'messengers' between an environmental cue and phenotypic responses in plants. A hormone is often synthesized in one part of the plant and translocated to another part where in very low concentrations, it causes a physiological response (Salisbury and Ross, 1985). Trewavas (1986) suggested that environmental variability necessitates specific plant tissues to acquire sensitivity to certain hormones in a developmental manner. There are also numerous occasions in which plant tissues have been shown to synthesize hormone(s) when challenged by an environmental cue, besides simply acquiring the sensitivity (Reid *et al.*, 1991). The function of hormones is believed to circumvent some of the metabolically constraining steps and enable growth and adaptation under otherwise unfavorable environments (Trewavas, 1986; Reid *et al.*, 1991).

Plant hormones mainly gibberellins (GA), auxins and ethylene play several important roles in regulating stem elongation (Davies, 1995a). A fundamental link between a major hormone and internodal elongation was first established by Phinney and coworkers (1961). They showed that the five dwarf mutants in maize d_1, d_2, d_3, d_5, and an_1 can be phenocopied to wildtypes after treatment with small amounts of GA_3. These mutants also contained less GA-like activity than the corresponding wild types when tested in a bioassay (Phinney, 1961). This led to the conclusion that GA_3 is important for normal stem elongation in maize. Other active forms of GAs (e.g. GA_1) have now been shown to influence stem elongation (Zeevaart *et al.*, 1993). Recently a large number of mutants that are deficient in GA biosynthesis and/or action has been identified in sweet pea, lettuce, wheat, spinach, tomato, *A. thaliana* and *Brassica spp.* (reviewed by Reid & Howell, 1995). Such GA mutants show severe alterations in their internodal elongation. Invariably, all of these mutants also reveal a complex response pathway involving other hormones especially ethylene and IAA (Reid and Howell, 1995).

From the classical experiments of Skoog and Miller (1989) the action of auxins has been closely linked to shoot growth. Auxin is well known for its strong effect in stimulating elongation in isolated stem segments (Cleland, 1995). However, inadequate or even conflicting evidence has made it difficult to establish the involvement of auxin in intact plants (Firn, 1994). Nevertheless, a close correlation between endogenous levels of indole-3-acetic acid (IAA), a predominant form of auxin in higher plants, and stem growth does exist, for example, in a range of genetic lines that

differ in plant height (Law and Davies, 1990). In the recent years, convincing evidence on the role of auxin on stem elongation has come from mutants such as *lka* and *lkb* in pea. These dwarf mutants with low levels of endogenous auxins display a greater response to low levels of exogenous IAA (Yang *et al.*, 1996). It is generally believed that auxin and GA promote stem elongation by different mechanisms. While IAA stimulates growth by cell extension, GAs promote growth by increasing the cell division and extension (Yang *et al.*, 1996).

Like in the case of auxin, the effect of ethylene on stem elongation has also been controversial. While a large number of studies indicate that exogenous ethylene inhibits stem elongation, ethylene has also been shown to promote stem elongation in some plant species. In fact, the earliest report on the physiological action of ethylene by Neljubov (1901), the so-called 'triple response' of young seedlings, involves a reduction in elongation. In plants that exhibit 'avoidance mechanism' such as deepwater rice (Kende, 1987), *Rumex spp.* (Voesenek *et al.*, 1992) and other semi-aquatic plants (Jackson, 1985), ethylene is essential for internodal elongation. Although the mechanism of ethylene action on stem elongation still remains unclear, ethylene has been implicated in determining the orientation of microtubules and microfibrils of cytoskeleton. The normal orientation of microtubules is along the circumference just inside the plasmalemma (like barrel hoops) and vertical to the plane of cell division (Abeles *et al.*, 1992). Evidence suggests that ethylene influences the cell elongation by changing the orientation of microtubules and microfibrils of the cytoskeleton (Steen and Chadwick, 1981; Lang *et al.*, 1982; Eisinger, 1983). Ethylene is also thought to influence cell elongation by mediating

some of the GA (Metraux and Kende, 1983) and auxin responses (reviewed by Firn, 1994).

In an effort to prime our understanding on the hormonal bases of stem elongation plasticity in *S. longipes*, Emery *et al* (1994b) found that ethylene is one of the major growth factors responsible in controlling stem elongation plasticity in both alpine and prairie ecotypes. In both ecotypes, ethylene production increases during rapid elongation. For example, when the plants are transferred from SDC to LDW, there is a gradual increase in ethylene production. In correlation with the degree of stem elongation, the amount of ethylene produced by alpine plants is less than that of prairie plants (Emery, 1994; Emery *et al.*, 1994b). Both ecotypes exhibit diurnal rhythmicity in ethylene production. While the prairie ecotype produces a strong daily ethylene rhythm, the alpine ecotype exhibits a much less pronounced rhythm for ethylene production (Emery *et al.*, 1994b). With regard to ethylene sensitivity, low amounts of exogenously applied ethylene promote stem elongation in prairie ecotype but inhibit stem elongation in alpine ecotype (Emery *et al.*, 1994b).

A comparative ecophysiological study on these two ecotypes also shows differences in sensitivity and production levels of ethylene in response to wind, an important environmental stress in both habitats. Alpine plants respond to wind with an increase in ethylene production and a reduction in stem elongation. Inhibition of ethylene synthesis or action can restore normal elongation in the presence of wind stress (Emery *et al.*, 1994b). In contrast, the increase in ethylene production levels during wind stress is relatively insignificant in prairie ecotypes as they can not afford

reduction in stem elongation. Applying inhibitors of ethylene synthesis or action in prairie ecotype causes reduction in stem elongation (Emery *et al.*, 1994b). Although an exact role for ethylene was not defined, these studies clearly showed the differences in the abilities of alpine and prairie ecotype to produce ethylene and indicated that such differences in ethylene production is a significant factor in governing the variation in stem elongation plasticity between alpine and prairie ecotypes. This necessitates an understanding of how these two ecotypes differentially regulate ethylene production in response to a common set of environmental cues, at the physiological, biochemical and molecular level. This forms the general objective of the present study.

Ethylene (C_2H_4) is a gaseous hormone. It is synthesized by almost all types of plant tissues (Abeles *et al.*, 1992). Being a simple gas, it easily moves from the site of synthesis by diffusion. It has a pronounced biological activity in plants and controls various aspects of growth and development (Abeles *et al.*, 1992). Ethylene production in most tissues can be induced by various biotic and abiotic stresses (Abeles *et al.*, 1992). The biosynthesis of ethylene has been extensively studied in higher plants (reviewed by Yang and Hoffman, 1984; Van Der Straeten and Van Montagu, 1991; Kende, 1993; Fluhr and Mattoo, 1996). Ethylene is synthesized from S-adenosyl methionine (SAM), a branch point for many other metabolic pathways such as methylation and polyamine biosynthesis. In the first step, SAM is converted to a cyclic non-protein amino acid called 1-aminocyclopropane carboxylic acid (ACC; Adams and Yang, 1979). This is catalyzed by an enzyme called ACC synthase (Kende, 1993). ACC is then oxidized to ethylene by ACC oxidase, formerly known as ethylene forming

enzyme (EFE, Hoffman and Yang, 1984). ACC can also be conjugated and sequestered in plant cells. Amrhein *et al.* (1981) showed that ACC can be reversibly malonylated to 1-malonylaminocyclopropane-1-carboxylate (MACC). The enzyme ACC N-malonyl transferase which catalyzes the malonylation of ACC has been partially purified from mungbean hypocotyls (Guo *et al.*, 1992) and tomato fruit pericarp (Martin and Saftner, 1995). Studies conducted on ACC N-malonyl transferase indicate that the same enzyme can catalyze the malonylation of other D-amino acids in a non-specific manner (Yang and Hoffman, 1984; Benichou *et al.*, 1995). Thus the relevance of malonyl transferase in the regulation of ethylene production is difficult to analyze (Yang and Hoffman, 1984; Fluhr and Mattoo, 1996). A new conjugate, 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid (GACC) has recently been identified in tomato (Martin *et al.*, 1995). The enzyme responsible for such conjugation has recently been identified as γ-glutamyl hydrolase/transpeptidase (Martin and Slovin, 1997). Like in the case of ACC N-malonyltransferase, γ-glutamyl hydrolase/ transpeptidase has also been shown to conjugate L-amino acids such as phenylalanine and methionine in a non specific manner (Martin and Slovin, 1997). It is presently unclear how many other types of ACC conjugate exist in plants and to what level they influence the ethylene production. The overall reaction is summarized in Fig. 1.1.

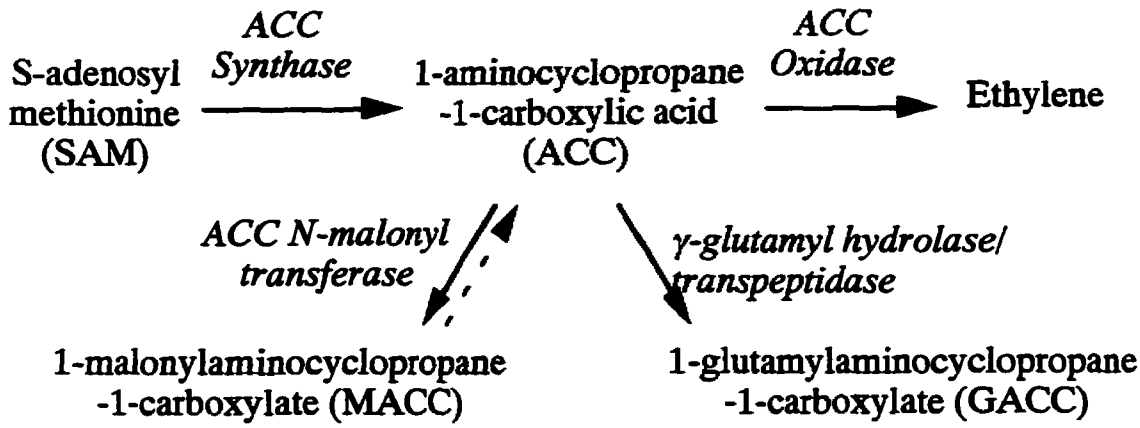

Fig. 1.1. A schematic diagram showing the biosynthetic pathway of ethylene in higher plants.

Ethylene production under various stress conditions and developmental stages is preceded by an induction of ACC synthase gene expression (Zarembinski and Theologis, 1994). Such ethylene production can be inhibited by chemicals that can inhibit the enzymatic activity of ACC synthase. Hence, ACC synthase is considered as a key regulatory step in ethylene biosynthesis (Abeles *et al.*, 1992). The genes and cDNAs encoding ACC synthase and ACC oxidase have been cloned in many plant species (reviewed by Fluhr and Mattoo, 1996) and their biochemical characteristics have been fairly well understood. The outstanding progress made on the biosynthetic aspects of ethylene during the past decade has now set the stage for important advances in our understanding of the regulatory mechanisms of ethylene production. The general goals of this Ph.D. work are to understand how ethylene biosynthesis is regulated at ACC synthase and ACC oxidase level in alpine and prairie ecotypes at the molecular level, and what are the possible roles of such regulation on phenotypic plasticity. To achieve these general goals, the following specific objectives were designed.

1. To clone and characterize ACC oxidase at the molecular and biochemical level
2. To study the regulation of ACC oxidase activity and mRNA abundance
3. To clone and characterize ACC synthase gene and/or cDNAs encoding ACC synthase
4. To study the regulation of ACC synthase mRNA accumulation

In an effort to gain some insights into cellular intermediates that are involved in the regulation of ethylene biosynthesis, γ- aminobutyric acid (GABA), a non-protein amino acid that is rapidly accumulated under those stress conditions in which ethylene is also produced (Bown and Shelp, 1997), was tested for its ability to stimulate ethylene production in *S. longipes*. GABA is Since this compound also possesses structural similarity to ACC, it is hypothesized that GABA might influence ethylene production and the ethylene-dependent stem elongation in *S. longipes*. Hence the following objective was introduced during the course of this research project.

5. To study the role of GABA on ethylene production and stem elongation.

CHAPTER TWO
MATERIALS AND METHODS

2.1. Materials

Plants were originally collected from the Chain Lakes area of Alberta (prairie ecotype) and the summit of Plateau mountain of Alberta (alpine ecotype). Plants were potted in a soil mix containing peat moss, terra green, sand, perlite and vermiculite (1:4:2:1:1) in growth chambers (Conviron, Winnipeg, Canada) under short day and cold (SDC; 8 h photoperiod and 8° C day/5° C night) for a minimum of 60 days to simulate winter (Macdonald *et al.*, 1984). The plants were then transferred to long day and warm (LDW; 16 h photoperiod and 22° C day/18° C night). Plants were maintained under Philips F48t12/C50/HO white bulbs (Philips Lighting Co., Sommerset, NJ, USA). Photosynthetically active radiation at plant height ranged from 225 to 250 μmol m^{-2} s^{-1} when measured with a Li-Cor quantum sensor (model LI-190SB; LI-COR Inc., Lincoln, NE, USA) After maturity, the plants were clonally propagated and cycled through SDC and LDW annually. Stem and leaf tissues were harvested and incubated for ethylene measurements or immediately frozen in liquid N_2 and stored at -80° C for later RNA, enzyme, ACC or GABA extractions.

For *in vitro* studies, a recently developed tissue culture system (Miranda *et al.*, 1997) was followed. Shoot tips consisting of a bud and 2 young leaves were collected from young ramets, surface sterilized with 70% ethanol, 10% commercial bleach and washed with sterile distilled water, and cultured in petridishes containing MS media (Murashige and

Skoog, 1962) supplemented with 0.1 mg/l thiamine, 100 mg/l myo-inositol, 30 g/l sucrose and 0.8% agar for 4 to 6 weeks to facilitate a continuous supply of sterile shoot tips for further experiments. Such shoot tips were then subcultured in a similar media and the growth measurements were made. The pH of all the media was adjusted to 5.7 before autoclaving. After 21 days in culture, plants were carefully removed and used for measurements of internodal and stem lengths or immediately frozen in liquid N_2 and stored at -70° C for later GABA extraction.

2.2. Methods

2.2.1. Nucleic Acids

2.2.1.1. DNA methods

2.2.1.1.1. Genomic DNA extraction

Genomic DNA from stem and leaf tissues were extracted using the protocol described by Doyle and Doyle (1987). The frozen tissues were ground in liquid N_2 and homogenized in 2 x CTAB buffer (2% hexadecyltrimethyl ammonium bromide (CTAB), 1.4 M NaCl, 0.2% (v/v) 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0). After incubating at 60° C for 1 h, the slurry was extracted with chloroform-isoamyl alcohol (24:1; v/v) and centrifuged at 10, 000 rpm for 15 min at room temperature. The aqueous phase was transferred to 2/3 volumes of cold isopropanol. The DNA was spooled with a glass tip and washed in a buffer containing 76% ethanol and 10 mM ammonium acetate. The DNA pellet was dried in air and dissolved

in distilled water. The DNA was treated with RNase A to a final concentration of 10 μg/ml for 30 min at 37° C and washed in 0.25 M ammonium acetate and 70% ethanol. The DNA was pelleted by centrifuging at 10,000 rpm for 10 min, dried and resuspended in TE buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA).

2.2.1.1.2. Southern blotting and hybridization

Ten μg of genomic DNA was digested overnight with appropriate restriction endonuclease(s) (Pharmacia, Uppsala, Sweden) at the recommended temperature. The digested DNA was separated on a 1.2% agarose gel overnight at 25V, and blotted onto a nylon membrane (Hybond-N, Amersham, UK) in 20x SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7). After blotting, the membranes were baked at 80° C for 2 h and prehybridized in Rapid-hyb buffer® (Amersham, UK) at 60° C for 2 h. Hybridization was also done in the same buffer at 60° C for at least 5 h. Membranes were washed once in 5x SSPE plus 0.1% SDS at room temperature, twice in 2x SSPE plus 0.1% SDS at 60° C for 20 min, twice in 1x SSPE plus 0.1% SDS at 60° C for 20 min and if necessary at 0.5x SSPE plus 0.1% SDS at 60° C for 15 min. The membranes were then exposed to Kodak XAR 5 film at -80° C.

2.2.1.1.3. Polymerase Chain Reaction (PCR)

Sequence specific primers were synthesized using Pharmacia DNA synthesizer (Pharmacia LKB Biotechnology, Uppsala, Sweden). All PCRs were carried out using a Robocycler (Stratagene, LaJolla, CA, USA) in 35

cycles of denaturation (1 min) at 95° C, annealing (1 min) at 50 to 52° C, and primer extension (2 min) at 72° C, using Taq DNA polymerase (Pharmacia) for anaytical purposes or Pwo DNA polymerase (Boehringer Mannheim, Mannheim, Germany) for cloning purposes.

2.2.1.1.4. DNA cloning

The DNA fragment (insert) to be cloned was digested with appropriate restriction nuclease to produce either sticky or blunt ends. In the case of PCR products, they were end filled with DNA Klenow polymerase (Pharmacia) before they were used for cloning. Such DNA fragments were ligated to pBluescript® (Stratagene, La Jolla, CA, USA) cut with corresponding restriction nuclease(s) using ligation buffers described by King and Blakesley (1986) at room temperature overnight. The ligation mix was used to transform competent cells of *Escherichia coli* (DH 5α) by applying heat shock (42° C) for 1 min (Sambrook *et al.*, 1989). The transformed bacterial cells were selected for their ability to produce blue color as described by the manufacturers (Stratagene).

2.2.1.1.5. DNA sequencing

DNA sequencing was done using the chain termination method described by Sanger *et al.* (1981). During the synthesis of new complementary *in vitro* DNA strands by T7 DNA Sequenase (Pharmacia), the elongation was arrested using either α^{32}P labeled dideoxy nucleotides or fluorescent dye labeled dideoxy nucleotides. The reaction products were separated in a high resolution polyacrylamide gel electrophoresis. The dye terminated

sequencing reactions were done at the automatic sequencing facility available at Department of Medical Biochemistry, University of Calgary.

2.2.1.1.6. Nucleotide and amino acid sequence analyses

Homology search for the DNA sequences were made at genbank using Advanced BLASTN Search program (Altschul *et al.*, 1990). Characterization of the nucleotide sequences and conceptual translation of nucleotide sequences were done using DNA Strider™ 1.2 (Centre d'Etudes de Saclay, France) and MacVector™ 6.0 DNA sequence analysis (Oxford Molecular group Inc., Campbell, CA, USA) programs. The alignment of identical or homologous amino acid sequences were done using CLUSTAL V multiple sequence alignment program (Higgins *et al.*, 1992) on Kyte-Doolittle scale and viewed using SeqVu version 1.0.1 (The Garvan Institute of Medical Research, Sydney, Australia).

2.2.1.2. RNA methods

2.2.1.2.1. RNA extraction

Total RNA was isolated from the tissues using hot phenol as described by Verwoerd *et al.* (1989). The frozen tissues were ground to powder and homogenized in 10 ml of hot (80° C) phenol and 2 ml of chloroform per g fw of tissues. The slurry was centrifuged at 10,000 rpm for 15 min at 4° C. The supernatant was extracted once with chloroform-isoamyl alcohol (24:1) and the total RNA was precipitated using 95% ethanol overnight at -20° C. The RNA pellet was dissolved in 2M LiCl and precipitated once

again at -20° C. The RNA pellet was then washed in 70% ethanol and 0.3 M sodium acetate, dried and dissolved in diethyl pyrocarbonate (DEPC) treated water. Poly $(A)^+$ RNA was isolated from total RNA using Poly A tract® mRNA isolation systems (Promega Corporation, Madison, USA).

2.2.1.2.2. Northern blotting and hybridization

For Northern analysis, 15 μg of total RNA or 10 μg of poly $(A)^+$ RNA was separated by electrophoresis in formaldehyde agarose gels and transferred to nylon membranes (Hybond-N+, Amersham, UK) in 20x SSPE (3.6 M NaCl, 0.2 M sodium phosphate, 0.02 M EDTA, pH 7.7). Hybridization was done in 50% formamide, 5x SSPE, 0.5% SDS, 5x Denhardt's solution, yeast tRNA (1.0 μg/ml) at 42° C using DNA probes labeled by random oligonucleotide priming (Feinberg and Vogelstein, 1983). Membranes were washed twice with 2x SSPE plus 0.1% SDS at RT, twice with 1x SSPE plus 0.1% SDS at 65° C for 30 min, and twice with 0.1x SSPE plus 0.1% SDS at 65° C for 30 min.

2.2.1.2.3. cDNA library construction

Alpine (genotype 1D) and prairie (genotype 7B) ecotypes were grown under LDW conditions. Stem and leaf tissues of 7-day-old ramets were harvested. The poly $(A)^+$ RNA extracted from these tissues was reverse transcribed in the presence of methylated dCTP and polyT-Xho I linker primers using Stratascript Reverse transcriptase® (Stratagene, La Jolla, CA, USA). To the 5' end of double stranded cDNAs, Eco RI linkers were ligated. The cDNA pool was then digested with Eco RI and Xho I and a

cDNA library was constructed using lambda Uni-ZAP™ XR vector arms (Stratagene, La Jolla, CA, USA) as described by the manufacturer (Stratagene; Huse and Hansen, 1988). The libraries made from 1D and 7B had a primary titer values of 712,500 and 1,415,000 pfu respectively. These libraries were then amplified (to an approximate titer of 10^{10} pfu/ml) and stored at -80° C.

2.2.1.2.4. cDNA library screening

Approximately 35,000 plaques per petri plate were screened as suggested by the manufacturers (Stratagene). The DNA from plaques were transferred to a nylon membrane (Hybond N+; Amersham), denatured in 1.5 M NaCl and 0.5 M NaOH for 2 min, neutralized for 5 min in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0), and fixed on to the membrane by baking at 80° C for 2 h. Prehybridization and hybridization of membranes were done with appropriate probes under the conditions described in section 2.2.1.2.2. Isolates from primary screening were subjected to secondary and tertiary screening in a similar manner. The cloned insert contained within the lambda vector was excised *in vivo* as a phagemid by coinfecting the host SOLR® (Stratagene) strain of *E. coli* with ExAssist helper phage® (Stratagene) as described by the manufacturers (Stratagene; Huse and Hansen, 1988).

2.2.1.2.5. Rapid amplification of 3' cDNA ends (3'RACE)

The protocol for amplification of 3' ends of mRNA has previously been described by Frohman *et al.* (1988). One microgram of total RNA in 10 µl

of water and 0.5 µl of 1 nM $(dT)_{18}$ - linker primer were vacuum dried and dissolved in 3 µl of water. The mixture was heated to 80° C, gradually cooled to 37° C, added to 2 µl of 5x First Strand buffer (Life Technologies, Gibco BRL, Burlington, Canada), 1 µl of 0.1 M dithiothreitol, 2 µl of 5 mM dNTPs, 1 µl of RNA guard (Pharmacia LKB Biotechnology, Uppsala, Sweden), and 10 units of Superscript II Reverse Transcriptase® (Life Technologies, Gibco BRL, Burlington, Canada), and incubated at 37° C for 2 h. The reaction mixture was then inactivated at 90° C and stored at -20° C. PCR was carried out using 2 µl of cDNA pool as template and forward antisense linker and ACC synthase degenerate oligonucleotides as primers.

2.2.2. Biochemical and physiological methods

2.2.2.1. ACC oxidase extraction and assay

Under N_2, the harvested tissue was ground in liquid nitrogen to powder and homogenized using 7.5 volumes of cold extraction buffer (100 mM BTP {1,3-bis[tris(hydroxymethyl)-methylamino]propane}-HCl, pH 6.8), 50 µM $FeSO_4$ and 20 mM sodium ascorbate. The homogenate was centrifuged at 10,000 rpm for 10 min (Micromax®; International Equipment Co., MA, USA), and the supernatant containing the enzyme extract was collected. Ten-ml pyrex tubes containing 1 ml of assay buffer (100 mM BTP-HCl, pH 6.6), 50 µM $FeSO_4$, 20 mM sodium ascorbate, 3 mM ACC and pre-incubated with 1.5% CO_2 (CO_2 was injected into the headspace of the tubes). Fifty µl of enzyme extract was added to these tubes and incubated at 37° C for 30 min with shaking. The ethylene content of 1- ml head space gas samples from these tubes was then analyzed using a Photovac 10S Plus gas

chromatograph (Photovac Inc., Markham, ON, Canada) equipped with a photo ionization detector and a 40/60 Carbopack B column (1.5% XE-60/1% H_3PO_4; Supelco Canada, Oakville, ON, Canada).

2.2.2.2. ACC extraction

About 1 g of tissue was extracted twice at 70° C in 5 ml of 80% ethanol for 15 min each. The extracts were then dried *in vacuo* at 40° C and extracted once with chloroform-distilled water (1:2; v/v). ACC was then determined as described by Saltveit and Yang (1987). Aliquots of each sample (500 µl) were transferred to 12x75 mm test tube containing 5 µmol $HgCl_2$. Using water, the total volume was brought to 900 µl, and the tube was sealed with rubber septum and kept on ice. A cold mixture of 5.25% NaOCl and saturated NaOH (2:1; v/v) was added to the tube and vortexed for 5 s. One ml of gas was immediately withdrawn from the headspace and analyzed for ethylene content using Photovac 10S Plus gas chromatograph.

2.2.2.3. Ethylene measurements

Since ethylene production in *S. longipes* oscillates in a rhythmic fashion during a day, the tissues were collected at 15:00 h the time the rhythm reaches a peak(Emery *et al.*, 1994b). Excised tissues were incubated in 10-ml syringes with the plungers adjusted to 6 ml. After 20 min of incubation, a 3-ml gas sample was transferred to a second syringe through a three-way valve, and analyzed for ethylene content using a Photovac 10S Plus gas chromatograph.

2.2.2.4. GABA extraction and estimation

Frozen samples (0.1 to 0.5 g) were pulverized under liquid N_2 and stirred in 10 ml of methanol:chloroform:water (6:2.5:1.5) with 5% polyvinyl poly pyrrolidone (w/v). The homogenate was centrifuged at 10,000 rpm for 10 min at 4° C. The supernatant was extracted once with an equal volume of chloroform and dried *in vacuo* at 40° C. The residue was dissolved in 1.5 ml of 5 mM HCl and passed through a Dowex-50W (H^+) resin column (Sigma Chemical Co. Mississauga, Ont., Canada). Using 2M NH_4OH, GABA was eluted and dried *in vacuo* at 40° C. The residue was dissolved in 1.5 ml of 100 mM PPi buffer and assayed for GABA content using GABAse assay (ICN Pharmaceuticals Canada Ltd., Montreal) as described by manufacturers. GABA was quantitatively determined by indirectly measuring the stoichiometric reduction of NADP by GABAse at 340 nm. In some cases, GABA levels were estimated using HPLC after ninhydrin derivatization (Baum *et al.*, 1996) at the Amino acid analysis Lab, Department of Medical Biochemistry, University of Calgary.

CHAPTER THREE

Molecular Cloning and Biochemical Characterization of 1-Aminocyclopropane-1-carboxylate Oxidase in *S. longipes*

3.1. Introduction

Long before the ethylene biosynthetic pathway was characterized in plants, it was known that a nitrogen atmosphere arrests ethylene production in pear (Hansen, 1942) and apple fruit tissues (Burg and Thimann, 1959), but that a surge of ethylene production occurs upon exposure to air. These studies suggested that an ethylene biosynthetic intermediate accumulates during anaerobic incubation and is subsequently converted to ethylene in the presence of oxygen. Adams and Yang (1979) compared the conversion of SAM to ethylene in air and in nitrogen atmosphere. In air, SAM was efficiently converted to ethylene whereas in nitrogen, SAM was metabolized to ACC but not ethylene indicating that conversion of ACC to ethylene is an oxygen dependent reaction. The putative enzyme mediating this reaction was called at that time as 'ethylene forming enzyme' (EFE; Yang and Hoffman, 1984). When ACC was applied to most of the plant tissues, a drastic increase in ethylene production was observed (Cameron *et al.*, 1979; Lurssen *et al.*, 1979) suggesting that EFE is largely constitutive. Earlier efforts to characterize EFE were faced with many hurdles since EFE activity is thermolabile, lacked the required catalytic discrimination for methyl-ACC and ethyl-ACC, and often exhibited Km values that were inconsistent with the internal ACC concentration (reviewed by Yang and Hoffman, 1984).

The search for EFE was cut short with the advent of molecular techniques. Based on the differential hybridization of mRNAs isolated from ripening tomato fruits, Slater *et al* (1985) identified a clone called pTOM13 the rapid appearance of which was also found in wounded tissues. pTOM13 is not a complete cDNA as it lacks the start codon. Nevertheless, a breakthrough in identifying EFE came when tomato plants was transformed with pTOM13 in antisense orientation fused to a constitutive promoter (CaMV 35S). Ethylene synthesis in such transgenic plants were reduced in a transgene dosage dependent manner even when ACC was applied exogenously (Hamilton *et al.*, 1990). Since the predicted amino acid sequence of pTOM13 is similar to flavanone 3-hydroxylases and 2-oxoglutarate-dependent dioxygenases, Hamilton *et al.* (1990) suggested that pTOM13 might encode EFE in tomato plants. The ultimate proof that pTOM13 indeed encoded EFE came from two independent lines of evidence. Yeast transformed with pRC13 (corrected pTOM13) expressed EFE activity that exhibited the proper stereospecificity and saturation kinetics (Hamilton *et al.*, 1991). Similarly oocytes of *Xenopus laevis* injected with pTOM13 gained the ability to convert ACC to ethylene (Spanu *et al.*, 1991). Following these findings, cDNAs encoding EFE (now known as 'ACC oxidase'; Kende, 1993) has been cloned in a large number of plant species such as apple (Dong *et al.*, 1992), carnation (Wang and Woodson, 1992), orchid (Nadeau *et al.*, 1993) and sunflower (Liu *et al.*, 1997).

The thermolabile nature and the difficulties involved in purifying ACC oxidase delayed the progress in our understanding of the protein. Since the deduced amino acid sequence of pTOM13 resembles flavanone 3-hydroxylase, Ververidis and John (1991) extracted and assayed ACC

oxidase in melon fruits under conditions that preserve flavanone 3-hydroxylase. By extracting ACC oxidase under N_2 gas and including Fe^{2+} and ascorbate in the assay medium they recovered maximum ACC oxidase activity which showed the proper stereospecificity (Ververidis and John, 1991). *In vitro* ACC oxidase activity can be enhanced by CO_2 and to a greater extent depends on the presence of CO_2 (Finlayson and Reid, 1993). Partially purified ACC oxidase from apple fruits is about 39 kDa by size and active as a monomer (Dong *et al.*, 1992).

The aims of this study are (i) to clone the cDNAs encoding ACC oxidase in alpine and prairie ecotypes of *S. longipes*, (ii) characterize the enzyme at the biochemical level and (iii) study the kinetic differences between the two ecotypes, if any.

3.2. Materials and Methods

3.2.1. Plant growth

Plants were kept under SDC for about 90 days and transferred to LDW. Stem and leaf tissues were collected from 7 to 10 days old plants grown under LDW, frozen in liquid N_2 and stored at -80° C for later extraction of ACC oxidase.

3.2.2. Isolation of ACC oxidase cDNA fragment

The primers used to isolate ACC oxidase cDNA fragment in this study were kindly provided by Dr. J.-H. Liu, Lethbridge Research Center, Agriculture

and Agri-Food Canada, Lethbridge. These primers were originally designed for the purpose of sequencing pS 2-1-5, the sunflower ACC oxidase cDNA clone (Genbank accession number L21976). Using an aliquot of cDNA library (1D; alpine) as template and two oligonucleotide primers namely FO 7 (5'-GGTTAAGCAACTACCCACC-3', positions: 480-497 in sunflower) and R1 (5'-CTGTTGCAACTGGACCC-3', positions: 956-973 in sunflower), a 480 bp fragment (SlACO-1) was amplified. The nucleotide as well as the predicted amino acid sequence of SlACO-1 (Genbank accession number- U30247) was found to have a high degree of similarity (80-95%) with ACC oxidase from other species.

3.2.3. Cloning of ACC oxidase cDNAs

About 40,000 plaques from each 1D (alpine) and 7B (prairie) cDNA libraries were screened by following the protocol described by Sambrook *et al.* (1989). SlACO-1 labeled with α^{32}P dCTP was used as a probe. Several hybridizing plaques were obtained. Two clones namely pAACO-18 (from alpine ecotype) and pPACO-26 (from prairie ecotype) exhibited very high homology (80 to 95%) to known ACC oxidase at the nucleotide and amino acid level.

3.2.4. ACC oxidase extraction and assay

Due to the thermolabile nature of the enzyme, the entire handling of ACC oxidase was done on ice. The enzyme was extracted and assayed as described in section 2.2.2.1. To test the effect of CO_2 on enzymatic activity, various concentrations of CO_2 were injected into the headspace of assay

tubes. To prevent the changes in the pH of the buffer due to the addition of CO_2, the buffer pH was adjusted prior to the addition of CO_2 so as to maintain the final pH of the buffer at a constant value of 6.6 in each treatment.

3.3. Results

3.3.1. Cloning strategy

In order to clone ACC oxidase cDNAs, two separate cDNA libraries were constructed from stem and leaf tissues of alpine and prairie plants grown under LDW. From the cDNA library of alpine ecotype, ACC oxidase cDNA fragment was amplified using primers designed from the conserved amino acid sequences of sunflower ACC oxidase cDNA (Liu and Reid, unpublished data). This partial ACC oxidase cDNA fragment was then used as a probe to isolate ACC oxidase clones from the cDNA libraries of alpine and prairie ecotype.

3.3.2. Construction of cDNA libraries

Two individual cDNA libraries were made from alpine (genotype; 1D) and prairie ecotype (genotype; 7B). Stem and leaf tissues of 7-day-old ramets under LDW conditions were harvested. Both cDNA libraries were constructed using Uni-ZAP™ XR vector (Stratagene, La Jolla, CA, USA). The cDNA libraries made from alpine and prairie ecotypes had primary titer values of 712,500 and 1,415,000 pfu respectively. These libraries were later amplified to an approximate titer of 10^{10} pfu/ml.

3.3.3. PCR amplification of a partial ACC oxidase cDNA fragment

Using two oligonucleotides that are homologous to conserved amino acid domains namely FO (nucleotide positions 480 to 497 in sunflower) and R1 (nucleotide positions 956 to 973 in sunflower) as primers and an aliquot of cDNA library from alpine ecotype as template, a 480 bp fragment was amplified. This 480 bp fragment was named as SlACO-1 (Fig. 3.1; Genbank accession no. U30247). Upon conceptual translation, SlACO-1 was found to have a high degree of similarity (80 to 90%) to ACC oxidase from other species at the amino acid level.

3.3.4. Cloning of ACC oxidase cDNAs

The cDNA libraries of alpine and prairie ecotypes were screened using SlACO-1 as a probe. Several clones that hybridized to SlACO-1 were isolated. Two cDNA clones named pAACO-18 (from alpine ecotype) and pPACO-26 (from prairie ecotype) were taken for further analyses. pAACO-18 is 1098 bp long (Fig. 3.2). It includes 27 bp 5' untranslated region (UTR), 966 bp coding region and 105 bp 3' UTR. The predicted amino acid sequence of pAACO-18 is highly homologous (80 to 95%) to that of other known ACC oxidases (Fig. 3.3). Hence pAACO-18 (Genbank accession no. AF011399) has been designated as a cDNA encoding ACC oxidase in alpine ecotype of *S. longipes*.

Fig. 3.1. The nucleotide sequences of alpine ACC oxidase cDNA fragment, SlACO-1. SlACO-1 (Genbank accession no. U30427) was isolated from cDNA library of alpine ecotype by PCR using FO and R1 primers (section 3.2.2). SlACO-1 is 482 bp in length and encodes 160 amino acids.

```
  1 GTT AGC AAC TAC CCA CCG CTA CCA AAG CCT GAC TTA ATT AAA GGC TTA AGT GCA CAT ACC  60
  1 V   S   N   Y   P   P   L   P   K   P   D   L   I   K   G   L   S   A   H   T   20

 61 GAT GCC GGA GGC ATC ATC CTT CTC TCT GAA GAC GAC AAG GTC AGT GGA CTT GAA CTC CTC 120
 21 D   A   G   G   I   I   L   L   S   E   D   D   K   V   S   G   L   E   L   L   40

121 AAG GAC AGT GAT TGG ATA GAC GTC CCG CCG ATG AGA CAT AGC ATA GTG GTC AAT TTA GGA 180
 41 K   D   S   D   W   I   D   V   P   P   M   R   H   S   I   V   V   N   L   G   60

181 GAT GAA CTC GAA GTC ATA ACA AAC GGC AAG TAC TGG AGT GTC ATG CAT CGA GTA CAG ATT 240
 61 D   E   L   E   V   I   T   N   G   K   Y   W   S   V   M   H   R   V   Q   I   80

241 GAT ACT AAC ATT ACG AGA ATG TCA ACG GCA AGT TTC TAT GTT CCG GGA AAC GAT CGT GTC 300
 81 D   T   N   I   T   R   M   S   T   A   S   F   Y   V   P   G   N   D   R   V  100

301 ATT GCT CCG GCT GAA GCT CTC CCT AAA GAG TTT AAG GAT GAC GAG GTC TAT CCA AAA TTC 360
101 I   A   P   A   E   A   L   P   K   E   F   K   D   D   E   V   Y   P   K   F  120

361 GTC TTT AAG AGC TAC ATG AAG GCT TAT GCT AAG TTG ATT CGT CAG AGT GGC CTC AAG TTC 420
121 V   F   K   S   Y   M   K   A   Y   A   K   L   I   R   Q   S   G   L   K   F  140

421 GAA GCG AAA GAA CCG CGG TTT GAG GCT ATG AAG GCA CAC CGA ATT GGG TCC AGT TGC AAC 480
141 E   A   K   E   P   R   F   E   A   M   K   A   H   R   I   G   S   S   C   N  160

481 AG                                                                            482
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Fig. 3.2. Nucleotide sequences of pAACO-18, a cDNA encoding ACC oxidase in alpine ecotype of *S. longipes*. Nucleotides representing the coding region are given in upper case letters and the 5'untranslated and 3'untranslated regions are given in smaller case letters.

```
   1 acgattggacggttttacgattagacg ATG GAG AAC TTT CCA ATA ATA AAC ATG GAA AAC ATA GAA  66
   1                             M   E   N   F   P   I   I   N   M   E   N   I   E   13

  67 CGG AAG AAC ACG CGA GAC ATG ATA AAA GAC GCG TGT GAA AAC TGG GGA TTT TTT GAA TTA 126
  14 R   K   N   T   R   D   M   I   K   D   A   C   E   N   W   G   F   F   E   L   33

 127 GTA AAC CAC GGA ATA CCA CAC TCT TTA CTA GAC AAA ATG GAA AAA CTC ACA AAA GAC CAC 186
  34 V   N   H   G   I   P   H   S   L   L   D   K   M   E   K   L   T   K   D   H   53

 187 GTA AGG AAG TGT TTA GAA GAA AGG TTT AAA GAA ATG GTA GCA AGC AAA GGA CTA GAA GCA 246
  54 V   R   K   C   L   E   E   R   F   K   E   M   V   A   S   K   G   L   E   A   73

 247 GTG GAA GCT GAA GTA ACA GAC CTA GAC TGG GAA AGC ATA TTT TTT CTA AAG CAC CTA CCA 306
  74 V   E   A   E   V   T   D   L   D   W   E   S   I   F   F   L   K   H   L   P   93

 307 GTA TCT TCA ATA TCT GAA CTA CCA GAC CTA GAC GAA GAA TAT CGA GAA GTA ATG ATA GAC 366
  94 V   S   S   I   S   E   L   P   D   L   D   E   E   Y   R   E   V   M   I   D  113

 367 TTT TCA AAG CGA AAA GAA AAG AAA GCA GAA ACA AAA AAA GAC AAA AAA TGT GAA AAC AAA 426
 114 F   S   K   R   K   E   K   K   A   E   T   K   K   D   K   K   C   E   N   K  133

 427 GGA AAA GAA AAG AGC TAT CTA AAA AAC ACA TTT TAT GGA AGC AAA GGA CCA AAC TTT GGA 486
 134 G   K   E   K   S   Y   L   K   N   T   F   Y   G   S   K   G   P   N   F   G  153

 487 ACA AGC GTT AGC AAC TAC CCA CCG CTA CCA AAG CCT GAC TTA ATT AAA GGC TTA AGT GCA 546
 154 T   S   V   S   N   Y   P   P   L   P   K   P   D   L   I   K   G   L   S   A  173

 547 CAT ACC GAT GCC GGA GGC ATC ATC CTT CTC TCT GAA GAC GAC AAG GTC AGT GGA CTT GAA 606
 174 H   T   D   A   G   G   I   I   L   L   S   E   D   D   K   V   S   G   L   E  193

 607 CTC CTC AAG GAC GGA GAT TGG ATA GAC GTC CCG CCG ATG AGA CAT AGC ATA GTG GTC AAT 666
 194 L   L   K   D   G   D   W   I   D   V   P   P   M   R   H   S   I   V   V   N  213

 667 TTA GGA GAT GAA CTC GAA GTC ATA ACA AAC GGC AAG TAC TGG AGT GTC GAG CAT CGA GTA 726
 214 L   G   D   E   L   E   V   I   T   N   G   K   Y   W   S   V   E   H   R   V  233

 727 CAG ATT GAT ACT AAC ATT ACG AGA ATG TCA ACG GCA AGT TTC TAT GTT CCG GGA AAC GAT 786
 234 Q   I   D   T   N   I   T   R   M   S   T   A   S   F   Y   V   P   G   N   D  253

 787 CGT GTC ATT GCT CCG GCT GAA GCT CTC CCT AAA GAG TTT AAG AAG GAC GAG GTC TAT CCA 846
 254 R   V   I   A   P   A   E   A   L   P   K   E   F   K   K   D   E   V   Y   P  273

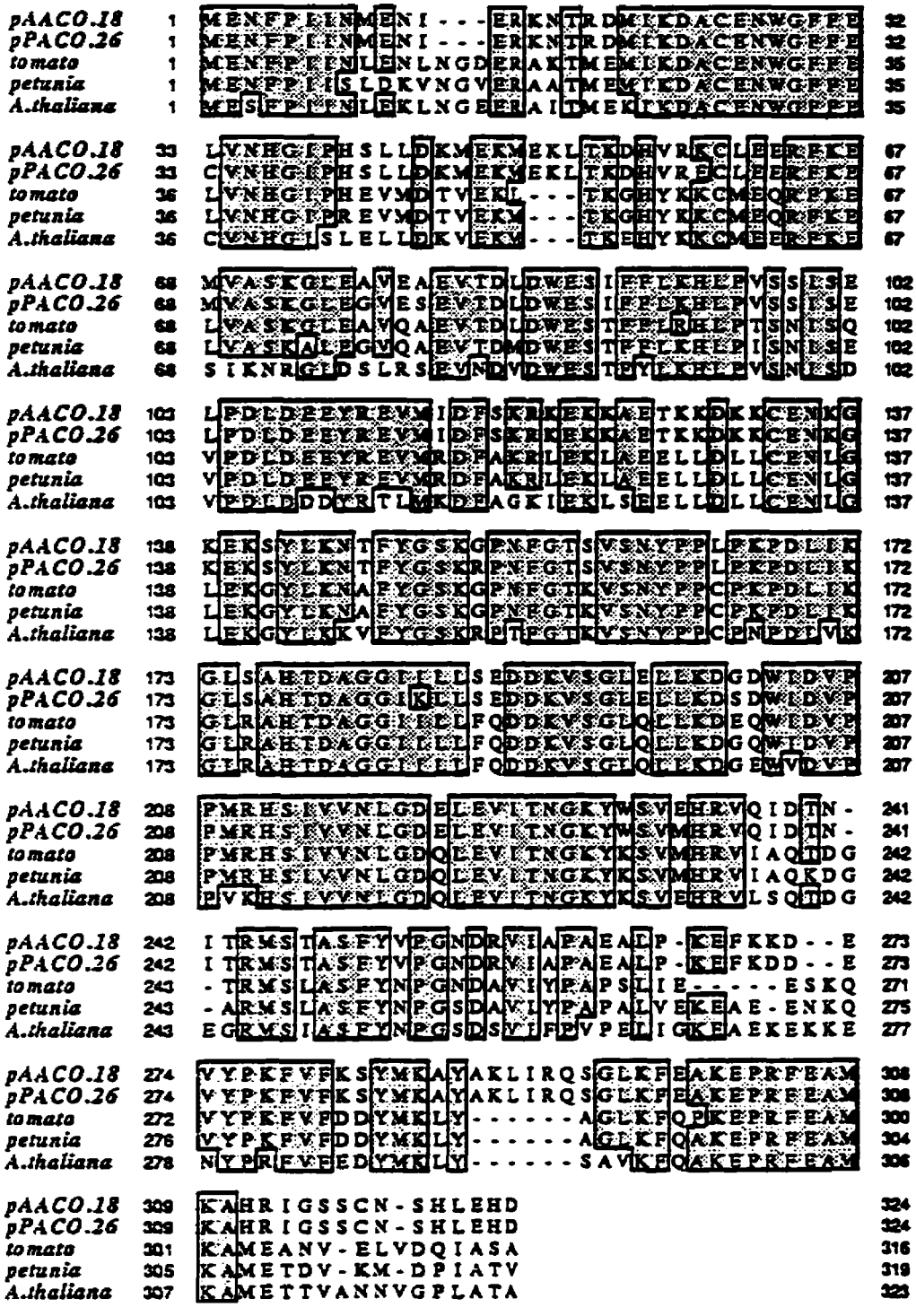
 847 AAA TTC GTC TTT AAG AGC TAC ATG AAG GCT TAT GCT AAG TTG ATT CGT CAG AGT GGC CTC 906
 274 K   F   V   F   K   S   Y   M   K   A   Y   A   K   L   I   R   Q   S   G   L  293

 907 AAG TTC GAA GCG AAA GAA CCG CGG TTT GAG GCT ATG AAG GCA CAC CGA ATT GGG TCC AGT 966
 294 K   F   E   A   K   E   P   R   F   E   A   M   K   A   H   R   I   G   S   S  313

 967 TGC AAC AGC CAC TTA GAA CAC GAC TGA ctgactagaacgtattacggctctagctcttgatcggactaggc 1037
 314 C   N   S   H   L   E   H   D   *                                               322

1038 ttagataataacgacgagagcgaggcagagaccgagacaaaaaaaaaaaaaaaaaaaaaaaa                  1098
```

Fig. 3.3. Amino acid sequence alignments of ACC oxidase from alpine ecotype (pAACO-18), prairie ecotype (pPACO-26) of *S. longipes*, tomato, petunia and *Arabidopsis thaliana*. Identical amino acid residues are presented in the shaded boxes.


pAACO.18 1 MENFPLINMENI---ERKNTRDMIKDACENWGFFE 32
pPACO.26 1 MENFPLINMENI---ERKNTRDMIKDACENWGFFE 32
tomato 1 MENFPFINLENLNGDERAKTMEMIKDACENWGFFE 35
petunia 1 MENFPFISLDKVNGVERAATMEMIKDACENWGFFE 35
A.thaliana 1 MESFPFINLEKLNGEERAITMEKIKDACENWGFFE 35
pAACO.18 33 LVNHGIPHSLLDKMEKMEKLTKDHVRKCLEERFKE 67
pPACO.26 33 CVNHGIPHSLLDKMEKMEKLTKDHVRECLEERFKE 67
tomato 36 LVNHGIPHEVMDTVEKL---TKGHYKKCMEQRFKE 67
petunia 36 LVNHGIPPREVMDTVEKM---TKGHYKKCMEQRFKE 67
A.thaliana 36 CVNHGISLELLDKVEKM---TKEHYKKCMEERFKE 67
pAACO.18 68 MVASKGLEAVQAEVTDLDWESIFFLKHFPVSSISSE 102
pPACO.26 68 MVASKGLEGVESEVTDLDWESIFFLKHFPVSSISSE 102
tomato 68 LVASKGLEAVQAEVTDLDWESTFFLRHFPTSNISQ 102
petunia 68 LVASKALEGVQAEVTDMDWESTFFLKHFPISNLSE 102
A.thaliana 68 SIKNRGLDSLRSEVNDVDWESTFYLKHFPVSNLSD 102
pAACO.18 103 LPDLDEEYREVMQIDFSKRKEKKAETKKDKKCENKG 137
pPACO.26 103 LPDLDEEYREVMQIDFSKRKEKKAETKKDKKCENKG 137
tomato 103 VPDLDEEYREVMRDFAKRLEKLAEELLDLLCENLG 137
petunia 103 VPDLDEEYREVMRDFAKRLEKLAEELLDLLCENLG 137
A.thaliana 103 VPDLDDDYRTLMKDFAGKIEKLSEELLDLLCENLG 137
pAACO.18 138 KEKSYLKNTFYGSKGPNFGTSVSNYPPLPKPDLIK 172
pPACO.26 138 KEKSYLKNTFYGSKRPNFGTSVSNYPPLPKPDLIK 172
tomato 138 LEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIK 172
petunia 138 LEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIK 172
A.thaliana 138 LEKGYLKKVFYGSKRPTPGTKVSNYPPCPNPDLVK 172
pAACO.18 173 GLSAHTDAGGLTLLLSEDDKEVSGLELLKDGDWIDVP 207
pPACO.26 173 GLSAHTDAGGIKLLLSEDDKVSGLELLKDSDWLDVP 207
tomato 173 GLRAHTDAGGITLLFQDDKVSGLQLLKDEQWLDVP 207
petunia 173 GLRAHTDAGGITLLFQDDKVSGLQLLKDGQWFDVP 207
A.thaliana 173 GIRAHTDAGGITLLFQDDKVSGLQLLKDGEWVDVP 207
pAACO.18 208 PMRHSIVVNLGDELEVITNGKYWSVEHRVQIDTN- 241
pPACO.26 208 PMRHSIVVNLGDELEVITNGKYWSVMHRVQIDTN- 241
tomato 208 PMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQTDG 242
petunia 208 PMRHSLVVNLGDQLEVLTNGKYKSVMHRVIAQKDG 242
A.thaliana 208 PVKHSIVVNLGDQLEVITNGKYKSVEHRVLSQTDG 242
pAACO.18 242 ITRMSLASFYVPGNDRVIAPAEALP-KEFKKD--E 273
pPACO.26 242 ITRMSLASFYVPGNDRVIAPAEALP-KEFKDD--E 273
tomato 243 -TRMSLASFYNPGNDAVIYPAPSLIE------ESKQ 271
petunia 243 -ARMSLASFYNPGSDAVLYPAPALVEKEAE-ENKQ 275
A.thaliana 243 EGRMSIASFYNPGSDSVIFPVPELIGKEAEKEKKE 277
pAACO.18 274 VYPKFVFKSYMKAYAKLIRQSGLKFEAKEPRFEAM 308
pPACO.26 274 VYPKFVFKSYMKAYAKLIRQSGLKFEAKEPRFEAM 308
tomato 272 VYPKFVFDDYMKLY------AGLKFQPKEPRFEAM 300
petunia 276 VYPKFVFDDYMKLY------AGLKFQAKEPRFEAM 304
A.thaliana 278 NYPRFVFEDYMKLY------SAVKFQAKEPRFEAM 306
pAACO.18 309 KAHRIGSSCN-SHLEHD 324
pPACO.26 309 KAHRIGSSCN-SHLEHD 324
tomato 301 KAMEANV-ELVDQIASA 316
petunia 305 KAMETDV-KM-DPIATV 319
A.thaliana 307 KAMETTVANNVGPLATA 323

pPACO-26 is 1095 bp long (Fig. 3.4) and consisted of 45 bp 5' UTR, 947 bp coding region and 75 bp 3' UTR. Like pAACO-18, pPACO-26 (Fig. 3.5. Genbank accession no. AF011398) also exhibited a high degree of homology (80 to 95%) to ACC oxidase from other species. The nucleotide homology between pAACO-18 and pPACO-26 is 90.4% and only 9 of 321 amino acid residues (2.89%) were dissimilar indicating that ACC oxidase is highly conserved between alpine and prairie ecotypes (Fig. 3.3).

3.3.5. Southern analysis

In an effort to determine the number of ACC oxidase gene copies in *S. longipes*, restriction endonuclease digested genomic DNA was hybridized with SlACO-1 (Fig. 3.5). When the alpine genome was digested with Eco RI, a ~3.0 Kb band hybridized to SlACO-1 (Fig. 3.5). In Cla I digestion, two hybridzing bands (~0.75 Kb and ~4.5 Kb) were observed (Fig. 3.5). The sizes of the two hybridizing bands (~1.8 Kb and ~0.8 Kb) in Eco RI/Cla I double digestion add to a total of ~2.6 Kb. These results suggest that ACC oxidase may be encoded by a single gene.

3.3.6. Biochemical characterization of ACC oxidase

ACC oxidase was extracted from stem and leaf tissues of alpine and prairie ecotypes under conditions previously shown to recover the activity *in vitro* in sunflower (Finlayson and Reid, 1993). The crude extracts of both ecotypes exhibited soluble ACC oxidase activity. The activity was proportional to the amount of added extract and inactivated by boiling. The

Fig. 3.4. Nucleotide sequences of pPACO-26, an ACC oxidase cDNA clone isolated from prairie ecotype. Upper case letters represent the coding region. The amino acid sequence is given below the corresponding nucleotide triplets. Lower case letters represent the 5' and 3' untranslated regions.

```
   1 gacgattaccacttgatccgcatgccgactaggcgttacgatata ATG GAA AAC TTT CCA ATA ATC AAC    69
   1                                               M   E   N   F   P   I   I   N     8

  70 ATG GAA AAT ATC GAG CGG AAG AAC ACG CGT GAC ATG ATA AAA GAC GCG TGT GAA AAC TGG  129
   9 M   E   N   I   E   R   K   N   T   R   D   M   I   K   D   A   C   E   N   W    28

 130 GGT TTT TTT GAA TGT GTT AAC CAC GGT ATA CCT CAC TCT TTA CTA GAC AAA ATG GAG AAA  189
  29 G   F   F   E   C   V   N   H   G   I   P   H   S   L   L   D   K   M   E   K    48

 190 ATG GAG AAA CTC ACA AAA GAC CAT GTA AGG GAA TGT TTA GAA GAG AGG TTT AAA GAA ATG  249
  49 M   E   K   L   T   K   D   H   V   R   E   C   L   E   E   R   F   K   E   M    68

 250 GTA GCA AGT AAA GGA CTA GAA GGT GTA GAA AGC GAA GTA ACA GAC CTA GAC TGG GAA AGC  309
  69 V   A   S   K   G   L   E   G   V   E   S   E   V   T   D   L   D   W   E   S    88

 310 ATA TTT TTT CTA AAG CAC CTA CCA GTA TCT TCA ATA TCG GAA CTA CCA GAT CTA GAC GAG  369
  89 I   F   F   L   K   H   L   P   V   S   S   I   S   E   L   P   D   L   D   E   108

 370 GAA TAT CGA GAG GTA ATG ATA GAC TTT TCA AAG CGA AAA GAA AAG AAA GCA GAA ACT AAA  429
 109 E   Y   R   E   V   M   I   D   F   S   K   R   K   E   K   K   A   E   T   K   128

 430 AAG GAC AAA AAA TGT GAA AAC AAA GGA AAG GAA AAG AGC TAT CTA AAA AAC ACA TTT TAT  489
 129 K   D   K   K   C   E   N   K   G   K   E   K   S   Y   L   K   N   T   F   Y   148

 490 GGA AGC AAA AGG CCT AAC TTC GGA ACA AGC GTT AGC AAC TAC CCA CCG CTA CCA AAG CCT  549
 149 G   S   K   R   P   N   F   G   T   S   V   S   N   Y   P   P   L   P   K   P   168

 550 GAC CTC ATT AAA GGC CTG TCA GCA CAT ACC GAT GCC GGA GGC ATC AAG TTA CTC AGT GAA  609
 169 D   L   I   K   G   L   S   A   H   T   D   A   G   G   I   K   L   L   S   E   188

 610 GAC GAC AAG GTC AGT GGA TTG GAA TTA TTA AAG GAC AGT GAT TGG ATA GAC GTC CCG CCG  669
 189 D   D   K   V   S   G   L   E   L   L   K   D   S   D   W   I   D   V   P   P   208

 670 ATG AGA CAT TCA ATA GTG GTC AAT CTG GGA GAT GAA TTA GAA GTC ATA ACA AAC GGC AAG  729
 209 M   R   H   S   I   V   V   N   L   G   D   E   L   E   V   I   T   N   G   K   228

 730 TAC TGG TCA GTC ATG CAT CGA GTA CAG ATT GAT ACT AAC ATT ACG AGA ATG AGT ACG GCA  789
 229 Y   W   S   V   M   H   R   V   Q   I   D   T   N   I   T   R   M   S   T   A   248

 790 AGT TTC TAT GTT CCG GGA AAC GAT CGT GTC ATT GCT CCG GCT GAA GCT TTG CCT AAA GAG  849
 249 S   F   Y   V   P   G   N   D   R   V   I   A   P   A   E   A   L   P   K   E   268

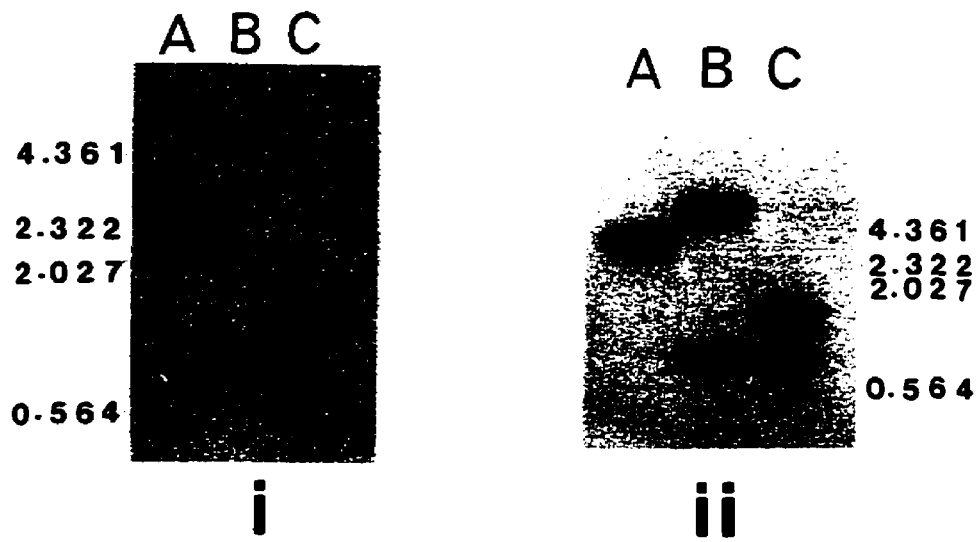
 850 TTT AAG GAT GAC GAG GTC TAT CCA AAA TTC GTC TTT AAG AGC TAC ATG AAG GCT TAT GCT  909
 269 F   K   D   D   E   V   Y   P   K   F   V   F   K   S   Y   M   K   A   Y   A   288

 910 AAG CTA ATT CGT CAG AGT GGC TTG AAG TTC GAA GCG AAA GAA CCG CGG TTT GAG GCT ATG  969
 289 K   L   I   R   Q   S   G   L   K   F   E   A   K   E   P   R   F   E   A   M   308

 970 AAG GCA CAC CGA ATT GGG AGT AGT TGC AAC AGC CAC TTA GAA CAT GAT TAA cgatatccttag 1032
 309 K   A   H   R   I   G   S   S   C   N   S   H   L   E   H   D   *               325

1033 gtcggttggcaataacggagtcatggcagtcattacgagactgagttaaaaaaaaaaaaaaaa                1095
```

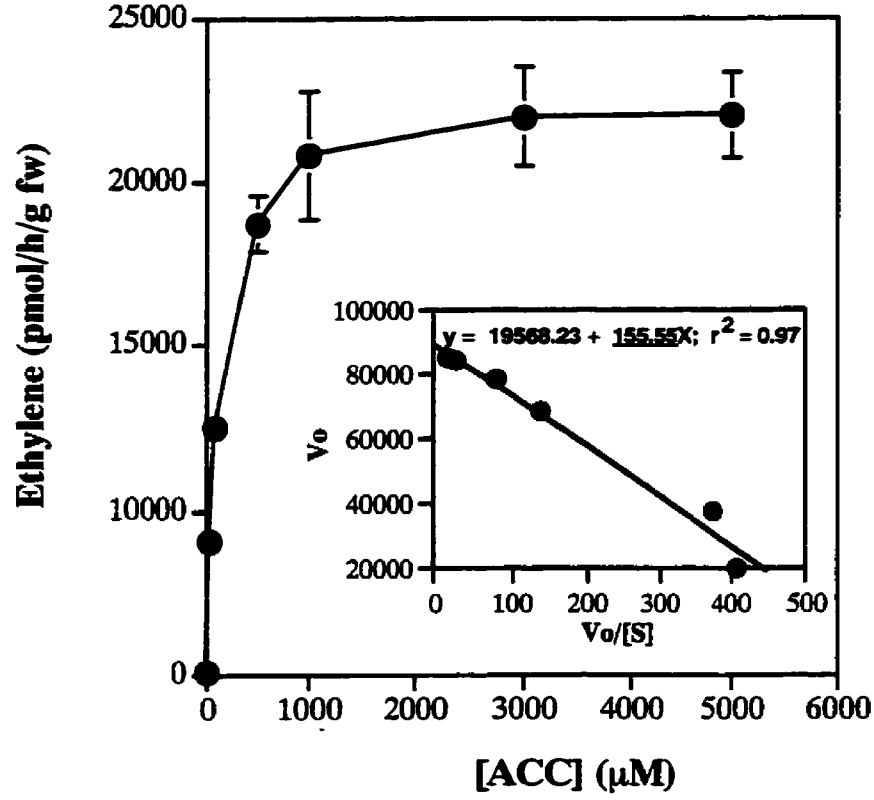
Fig. 3.5. Southern blot analysis of ACC oxidase in alpine ecotype of *S. longipes*. Ten μg of genomic DNA was digested with Eco RI (a), Cla I (b), and Eco RI/Cla I (c), separated on 1.2% agarose gel, transferred to a nylon membrane and hybridized with SlACO.1.


A B C
4.361
2.322
2.027
0.564
i
A B C
4.361
2.322
2.027
0.564
ii

effect of ACC concentration on ethylene production by the crude extracts of alpine and prairie ecotypes is shown in Fig. 3.6. In both ecotypes, the enzyme exhibited an apparent Km of ~155 μM for ACC. Fig. 3.7A shows the pH dependence of ACC oxidase activity. While the enzyme was active in a pH range of 6.0 to 8.0, its activity was optimal at pH 6.6. ACC oxidase activity was strongly promoted by the presence of ascorbate (Fig. 3.7B) and Fe^{2+}(Fig. 3.8A). ACC oxidase activity was inhibited by the presence of divalent cations such as Cu^{2+} (Fig. 3.8B) and Co^{2+} (Fig. 3.8C). The activity of the enzyme was also inhibited by the presence of AIBA (Fig. 3.9A). A Ki of 160 μM was observed for AIBA (Fig. 3.9B). ACC oxidase activity was stimulated by the presence of CO_2. The Vmax of ACC oxidase increased under high CO_2 and saturated after 5% CO_2 (Fig. 3.10A). The Km for ACC also increased from 155 μM under ambient CO_2 to 404 μM in the presence of 5% CO_2 (Fig. 3.10B).

Ammonium sulfate fractionation (50 to 70%) of crude extract recovered the activity of ACC oxidase (Fig. 3.11) the kinetics of which did not show any difference from that of the crude extract described above. The observed Km for ACC in crude extracts of the stem and leaf tissues harvested on day 8 (early vegetative stage) was different from those tissues harvested on day 24 (flowering stage) under LDW in alpine and prairie ecotypes (Table. 3.1). Such differences however were not found when the eluates of anion exchange columns were assayed (Table. 3.1).

Fig. 3.6. Saturation kinetics of ACC oxidase extracts from alpine (A) and prairie (B) ecotypes. Each point represents the mean of seven observed values, and is representative of at least three separate assays. The enzyme activity was assayed at ambient CO_2 concentration. Vertical bars indicate ± S.E. where larger than symbols. *Inset*: Eadie-Hofstee plot showing the apparent Km value (underlined) of enzyme for ACC (μM).


A
Ethylene (pmol/h/g fw)
25000
20000
15000
10000
0
0
1000
2000
3000
4000
5000
6000
[ACC] (µM)
y = 19568.23 + 155.55X; r2 = 0.97
Vo
100000
80000
60000
40000
20000
0
100
200
300
400
500
Vo/[S]

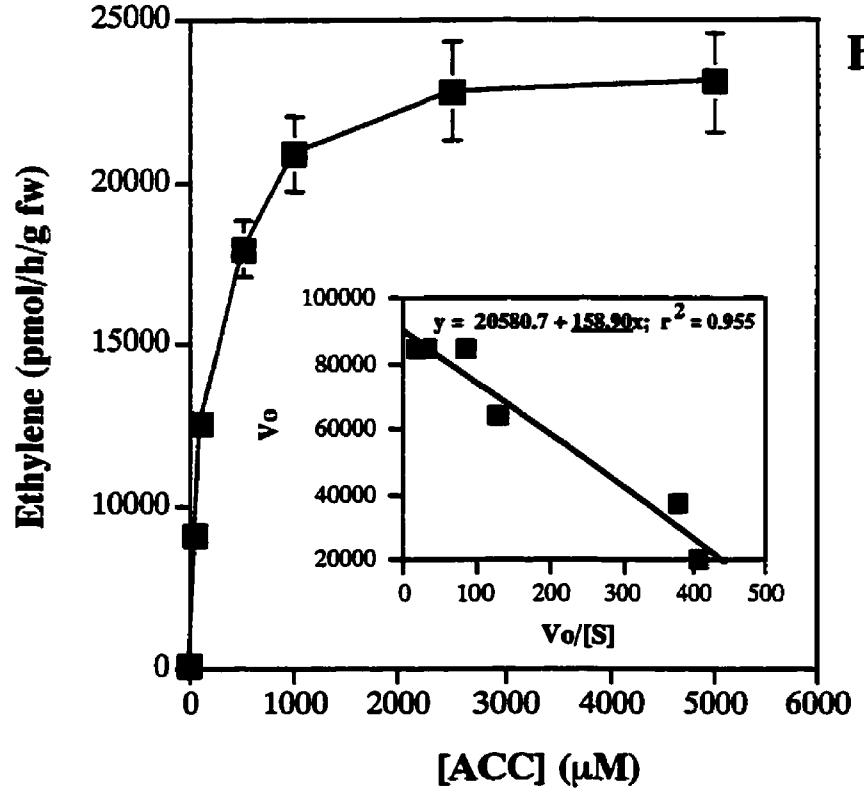
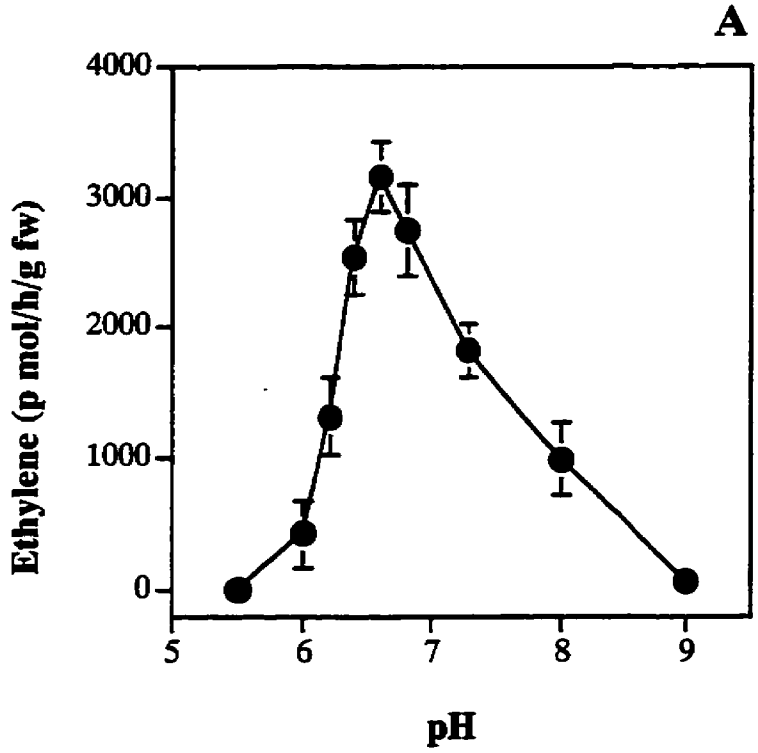

B
Ethylene (pmol/h/g fw)
25000
20000
15000
10000
0
0
1000
2000
3000
4000
5000
6000
[ACC] (µM)
y = 20580.7 + 158.90x; r2 = 0.955
Vo
100000
80000
60000
40000
20000
0
100
200
300
400
500
Vo/[S]

Fig. 3.7 (A and B). (A) pH dependence of ACC oxidase activity. ACC oxidase activity was assayed at differing pH values at ambient CO_2 concentration in the presence of 3 mM ACC. Each point represents the mean value of three or more assays. (B) Effect of ascorbate concentration on ACC oxidase activity. The enzyme was assayed at ambient CO_2 concentration in the presence of saturating concentration (3 mM) of ACC. Each point represents the mean of six observed values. Vertical bars indicate ± S.E. where larger than symbols.


A
4000
3000
2000
1000
0
Ethylene (p mol/h/g fw)
5
6
7
8
9
pH

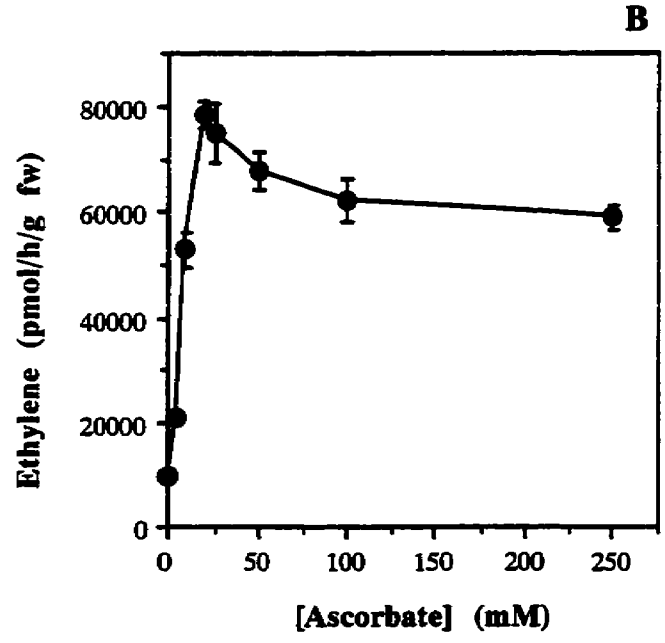
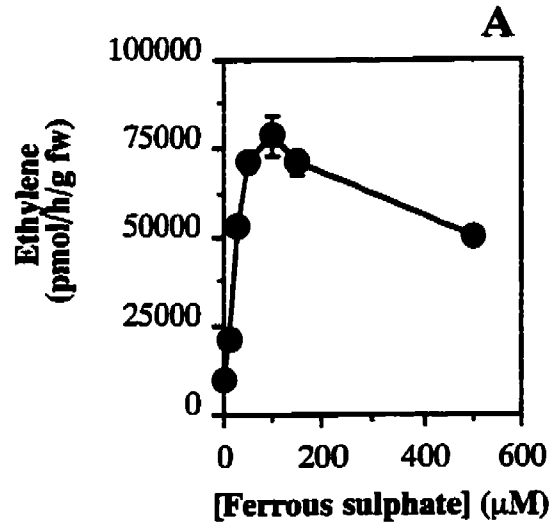
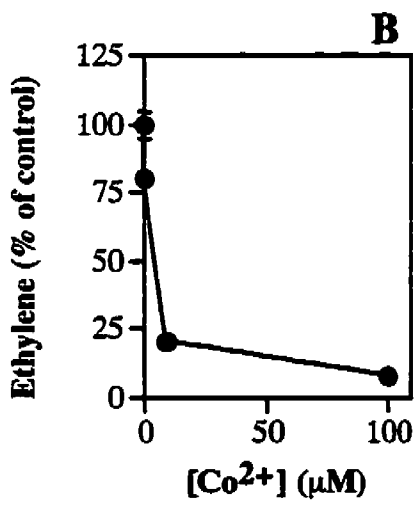

B
80000
60000
40000
20000
0
Ethylene (pmol/h/g fw)
0
50
100
150
200
250
[Ascorbate] (mM)

Fig. 3.8. *Upper panel*: Fe(II) dependence of ACC oxidase activity (A). Fe(II) was added in the assay buffer in the form of $FeSO_4$ and assayed for enzymatic activity at ambient CO_2 concentration in the presence of saturating ACC concentration (3 mM). *Lower panels*: Effect of divalent cations on ACC oxidase activity. Cobalt (B) and copper (C) metals were added as sulfate salts, and the enzyme was assayed at ambient CO_2 concentration in the presence of 3 mM ACC. Vertical bars indicate ± S.E. where larger than symbols.


A
100000
75000
50000
25000
0
Ethylene (pmol/h/g fw)
0
200
400
600
[Ferrous sulphate] (μM)


B
125
100
75
50
25
0
Ethylene (% of control)
0
50
100
[Co2+] (μM)

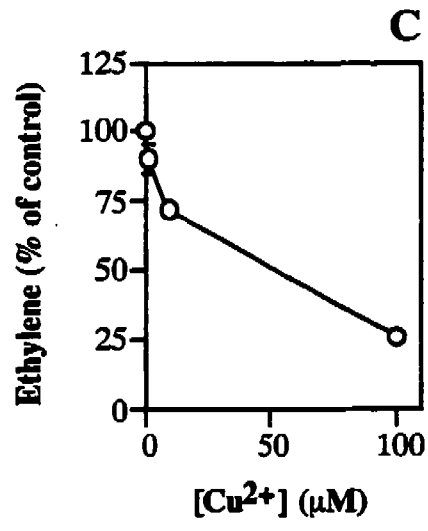
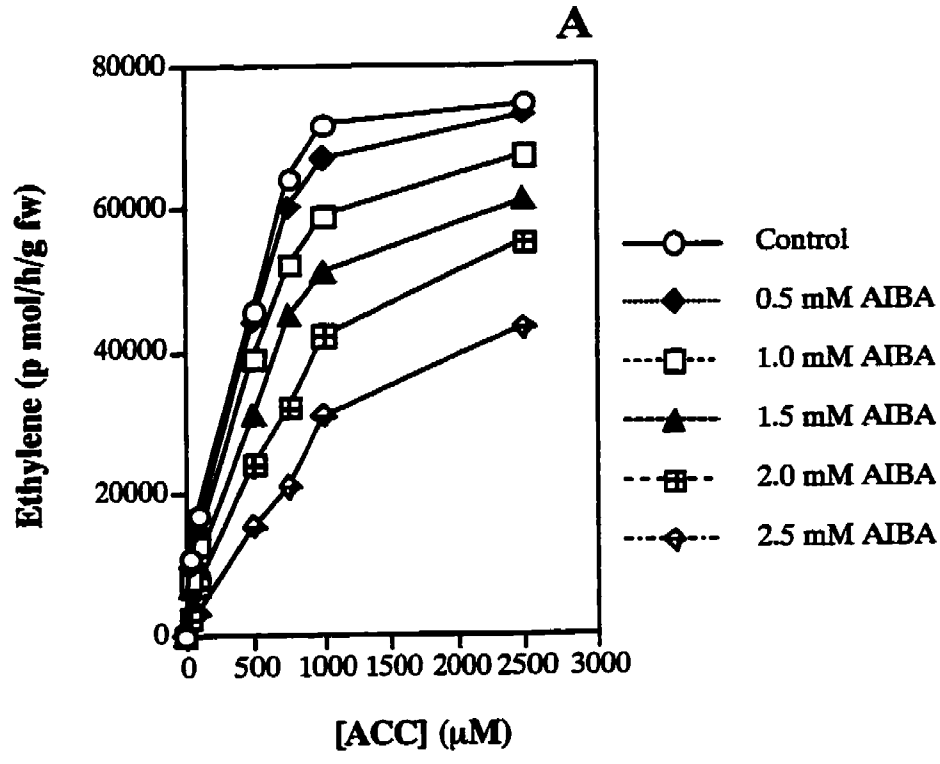

C
125
100
75
50
25
0
Ethylene (% of control)
0
50
100
[Cu2+] (μM)

Fig. 3.9. Competitive inhibition of ACC oxidase activity by AIBA (A). Each point represents the mean value of three or more assays. *Lower panel*: Eadie-Hofstee plot indicating apparent Ki value (underlined) for AIBA.


A
80000
60000
40000
20000
0
Ethylene (p mol/h/g fw)
0
500
1000
1500
2000
2500
3000
[ACC] (μM)
Control
0.5 mM AIBA
1.0 mM AIBA
1.5 mM AIBA
2.0 mM AIBA
2.5 mM AIBA

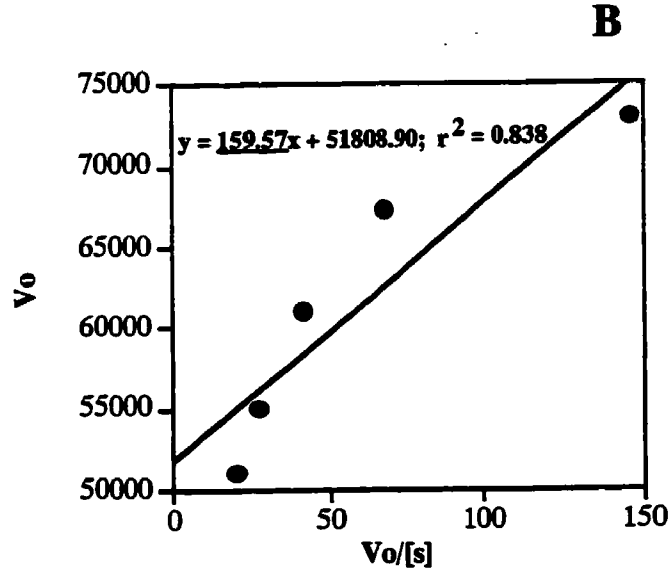
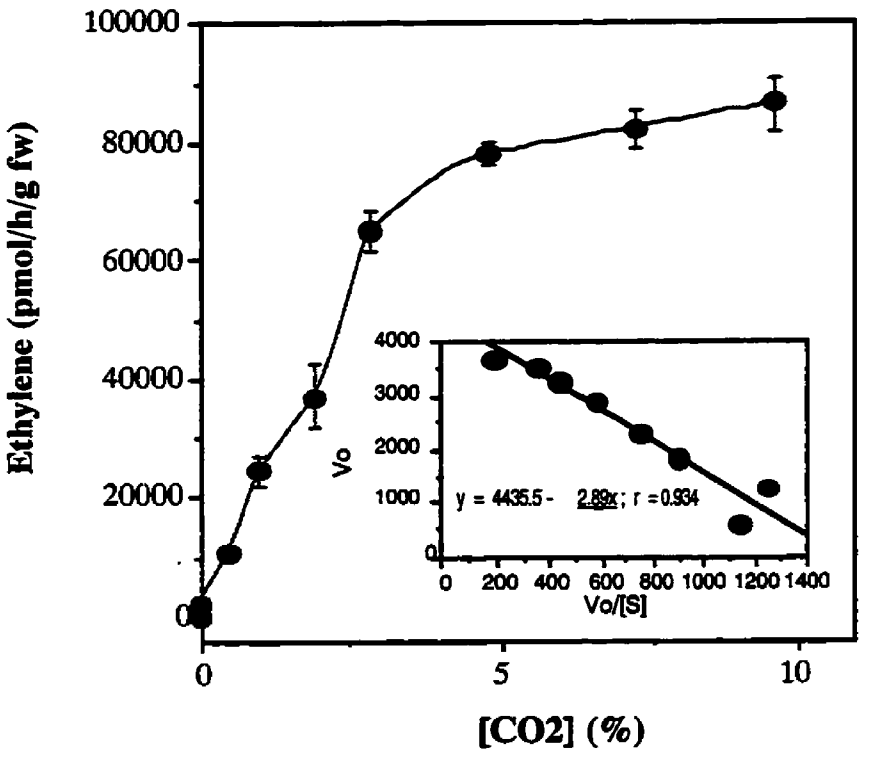

B
75000
70000
65000
60000
55000
50000
Vo
y = 159.57x + 51808.90; r 2 = 0.838
0
50
100
150
Vo/[s]

Fig. 3.10 (A and B). (A) Effect of CO_2 on ACC oxidase activity. *Inset*: Eadie-Hofstee plot indicating the Km value (underlined) for CO_2. (B). Saturation kinetics of ACC oxidase at 5% CO_2. *Inset*: Eadie-Hofstee plot indicating the increased Km value (underlined) of the enzyme for ACC.

A


Ethylene (pmol/h/g fw)
100000
80000
60000
40000
20000
0
0
5
10
[CO2] (%)
Vo
4000
3000
2000
1000
0
y = 4435.5 - 2.89x ; r = 0.934
0 200 400 600 800 1000 1200 1400
Vo/[S]

B

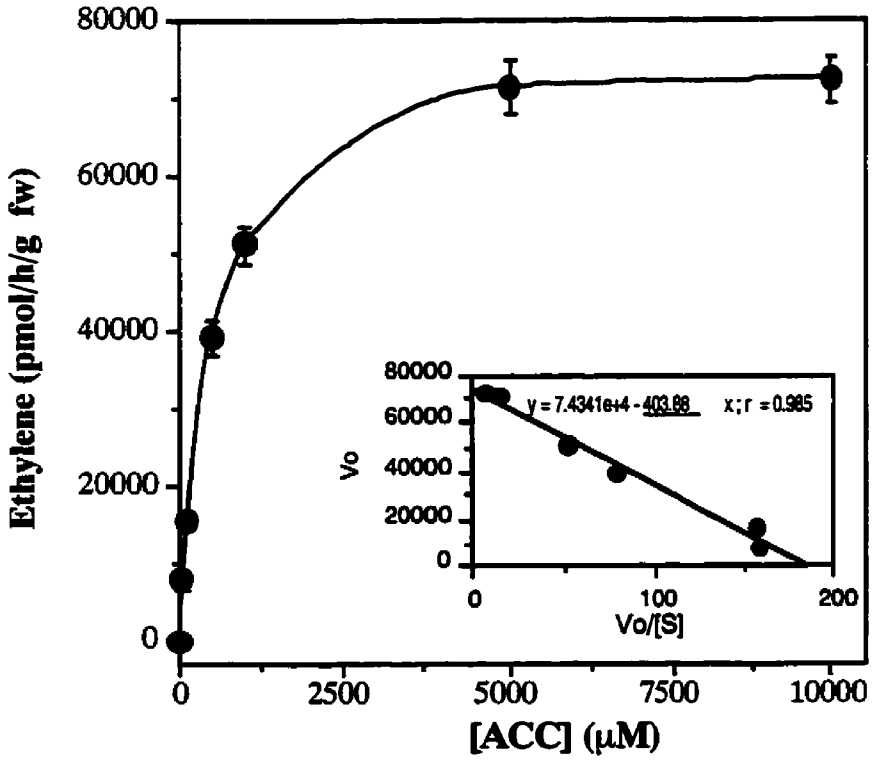

Ethylene (pmol/h/g fw)
80000
60000
40000
20000
0
0
2500
5000
7500
10000
[ACC] (µM)
Vo
80000
60000
40000
20000
0
y = 7.4341e+4 - 403.88 x ; r = 0.985
0
100
200
Vo/[S]

Fig. 3.11. Elution profile of ACC oxidase activity in various fractions of ammonium sulfate precipitation. Crude extracts of ACC oxidase from alpine plants were precipitated with different concentrations of ammonium sulfate on ice and 250 µl of each fraction was assayed for enzymatic activity at ambient CO_2 concentration in the presence of 3 mM ACC.

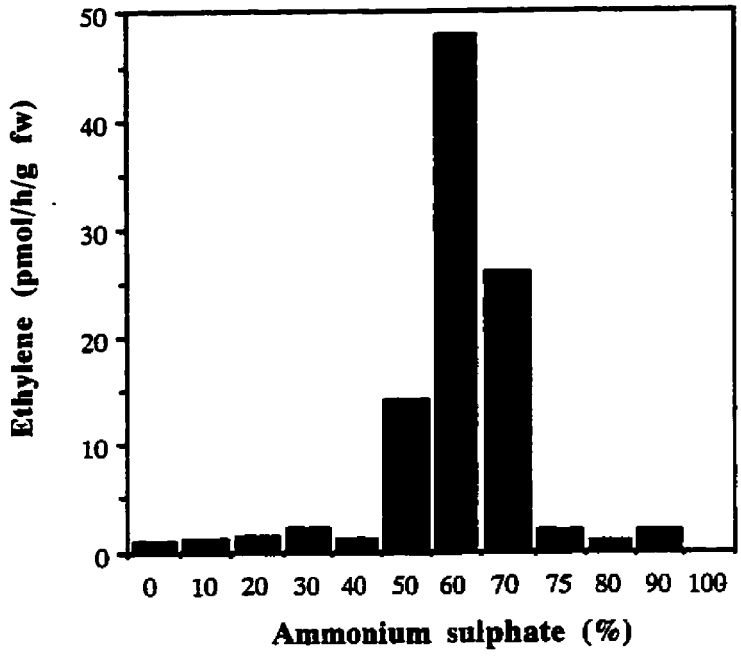

Ethylene (pmol/h/g fw)
50
40
30
20
10
0
0 10 20 30 40 50 60 70 75 80 90 100
Ammonium sulphate (%)

Table. 3.1. Differences in Km values ± S.E. of ACC oxidase crude extracts and anion exchange eluates for ACC at vegetative (day 8; A) and flowering (day 24; B) stages of alpine and prairie ecotypes. For assay of enzyme activity, 220 µl of the fractions were added to the standard assay mixture and measured at ambient CO_2 concentration in the presence of 3 mM ACC.

A

Type of preparation	Alpine	Prairie
Crude extract	151 ± 20	160 ± 19
Anion exchange eluate	149 ± 8	153 ± 19

B

Type of preparation	Alpine	Prairie
Crude extract	405 ± 21	378 ± 36
Anion exchange eluate	152 ± 12	157 ± 9

3.4. Discussion

The history of identification of ACC oxidase in plants was a typical case of 'reverse biochemistry'. While studying the differential gene expression in tomato fruits, pTOM 13 was cloned and later shown to have the ability to convert ACC to ethylene (Hamilton *et al.*, 1991). Since then ACC oxidase has been cloned in a large number of plant species. Amino acid sequence analysis of ACC oxidase from various plants shows very limited divergence (Zarembinski and Theologis, 1994). The two cDNA clones isolated in the present study exhibit high homology to known ACC oxidases of other plant species such as tomato, petunia and *A. thaliana*.

Although ACC oxidase has been shown to be encoded by a single gene in many plant species, recent studies in mungbean (Kim and Yang, 1994) tomato (Barry *et al.*, 1996) show that it is encoded by a small gene family comprised of two and three copies respectively. The three ACC oxidase members in tomato exhibit 88% sequence similarity (Barry *et al.*, 1996). All the three genes are differentially expressed in various tissues of tomato. Southern analyses done in the present study however suggest that ACC oxidase may be encoded by a single gene in *S. longipes*. Alpine and prairie ecotypes did not exhibit any polymorphism although previous studies have shown a substantial genetic divergence between these two ecotypes (Cai *et al.*, 1990; Emery *et al.*, 1994a). Since Southern analyses do not always clearly reflect the number of gene copies especially if there are any recent gene duplication, one should not ignore the possibility of existence of ACC oxidase multigene family in *S. longipes*.

The oxidation of ACC to ethylene was initially thought to be mediated by various enzymes such as peroxidase, IAA oxidase and lipoxygenase. Although these enzymes could convert ACC to ethylene, their Km for ACC were higher than the *in vivo* ACC levels (Yang and Hoffman, 1984). Ververidis and John (1991) were the first to demonstrate authentic ACC oxidase activity in melon fruit tissues which exhibited an apparent Km of 85 μM for ACC. Results from this study show that crude extracts of ACC oxidase of *S. longipes* has a Km of 155 μM. This is within the range (60 to 180 μM) observed in various plant species (reviewed by Abeles *et al.*, 1992).

The inhibition of enzyme activity by AIBA and divalent cations such as Cu^{2+} and Co^{2+} confirmed the authenticity of ACC oxidase. Earlier works had shown that AIBA, being a structural analog of ACC, inhibits ACC oxidase activity by competing with ACC (McGarvey and Christoffersen, 1992). The requirement of Fe^{2+} suggests that the enzyme might use iron as an electron acceptor during the oxidation of ACC (Smith *et al.*, 1992). Ascorbate has been proposed to protect the iron from fortuitous oxidation of iron (Smith *et al.*, 1992). Since many non-heme-iron containing enzymes typically incorporate one or both atoms of dioxygen into their substrates (Ingraham and Meyer, 1985), McGarvey and Christoffersen (1992) suggested that ascorbate may also be directly involved in the reaction by contributing electrons to the dioxygen during oxidation of ACC. Experiments with purified enzyme should be able to determine the validity of these schemes.

The dependence of ACC oxidase activity on the presence of Fe^{2+} and the abolition of ACC oxidase activity by the equimolar concentrations of Cu^{2+} and Co^{2+} with Fe^{2+} suggest that these metals may physically associate with the enzyme. The interference from such divalent cationic interactions could have been the cause for the differences in observed Km values in crude extracts and anion exchange eluates in the present study. Alternatively non-specific transfer of electrons from Fe^{2+} and/or ascorbate by other enzymes or non-protein oxidants could also have reduced the affinity of the enzyme for ACC in the crude extracts.

In summary, cDNAs encoding ACC oxidase in alpine and prairie ecotypes were isolated. They exhibit a high degree of similarity at the nucleotide (90.4%) and amino acid levels (97.19%) between each other. Southern analyses suggest that ACC oxidase may be encoded by a single gene copy in both ecotypes. Biochemical studies do not show any substantial difference in enzyme kinetics between the two ecotypes. The enzyme has an apparent Km of 155 μM for ACC. ACC oxidase activity depends on the presence of Fe^{2+} and ascorbate. The authenticity of ACC oxidase was confirmed by the inhibition of enzymatic activity by the Cu^{2+}, Co^{2+} and AIBA.

CHAPTER FOUR

Temporal Regulation of Activity and mRNA Accumulation of 1-Aminocyclopropane-1-carboxylic acid Oxidase in *S. longipes*

4.1. Introduction

One of the important features of ethylene dynamics in *S. longipes* is its ability to produce ethylene in a rhythmic fashion. Emery *et al.* (1994b and 1997) showed that the prairie ecotype exhibits a strong and reproducible ethylene rhythm, whereas the rhythm in alpine ecotype is often less pronounced. Rhythmic oscillations have been observed in several biological processes in plants (Piechulla, 1993). In general, there are two types of rhythm namely 'diurnal' and 'circadian' rhythms. While diurnal rhythms are governed strictly by external changes in light period, circadian rhythms are governed endogenously (hence often refered to as endogenous rhythms). So circadian rhythms, unlike diurnal rhythms, are persistent even under constant dark or constant light conditions (Sweeney, 1987). Another important difference is in their time interval (period). Typically, most circadian rhythms exhibit a period of ca. 24 h. whereas the period of diurnal rhythms can be changed and dependent on the length of day light (Sweeney, 1987). Even though circadian rhythm is prevalent in a wide range of organisms including several prokaryotes, the cellular machinery responsible for such rhythms, commonly known as 'biological clock', still remains largely unknown (Reppert and Weaver, 1997).

Rhythms in ethylene evolution has been reported previously in other plant systems including cotton (Lipe and Morgan, 1973; Rikin *et al.*, 1984), tomato (El-Beltagy *et al.*, 1976), bean (Morgan *et al.*, 1990), and rice (Michiyama and Saka, 1988). Ievenish and Kreicbergs (1992) reported endogenous rhythmicity of ethylene production in growing intact seedlings of barley, wheat and rye. Despite many reports on the occurrence of ethylene rhythm, there was no information available on whether there are any changes in the levels of precursor(s) and/or enzyme(s) involved in ethylene biosynthetic pathway. The objective of this work is to study the temporal regulation of ACC oxidase in alpine and prairie ecotypes.

4.2. Materials and methods

4.2.1. Plant material and growth conditions

The plants were transferred from SDC to one of the following treatments: (i) alternate day and night cycle (LD; 16h photoperiod) with 22° C day and 18° C night temperature cycle or constant 18° C, (ii) continuous light (LL) with 22° C (16h) and 18° C (8h) temperature cycle or constant 18° C, (iii) continuous dark (DD) with 22° C (16h) and 18° C (8h) temperature cycle, (iv) 15 min of red light pulse (R) in continuous dark with 22° C (16h) and 18° C (8h) temperature cycle, and (v) 15 min of blue light pulse (B) in continuous dark with 22° C (16h) and 18° C (8h) temperature cycle. Red light had a λmax of 660 nm, and blue light had a λmax of 460 nm at 23 to 27 μmol m^{-2} s^{-1}. Stem and leaf tissues were harvested at approximately every 4 h intervals and frozen in liquid nitrogen.

4.2.2. Northern analysis

The control 18S RNA probe was made by labeling a 1.6 Kb Xba I-Eco RI fragment of pGmr-1 (from Dr. E.A. Zimmer, National Museum of Natural History, Smithsonian Institution, Suitland, Md., USA). Probe for ACC oxidase was prepared by labeling SlACO-1. After hybridization, membranes were washed twice with 2xSSPE plus 0.1% SDS at RT, twice with 1xSSPE plus 0.1% SDS at 65° C for 30 min and twice with 1xSSPE plus 0.1% SDS at 65° C for 30 min. Quantification of the resulting autoradiographs was done using LKB 2222-020 Ultrascan XL Laser densitometer (LKB Produkter AB, Bromma, Sweden).

4.2.3. ACC oxidase extraction and assay

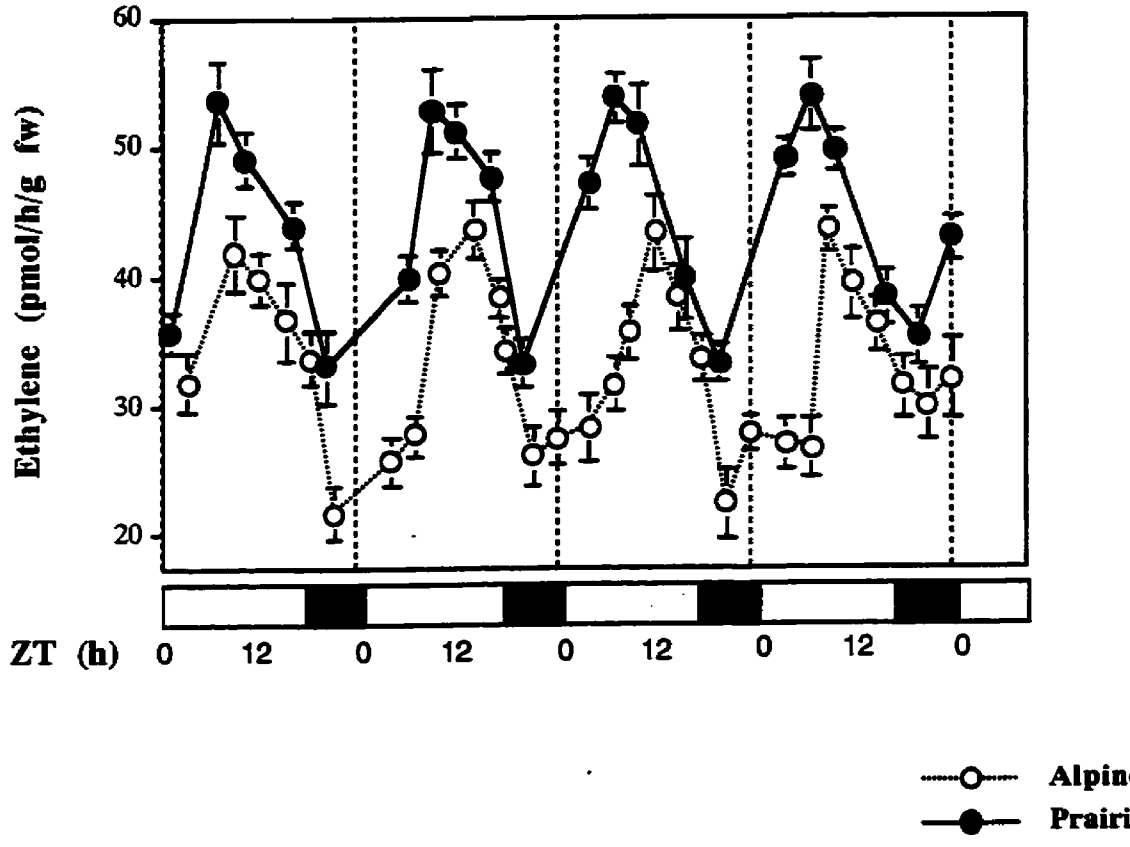
The enzyme was extracted as described in section 2.2.2.1. Fifty µl of enzyme extract was added to assay tubes and incubated for 30 min at 37° C with shaking. The ethylene content of 1- ml head space gas samples from the assay tubes was analyzed using a Photovac 10S Plus gas chromatograph.

4.3. Results

4.3.1. Circadian regulation of ACC oxidase activity *in vitro*

In plants that were grown under LD, there were daily fluctuations in the maximum ACC oxidase activity *in vitro* in both ecotypes (Fig. 4.1). Although there were differences in the amplitude of the rhythm observed in alpine and prairie ecotypes (Fig. 4.1), the characteristics of rhythm was

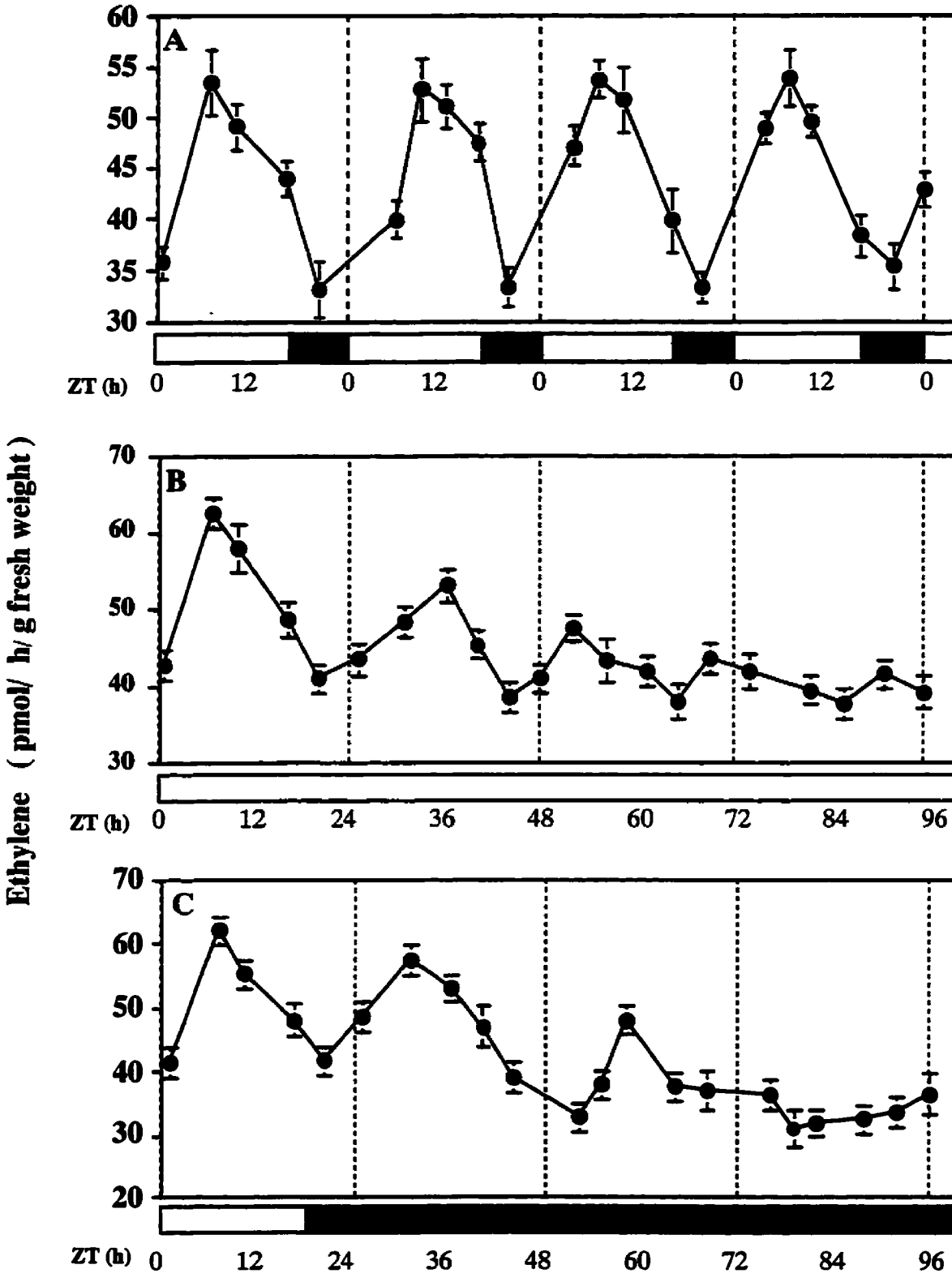
Fig. 4.1. Circadian oscillations in ACC oxidase activity in alpine and prairie ecotypes. Zeitgeber time (ZT) 0 indicates the time at which lights were turned on. The open and filled bars below the X axis indicate light and dark regimens. The data are mean values of six observed values and are representative of at least three separate assays. Vertical bars indicate ± S.E. where larger than symbols.


Ethylene (pmol/h/g fw)
60
50
40
30
20
ZT (h)
0
12
0
12
0
12
0
12
0
Alpine
Prairie

very similar in these ecotypes. The details of the characteristics of circadian regulation in prairie ecotype are elaborated here. When the lights were turned on (Zeitgeber time; ZT0), the enzyme activity was at 41.5 pmol of ethylene $h^{-1} g^{-1}$ fresh weight (Fig. 4.2A). The activity reached its peak (62.3 pmol of ethylene $h^{-1} g^{-1}$ fresh weight) by ZT6 (Fig. 4.2A). The activity then gradually decreased and reached its minimum by ZT20. The second peak appeared after 24 h at ZT6 on the second day and reached a similar level as before. Similarly, it took ca. 24 h for the minimal stage of activity to reoccur (Fig. 4.2A). Thus the period of the rhythm was about 24 h. The apparent Km of all the extracts remained at ~155 μM for ACC. In most of the cycles, the maximum activity observed was about 1.5 fold higher than the minimum activity (Fig. 4.2A).

The persistence of rhythm in enzymatic activity was examined by transferring plants that had been grown in LD to LL or DD for an extended period of time. The rhythms persisted in both LL (Fig. 4.2B) and DD (Fig. 4.2C). The first peak of activity in LL was at the expected time from the previous LD cycle and the amplitude of rhythm remained unaffected (Fig. 4.2B). The second peak of activity observed at ZT31 was reduced to 85% of LD maximum. The third peak of activity which was further reduced to 76% of the LD maximum occurred 3 h in advance at ZT52. The fourth peak of activity also appeared earlier at ZT69. Thus, the period of the rhythm under LL appears to be shorter (ca. 21 h) than under LD. Although the amplitude of rhythm under DD also became progressively reduced in a manner similar to that under LL, the period of rhythm seems to be broader (Fig. 4.2C).

Fig. 4.2 (A-C). Circadian oscillations in ACC oxidase activity *in vitro* under 16-h photoperiod (A), constant light (B), and constant dark (C) with 22° C (16h) and 18° C (8h) temperature cycle. The light period (day) is indicated by open bars and the dark period (night) is indicated by closed bars. The data shown are from six replicates of the same extract from a single ramet of an alpine plant, but are representative of data from four other alpine plants. Vertical bars indicate ± S.E.


A
B
C
Ethylene (pmol/ h/ g fresh weight)
ZT (h)
0
12
24
36
48
60
72
84
96

4.3.2. Circadian oscillations in ACC oxidase mRNA accumulation

Plants that were grown in SDC conditions were subsequently subjected to different photoperiodic cycles. Changes in ACC oxidase mRNA levels were monitored for 4 days in each treatment. Under LD (16 h photoperiod), the abundance of mRNA fluctuated strongly across each day in alpine (Fig. 4.3A) and prairie ecotype (Fig. 4.4A). In contrast, nearly constant transcript levels were observed with rDNA probe (Figs. 4.3B, 4.4B). The maximum level of ACC oxidase mRNA accumulation was observed at about 5 h after the lights were turned on (ZT5), and the time interval (period) for the re-occurrence of this maximum abundance was ca. 24 h (Fig. 4.4A). These oscillations in ACC oxidase transcript levels persisted even in the plants subjected to continuous light (Figs. 4.3C, 4.4C) or continuous darkness (Figs. 4.3D, 4.4D). Cycling of transcript levels however, showed damping under these regimens (Figs. 4.3C, 4.3D, 4.4C and 4.4D). These results are positively correlated with the oscillations observed for ACC oxidase activity observed *in vitro* (Figs. 4.2A through C).

4.3.3. Effect of temperature on circadian oscillations of enzymatic activity *in vitro*

Although transfer of plants from LDW to SDC altered the amplitude of rhythm, it did not affect the cycling of ACC oxidase activity *per se* in both ecotypes. For example, under LD conditions with a square-wave temperature cycle of 22° C day/18° C night, the rhythm was pronounced with an amplitude of 10.8 pmol of ethylene h^{-1} g^{-1} fresh weight in prairie ecotype (Fig. 4.2A). When the temperature cycle was modified to 8° C

Fig. 4.3 (A-D). Northern analysis of circadian regulation of ACC oxidase mRNA abundance. Northern blots containing 10 μg per lane of total RNA from alpine plants grown under 16-h photoperiod with 22° C day and 18° C night temperature cycle was probed with SlACO 1 (A). After stripping the SlACO 1 probe, the same blot was rehybridized with an 18S rDNA probe (B). Northern blots containing 10 μg of total RNA per lane from plants grown under continuous illumination (C) and continuous dark (D) with 22° C (16h) and 18° C (8h) temperature cycle were probed with SlACO 1. The size of the ACC oxidase transcript in A, C and D is 1.2 Kb and that of 18S rRNA is about 1.8 Kb. Bars below the blots show the light regimens: the open and filled bars indicate light and dark phases respectively. Zeitgeber time (ZT) is indicated below the bars.

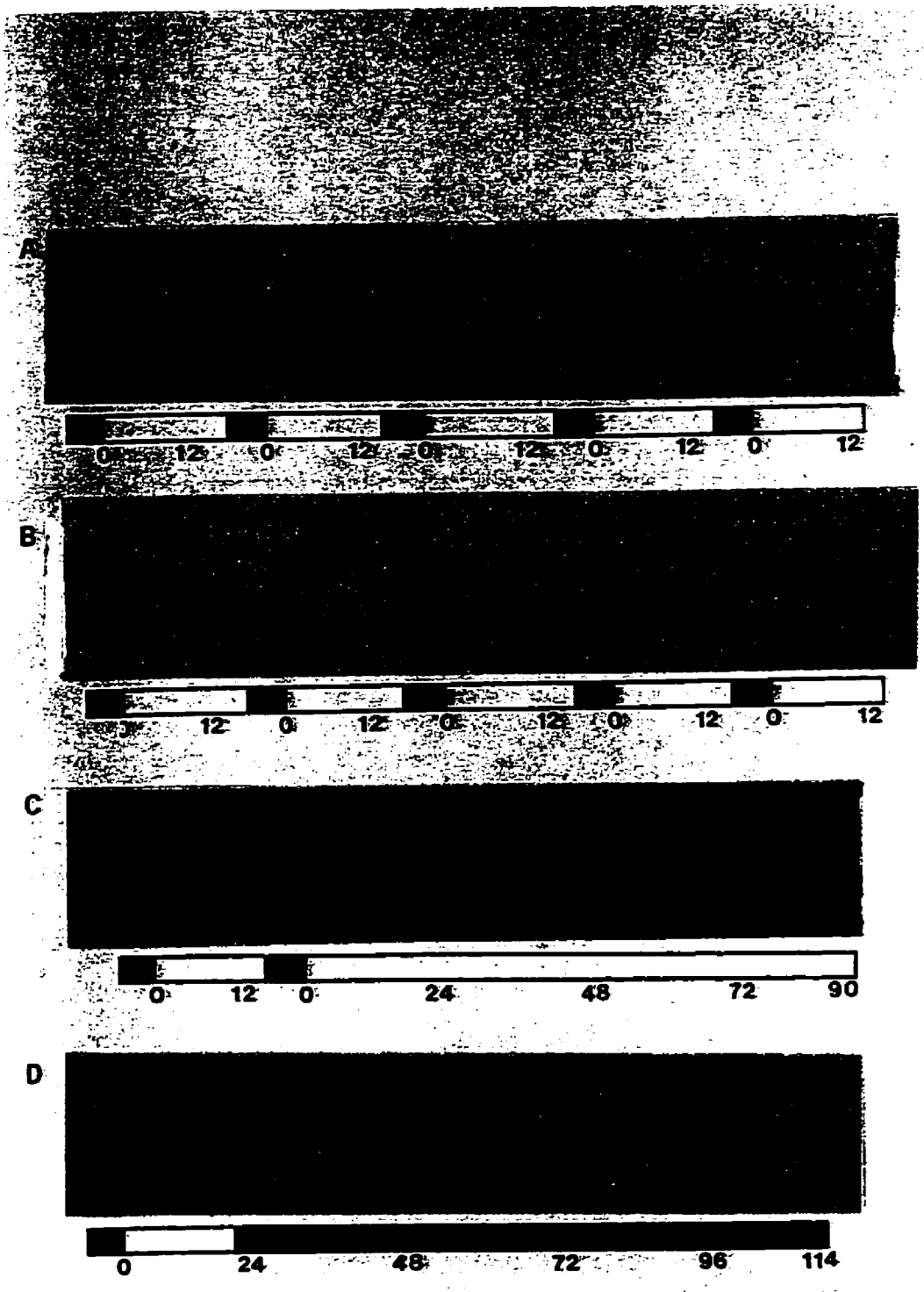
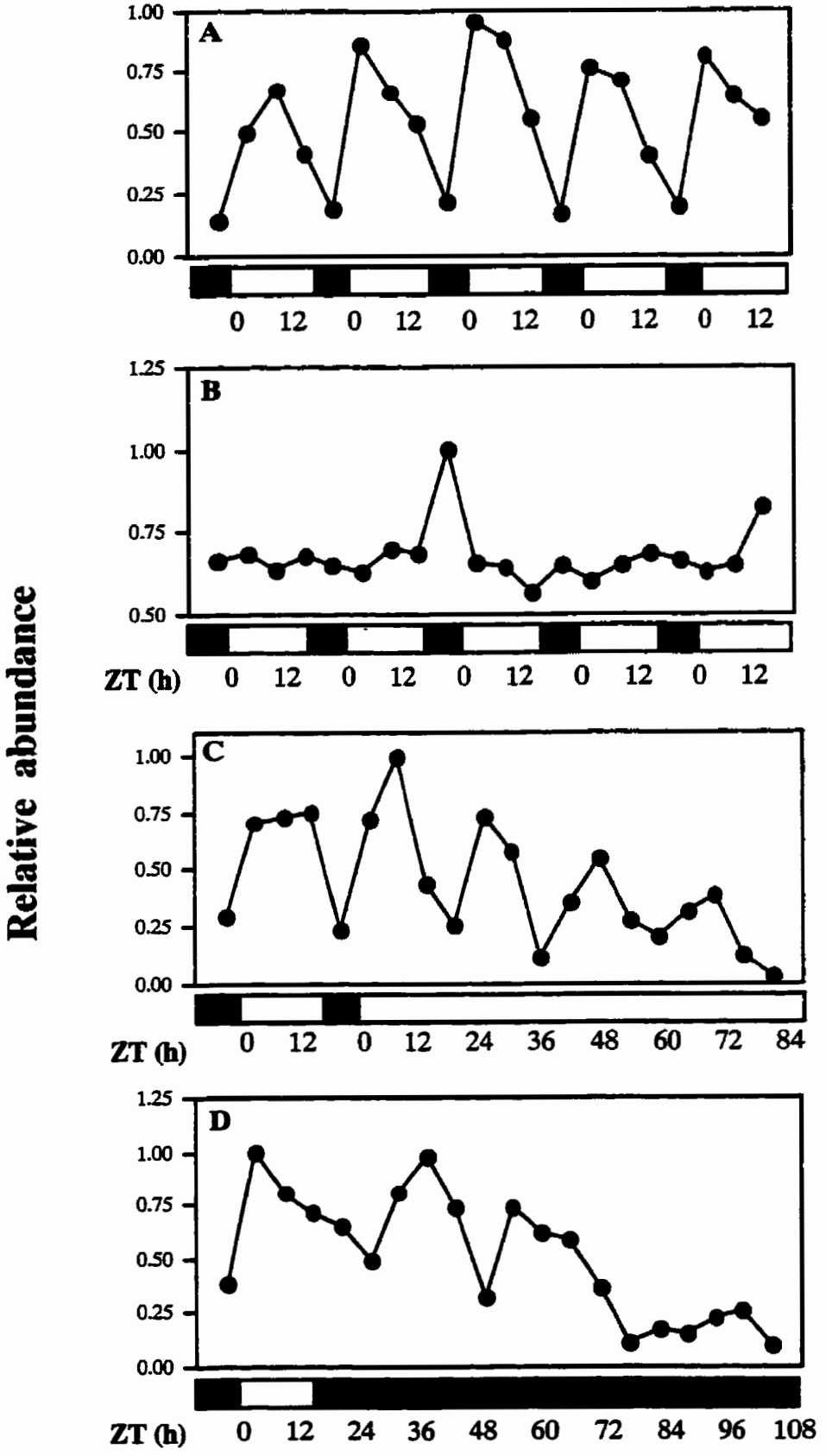

A
0 12 0 12 0 12 0 12 0 12
B
0 12 0 12 0 12 0 12 0 12
C
0 12 0 24 48 72 90
D
0 24 48 72 96 114

Fig. 4.4 (A-D). Quantification of Northern blots showing circadian regulation of ACC oxidase transcript. Northern blot containing total RNA of prairie plants grown under 16-h photoperiod with 22° C day and 18° C night temperature cycle was probed with SlACO 1 (A), stripped and reprobed with an 18S rDNA probe (B). The autoradiographs were scanned using Laser densitometer and the maximum value of relative mRNA abundance was adjusted to 1.0 for each experiment. Results from plants grown under continuous light (C), and continuous dark (D) with 22° C (16h) and 18° C (8h) temperature cycle also show circadian oscillations in ACC oxidase transcript. ZT is indicated below the bars.


A
B
C
D
Relative abundance
1.00
0.75
0.50
0.25
0.00
1.25
ZT (h)
0 12 0 12 0 12 0 12 0 12
0 12 0 12 24 36 48 60 72 84
0 12 24 36 48 60 72 84 96 108

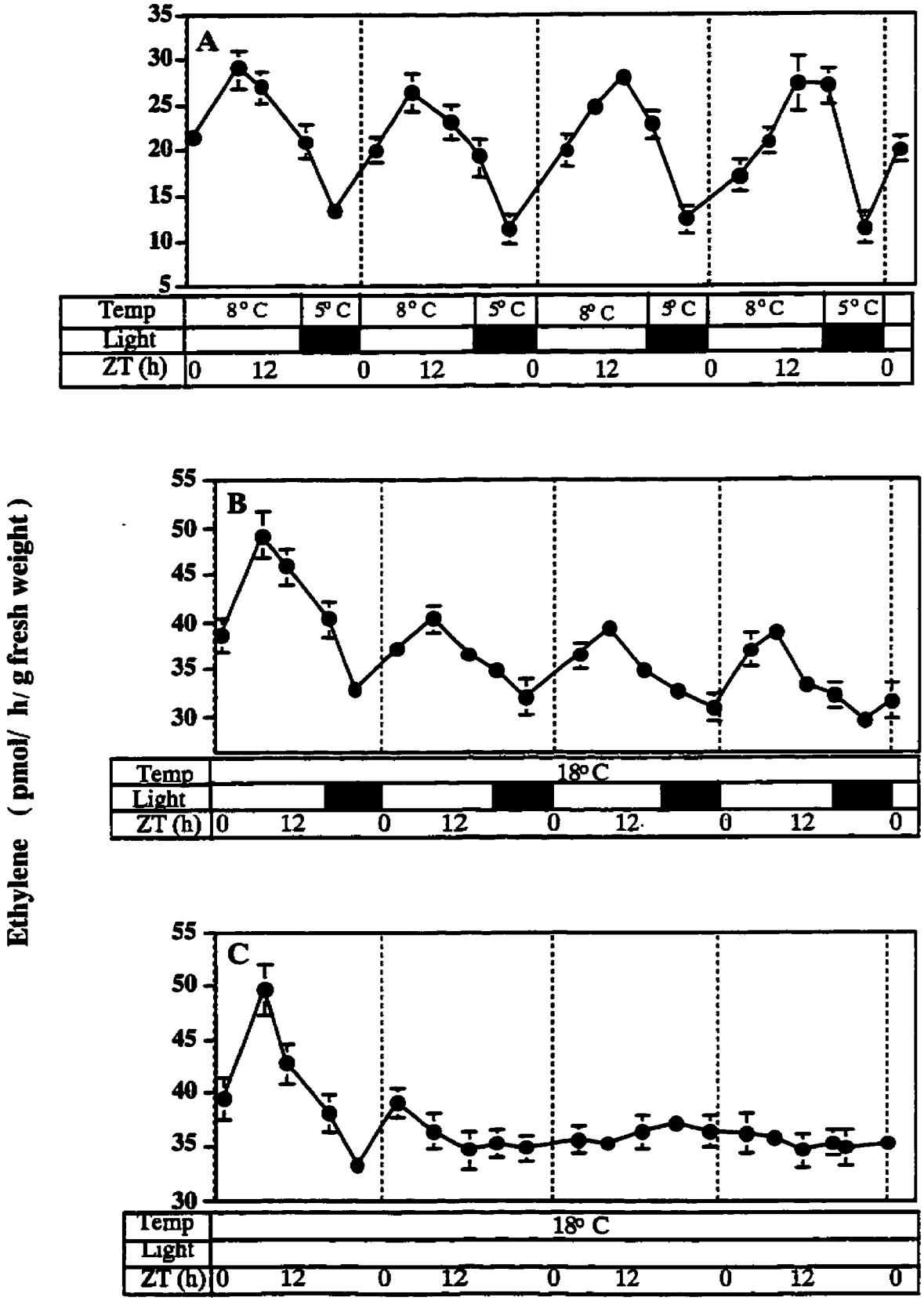
day/5° C night, not only the minimum and maximum enzyme activity levels were reduced, but also the amplitude of rhythm was reduced to 7.8 pmol of ethylene $h^{-1} g^{-1}$ fresh weight (Fig. 4.5A). However, the period of rhythm remained unchanged (Fig. 4.5A).

Constant temperature of 18° C accompanied by 16-h photoperiod also had a severe effect on the amplitude of rhythm. While the peak of the first cycle was only at 80% of the LD maximum observed under the temperature cycle of 22° C/18° C, the second, third and fourth peaks were further reduced but were maintained at ca. 40% (Fig. 4.5B). Nevertheless the peaks of all the four cycles appeared at 24 h intervals indicating that the period is not affected by constant temperature. The most dramatic effects were observed in plants that were kept under constant light at a constant temperature of 18° C. In the first cycle, the peak appeared at the expected time (ZT6) but with only 80% of LD maximum and the minimum activity was observed at ZT20 (Fig. 4.5C). While the amplitude of the rhythm was drastically reduced in the second cycle, the cycling of enzyme activity dampened out by the third and fourth cycle (Fig. 4.5C).

4.3.4. Effect of red and blue light pulses on circadian rhythm

The effect of red and blue light pulses on rhythmic ACC oxidase mRNA accumulation and enzymatic activity was studied under continuous dark. Plants that were grown under LD conditions with 22° C day/18° C night temperature cycle were transferred to continuous dark for four days. On the fifth day, light pulses were given for 15 min. A 15 min pulse of red light was able to reset the rhythm in ACC oxidase transcript abundance in

Fig. 4.5 (A-C). Oscillations in ACC oxidase activity *in vitro* under 16 h photoperiod accompanied by 8° C day and 5° C night temperature cycle. The data shown are from six replicates of the same extract from a single ramet of a plant, but are representative of data from four other plants (A). (B-C) Effect of constant temperature on rhythmic oscillations of ACC oxidase activity *in vitro*. The plants were grown under 8° C day and 5° C night temperature cycle and then transferred to constant temperature of 18° C with 16 h photoperiod (B) or constant temperature of 18° C accompanied by constant illumination (C). Vertical bars indicate ± S.E. where larger than symbols.


A
B
C
Ethylene (pmol/ h/ g fresh weight)
35
30
25
20
15
10
5
55
50
45
40
35
30
Temp
Light
ZT (h)
8° C
5° C
18° C
0
12

both ecotypes (Figs. 4.6A and 4.7A). The reset rhythm however, quickly faded under the continuous dark conditions. In contrast, a blue light pulse could not reset the rhythm in mRNA accumulation (Figs. 4.6B and 4.7B). In parallel with the changes in transcript level, the enzymatic activity also showed resetting of rhythm after treating with red light but not blue light pulse (Fig. 4.8) in both ecotypes.

4.4. Discussion

A growing number of molecular events in higher plants have been shown to exhibit circadian rhythm. For instance, transcriptional regulation of several plant genes encoding proteins involved in both photosynthetic (Giuliano *et al.*, 1988; Kay and Millar, 1993; Pilgrim and McClung, 1993) and non-photosynthetic (McClung, 1993; Piechulla, 1993) processes has been demonstrated to be under the control of circadian clock. At the biochemical level, the activities of several enzymes display endogenous circadian rhythm. For example, nitrate reductase activity oscillates rhythmically in *Chenopodium rubrum* (Cohen and Cumming, 1974), and *A. thaliana* (McClung, 1993).

Circadian regulation has also been observed for a wide range of physiological processes in higher plants (Sweeney, 1987). Even complex physiological processes such as hormone metabolism display rhythms in higher plants. Daily changes in levels of ABA (Kannangara *et al.*, 1982; Lecoq *et al.*, 1983; Henson *et al.*, 1982), cytokinin (Hewett and Wareing, 1973), IAA (Kannangara *et al.*, 1982; Wessler and Wild, 1993), and GA (Foster and Morgan, 1995) have been reported.

Fig. 4.6 (A-B). Light-specific induction of rhythmic ACC oxidase mRNA accumulation. Alpine plants that were grown under 16-h photoperiod were subsequently transferred to continuous dark for four days, and the light pulses were given for 15 min (white slit in the dark bar placed below the figures). Northern blots contain 10 μg of total RNA per lane and were probed with SlACO 1. RNA was isolated from plants that were given 15 min of red light pulse (A) and blue light pulse (B). ZT is indicated below the bars.

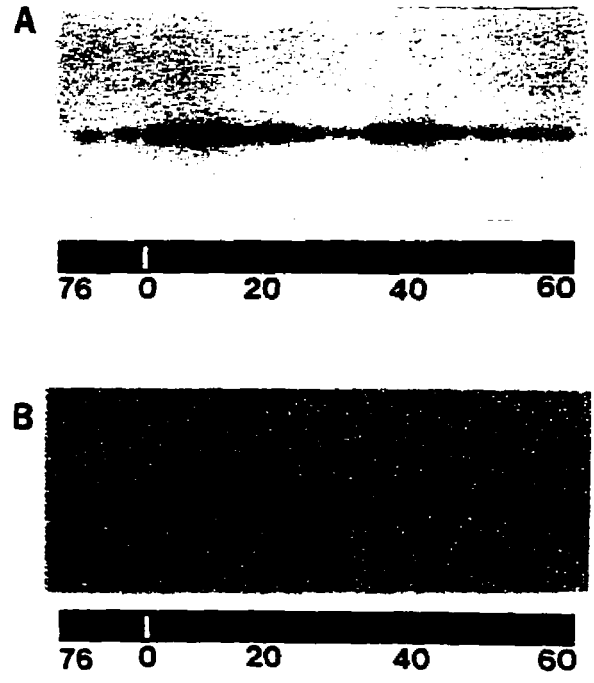

A
76
0
20
40
60
B
76
0
20
40
60

Fig. 4.7 (A-B). Quantification of Northern blots showing the effect of red, and blue light on circadian rhythm. Northern blots containing total RNA from prairie plants that were exposed to 15 min of red light (A) and blue light (B) in continuous dark were probed with SlACO 1. The maximum value of relative mRNA abundance of each blot was adjusted to 1.0. Small white inset bars indicate the time at which pulses were given (ZT 0).

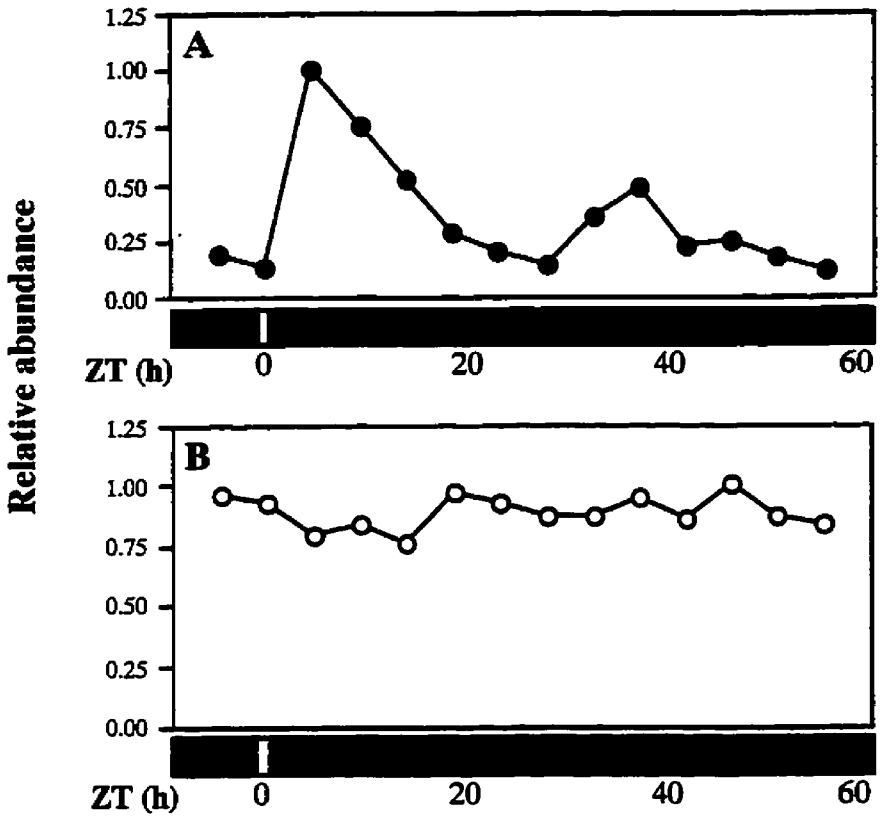
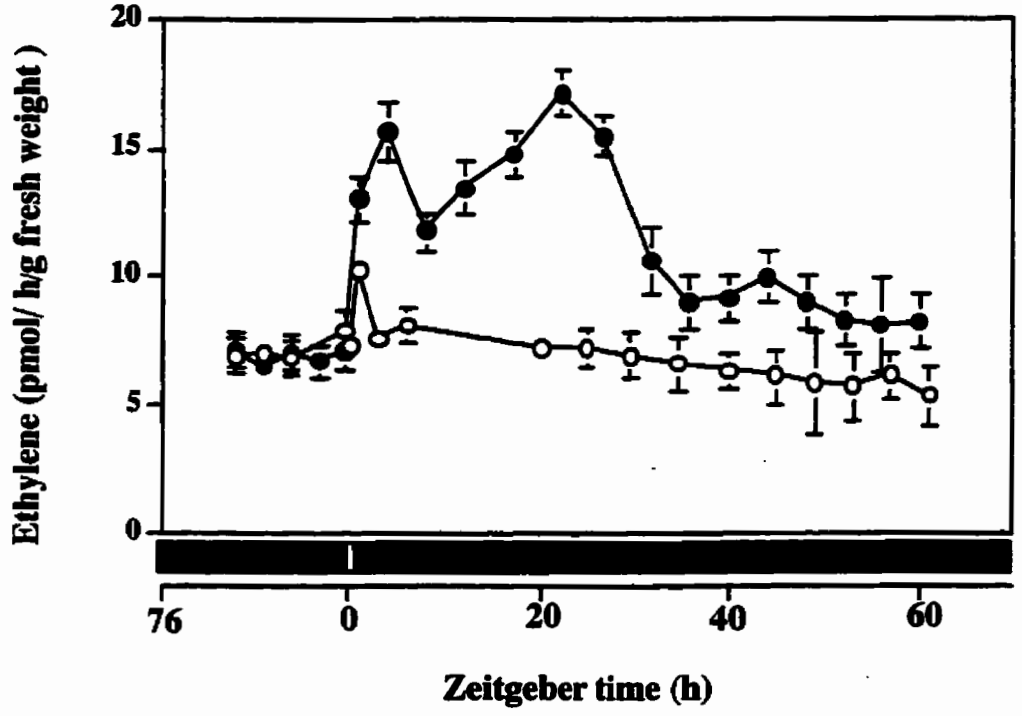

Relative abundance
A
B
1.25
1.00
0.75
0.50
0.25
0.00
ZT (h)
0
20
40
60

Fig. 4.8. Effect of red light (●) and blue light (O) pulses under continuous dark with 22° C (16h) and 18° C (8h) temperature cycle on ACC oxidase activity *in vitro*. The alpine plants were grown in continuous dark for four days and on the fifth day, light pulses were given (ZT0). Vertical bars indicate ± S.E. where larger than symbols.


Ethylene (pmol/ h/g fresh weight)
20
15
10
5
0
76
0
20
40
60
Zeitgeber time (h)

Rikin *et al.* (1984) suggested that the endogenous rhythm in ethylene evolution in cotton seedlings may be influenced by a combination of rhythmical changes in the biosynthetic pathway from methionine to ACC mediated by ACC synthase and the light effect on the conversion of ACC into ethylene mediated by ACC oxidase. For the first time, this investigation provides direct evidence for the existence of a circadian rhythm and the positive correlation between ACC oxidase mRNA transcript levels and enzymatic activity (Kathiresan *et al.*, 1996).

One of the salient features of all circadian rhythms is their persistence under constant environmental conditions. In the present study, oscillations in ACC oxidase transcript accumulation continued even under continuous light (LL) and continuous dark (DD). The damping of the rhythm under continuous light or dark conditions suggests that the external time cues are necessary for the expression of rhythm and that a circadian rhythm in ACC oxidase may not occur spontaneously, but must be initiated and coordinated by an external 'Zeitgeber' such as light. Interestingly the oscillation patterns for the ACC oxidase transcript levels and the *in vitro* ACC oxidase activity are similar. This result suggests that the rhythmic ACC oxidase activity *in vitro* may reflect possible underlying changes in *de novo* protein synthesis. Similar predictions have been established for nitrate reductase (Remmler and Campbell, 1986) and catalase (Redinbaugh *et al.*, 1990).

The period of free running rhythm in ACC oxidase activity is shorter under LL (about 21 h) but longer under DD (about 28 h). Millar *et al.* (1995) suggested that continued activation of phototransduction pathway under LL may advance the clock while the reduced activation under DD

may delay the clock. The rhythm that faded under DD was reset by a 15 min pulse of red light but not blue light. This result suggests the involvement of a red light signal transduction pathway in the circadian regulation of ACC oxidase. In contrast, the induction of rhythmic CAB gene expression by both red light- and blue light- responsive photoreceptor pathways was demonstrated in *A. thaliana* (Millar *et al.*, 1995). Cycling of both the transcripts and *in vitro* activity of ACC oxidase that was reset by red light pulse showed damping. The factor(s) responsible for such a fading of rhythm under continuous dark still remains to be identified.

Light inhibits the conversion of ACC to ethylene in leaf tissues of oat (Gepstein and Thimann, 1980), tobacco (de Laat *et al.*, 1981), and wheat (Wright, 1981). Kao and Yang (1982) demonstrated that the inhibitory effect in rice and tobacco leaves is mediated through lowering internal CO_2 levels under light conditions. Perhaps to counteract such an inhibitory effect of light, *S. longipes* has evolved a self-sustaining strategy to raise ACC oxidase activity levels during the light phase, possibly via *de novo* protein synthesis as judged by mRNA abundance. Fluctuations in ACC oxidase activity levels may cause oscillations in ethylene production if the endogenous ACC levels are not limiting. The resulting rhythmic ethylene production can, in turn, cause a rhythm in ethylene-induced responses such as stem elongation (Abeles *et al.*, 1992).

In addition to light, temperature cycles can also entrain rhythms (Sweeney, 1987). Under LD, both the low temperature cycle (8° C day and 5° C night) and the constant temperature of 18° C, severely reduced the amplitude of the rhythm in ACC oxidase activity in *S. longipes*, leaving the

period unaffected. This may be because of the reduced rates of metabolic functions under suboptimal temperatures (Palmer, 1976). Thus the circadian clock seems to have a temperature co-efficient (Q_{10}) of around 1.0 indicating that it is quite independent of temperature. When constant temperature (18° C) was accompanied by continuous light, the enzymatic activity dampened out in the third and fourth cycles. This result indicates that the entraining effects of light-dark cycles are more important than that of temperature cycles. Given the large temperature fluctuations observed in the prairie habitat (Emery *et al.*, 1994a), less dependence of the clock on temperature may offer greater adaptive values for *S. longipes* .

It is important to note that since both ecotypes produce rhythm in ACC oxidase activity (this study) and only prairie ecotype produces a reproducible ethylene rhythm (Emery *et al.*, 1994b), it is less likely that ACC oxidase alone is responsible for diurnal rhythm in ethylene production. Recently Emery *et al.* (1997) showed that ACC accumulation does not oscillate in concert with ethylene evolution and remains fairly constant in both ecotypes. These observations therefore suggest spatial regulation such as compartmentalization and/or transport of ACC to ACC oxidase could also play a major role in ethylene rhythmicity (Emery *et al.*, 1997).

In summary, stem and leaf tissues of alpine and prairie ecotypes exhibit circadian rhythm in the activity and mRNA abundance for ACC oxidase. Although the amplitude of rhythm in ACC oxidase activity is lower than that in prairie ecotype, the characteristics of circadian regulation are similar. The steady-state mRNA levels and enzymatic activity levels

fluctuated with a period of approximately 24 h and reached their maxima by the middle of the light phase and minima by the middle of the dark phase. The oscillations showed damping under constant light, constant dark and constant temperature conditions indicating that the rhythm is entrained by external signal. The results indicate that light/dark cycles have greater entraining effects than temperature cycles. A 15 min red light pulse, but not blue light pulse, could reset the rhythm in the continuous dark suggesting the possible role of red light signal transduction pathway in circadian regulation of ACC oxidase.

CHAPTER FIVE

Differential Regulation of 1-Aminocyclopropane-1-carboxylate Synthase Gene Family

5.1. Introduction

Since most tissues readily oxidize exogenously applied ACC, ACC synthase is often considered as the rate limiting step in ethylene biosynthesis (Yang and Hoffman, 1984). ACC synthase activity is highly regulated and induced by a wide range of internal and external cues. Such induction in ACC synthase activity is often mediated by an increase in transcript accumulation (Kende, 1993). ACC synthase is less conserved at the molecular level than ACC oxidase and is encoded by a gene family in several plant species (Zarembinski and Theologis, 1994). Expression patterns of various members of the ACC synthase gene family in response to developmental, hormonal and stress cues have been reported in species such as zucchini (Huang *et al.*, 1991), tomato (van Der Straeten *et al.*, 1990; Yip *et al.*, 1992), potato (Destefano-Beltran *et al.*, 1995), mung bean (Botella *et al.*, 1992), *A. thaliana* (Liang *et al.*, 1992; Rodrigues-Posada *et al.*, 1993), rice (Zarembinski and Theologis, 1993), and wheat (Subramaniam *et al.*, 1996).

Proteins encoded by multigene family members are known to share similar functions but differ in subtle characteristics that might have selective advantage to the cells or tissues in which they are expressed. Based on evidence collected from a narrow and selected range of key regulatory proteins that are encoded by gene families, Smith (1990) postulated that differential expression of family members may serve as a molecular basis of

phenotypic plasticity in plants. Thus besides its implication as the rate limiting step in ethylene production, studies on regulation of ACC synthase gene family may also provide valuable insights into the molecular mechanisms of phenotypic plasticity. The objectives of this investigation are (i) to clone ACC synthase cDNAs from alpine and prairie ecotypes and (ii) to study their regulation under various temperature and photoperiodic regimens.

5.2. Materials and Methods

5.2.1. Plant growth conditions

The plants were initially grown under SDC and transferred to one of the following conditions; (i) LDW, (ii) short day and warm (SDW; 8 h photoperiod and 22° C day/18° C night) or (iii) long day and cold (LDC; 8° C day/5° C night).

5.2.2. Cloning of ACC synthase gene fragment

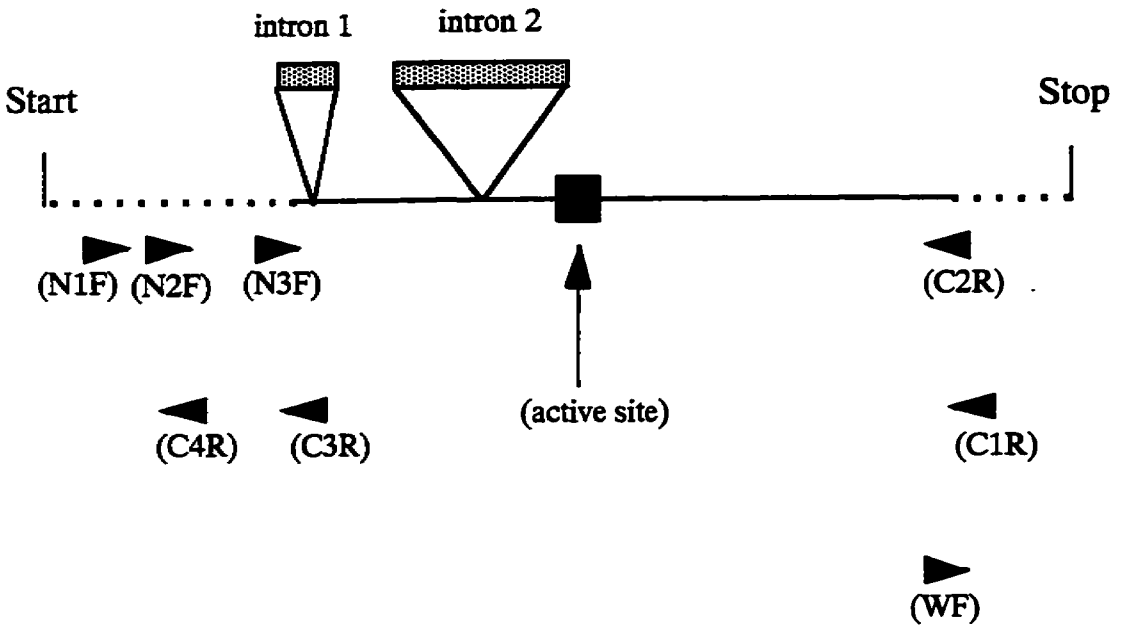
Genomic DNA was extracted from stem and leaf tissues by following the protocol described in section 2.2.1.1.1. Degenerate oligonucleotides corresponding to the following conserved amino acid sequences of ACC synthase; YFDGWK (N1F; positions 29-34 on *Brassica napus*), FQDYHG (N2F; positions 92-97 on *B. napus*), FRVCFA (C1R; positions 412-417 on *B. napus*), and CFANMD (C2R; positions 415-420 on *B. napus*) were synthesized using Pharmacia DNA synthesizer (Pharmacia LKB Biotechnology, Uppsala, Sweden). A schematic representation of the

relative positions of these primers on a model ACC synthase gene is shown in Fig. 5.1. The nucleotide sequences of these primers are as follows: N1F, 5'-TAYTTYGAYGGNTGGAARGC-3'; N2F, 5'-TTYCARGAYTAYCAY GG-3'; C1R, 5'-RTCCATRTTNGCRAARCA-3'; C2R, 5'-AGCRAARCAN ACNCKRAA-3'. First, a primary polymerase chain reaction was done using genomic DNA as template and N1F and C1R as primers. A secondary PCR performed using an aliquot of primary PCR products as templates and N2F and C2R as primers, yielded a 1.1 Kb fragment. Both PCRs were carried out using a Robocycler (Stratagene, LaJolla, CA, USA) in 35 cycles of denaturation (1 min) at 95° C, annealing (1 min) at 52° C, and primer extension (2 min) at 72° C. The secondary PCR product (SACS-1) was ligated into pBluescript (Stratagene, La Jolla, CA, USA) and sequenced using the automatic sequencing facility available at Department of Medical Biochemistry, University of Calgary. Upon conceptual translation, SACS-1 shows a high degree of similarity (70 to 85%) to other cloned ACC synthases.

5.2.3. Southern hybridization

The blot containing ten μg of digested genomic DNA per lane was hybridized in Rapid-hyb buffer® (Amersham, UK) at 60° C using exon III (Bgl II/ Sal I fragment) of SACS-1 as probe. Exon III encodes conserved amino acid residues that have been shown to form active site regions in other ACC synthases (Yip *et al.*, 1990; White *et al.*, 1994). Membranes were washed twice with 2x SSPE and 0.1% SDS at RT for 15 min, twice with 1x SSPE plus 0.1% SDS at 65° C for 30 min and twice with 0.2x SSPE plus 0.1% SDS at 65° C for 30 min.

Fig. 5.1. A schematic diagram showing positions of nested primers used in cloning a partial ACC synthase gene fragment (SACS-1) from genomic DNA. SACS-1 is 1835 bp long, contains 2 introns and 3 exons. The presence of active site in exon III is indicated.


intron 1
intron 2
Start
Stop
(N1F)
(N2F)
(N3F)
(C2R)
(active site)
(C4R)
(C3R)
(C1R)
(WF)

5.2.4. cDNA library screening

Approximately 500,000 clones from the cDNA libraries of alpine and prairie ecotype were screened using standard protocols described in section 2.2.1.2.4. Filters were prehybridized with 50% formamide, 5x SSPE, 0.5% SDS, 5x Denhardt's solution, 1.0 μg ml^{-1} yeast tRNA at 42° C for 5 h and hybridized in a similar buffer using exon III of SACS-1 as probe. The cDNA inserts were sequenced as described in section 2.2.1.1.5.

5.2.5. RNA extraction

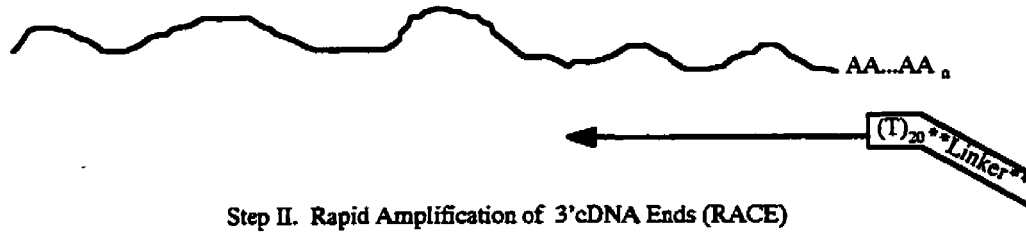
Total RNA was extracted from the stem and leaf tissues using hot phenol as described in section 2.2.1.2.1. Poly $(A)^+$ RNA was isolated from total RNA using Poly A tract R mRNA isolation systems (Promega Corporation, Madison, USA).

5.2.6. 3'RACE technique

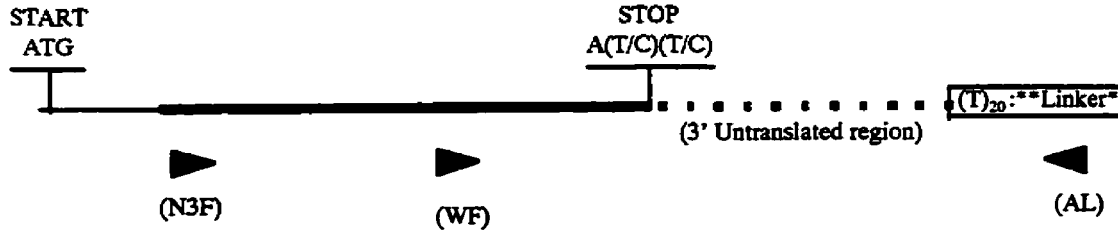
The protocol for amplification of 3' ends of mRNA is described in section 2.2.1.2.5. RT-PCR was carried out using 2 μl of cDNA pool as template and linker primer (AL) and degenerate oligonucleotide corresponding to the conserved domain VCFANMD (ZF; positions 414-420 on Canola) or GWFRVCFA (WF; positions 410-417 on Canola) as primers (Fig. 5.2). The nucleotide sequence of ZF is 5'-CANACRAARCGNCTRTACCT-3'; and that of WF is 5'-CCNACCAARKGNCANACRAARCG-3'. PCR conditions were the same as described for genomic PCR before. The

Fig. 5.2. A schematic diagram showing steps involved in cloning 3' untranslated region of ACC synthase transcripts using RACE technique. About 15 μg of total RNA was reverse transcribed using dTL primer. The pool of single stranded cDNAs were then used as template to amplify 3' ends of ACC synthase using a degenerate primer (N3F or WF) and antilinker (AL) primer. The 3'RACE products were then cloned and sequenced.

Step I. Reverse transcription



Step II. Rapid Amplification of 3'cDNA Ends (RACE)



Step III. Cloning of RACE products

Step IV. Sequencing of RACE products

3'RACE products were cloned using pBluescript® as vector and the inserts were sequenced.

5.2.7. Northern hybridization

Gene specific probes were prepared by amplifying the 3'untranslated regions (UTRs) using sequence specific primers. The amplified fragments were then labeled using random oligonucleotide primers (Feinberg and Vogelstein, 1983). Histone 2A variant (H2Av) of *Arabidopsis thaliana* (Genbank accession no. Z26465) was obtained through Arabidopsis Biological Resource Center, Columbus, OH, USA. The probe for ACC oxidase was synthesized by labeling SlACO-1, the 480 bp cDNA fragment encoding ACC oxidase in alpine ecotype.

5.2.8. ACC estimation and ethylene measurement

Due to the rhythmic nature of ethylene production in *S. longipes*, stem and leaf tissues were collected for ACC and ethylene measurements at 15 h, the time at which the ethylene production reaches its peak during a day (Emery *et al.*, 1994b). ACC was extracted from frozen stem and leaf tissues by following the method of Saltveit and Yang (1987). Ethylene measurements were made by incubating the ramets for 20 min in a 10 ml gas tight syringe with plungers adjusted to 6.0 ml. After incubation, 3 ml of gas sample was drawn via a three-way valve and analyzed for ethylene in a Photovac 10S plus gas chromatograph.

5.3. Results

5.3.1. Isolation of a genomic fragment encoding ACC synthase and Southern hybridization

A genomic fragment was amplified using degenerate primers, N2F and C2R, that correspond to conserved domains of known ACC synthases (Fig. 5.1). The amplified fragment (SACS-1) is 1741 bp long and contains 2 introns (Figs. 5.3). The exonic regions of SACS-1 have a high degree of nucleotide homology (70 to 85%) to ACC synthase cloned from other species. Conceptual translation of the exons also shows 75 to 91% homology to predicted amino acid sequences of several cloned ACC synthases. Exon III of SACS-1 encodes conserved amino acid residues that have been proposed to constitute the active site of ACC synthase in tomato (Yip *et al.*, 1990) and apple (White *et al.*, 1994).

Since the exon III contains unique restriction sites for Bgl II and Sal I (Fig. 5.3), genomic DNA of alpine and prairie ecotypes was digested using Bgl II and/or Sal I restriction enzymes and hybridized with exon III. The presence of four hybridizing bands in double-digested (Bgl II/Sal I) genomic DNA suggests that the genome may contain about 4 copies of the ACC synthase gene (Figs. 5.4A, B). Genomic DNA of alpine and prairie ecotypes did not reveal any polymorphism for Bgl II and/or Sal I (Figs. 5.4A, B)

Fig. 5.3. The nucleotide sequences of SACS-1. The sequences of introns are presented in lowercase letters and that of exons are shown in uppercase letters. The amino acid residues encoding ACC synthase active site are underlined. The 10 of 11 amino acid residues conserved between known ACC synthases and aminotransferases (Zarembinski and Theologis, 1994) are boxed. The restriction nucleotides for Bgl II and Sal I are shown in boxes.

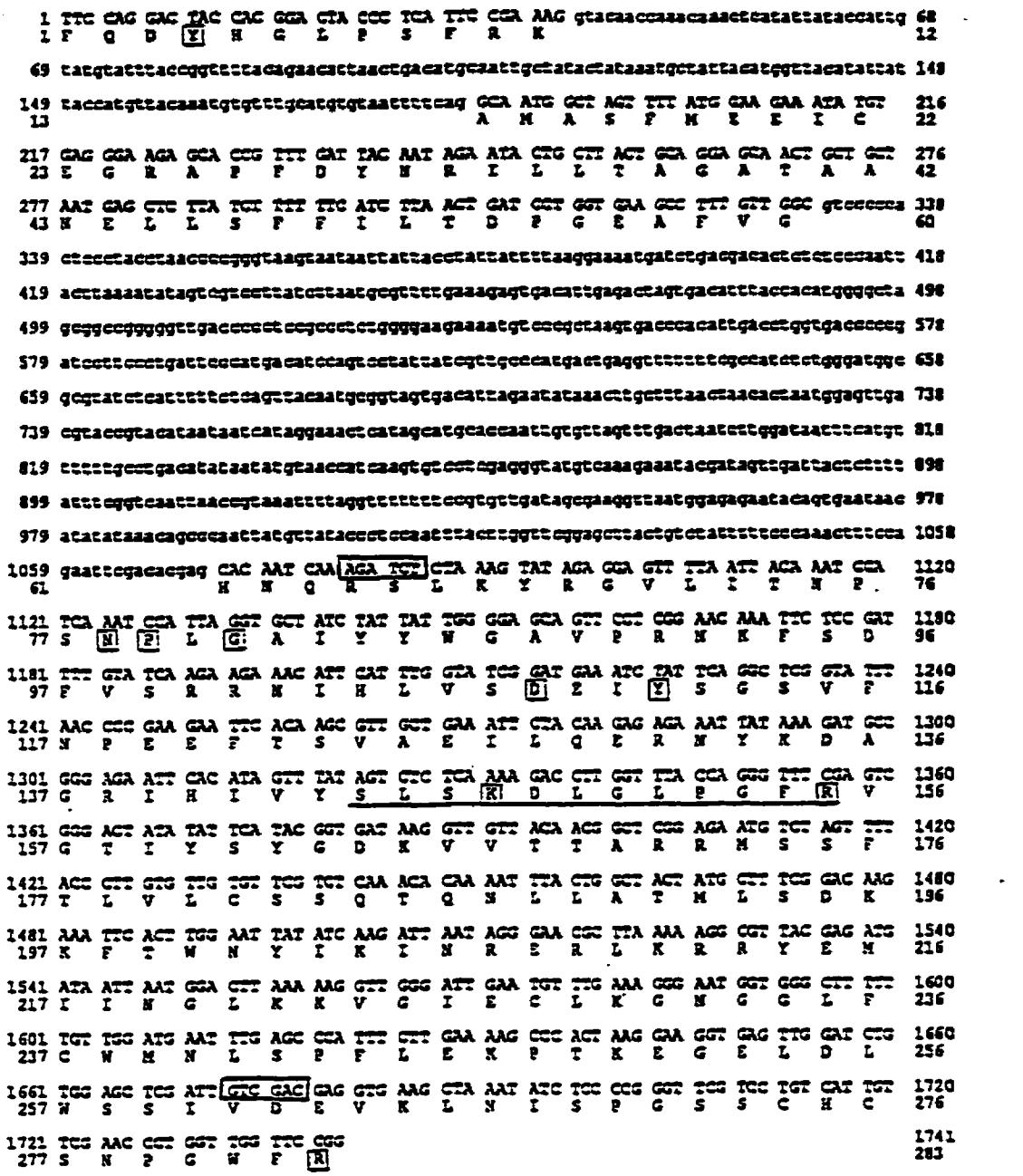
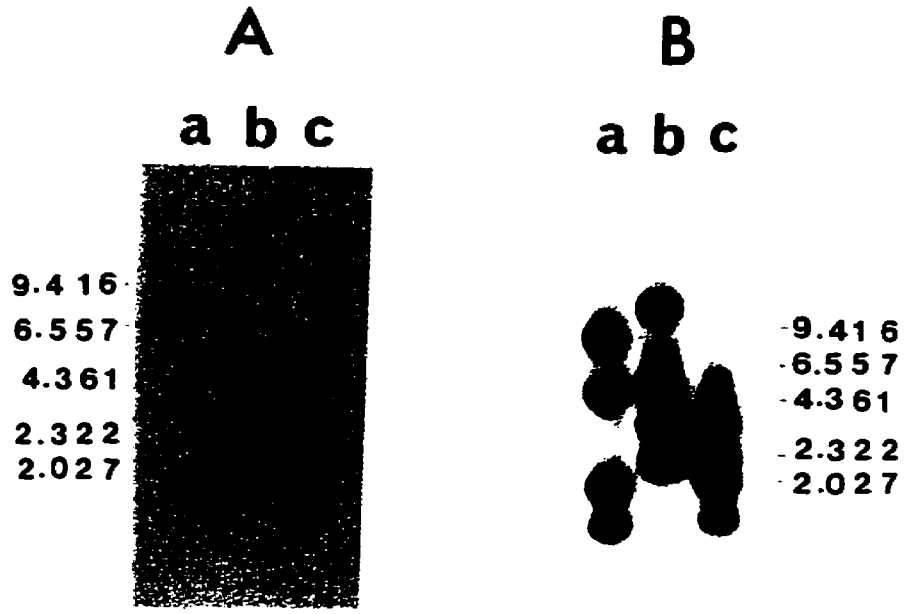


Fig. 5.4 (A-B). Southern analysis of genomic DNA from alpine (A) and prairie (B) ecotype of *S. longipes*. Ten μg of DNA was digested with Bgl II/Sal I (a), Bgl II (b) and Sal I (c), blotted onto a nylon membrane and hybridized with Sal I/Bgl II fragment of SACS-1.


A
a b c
9.416
6.557
4.361
2.322
2.027
B
a b c
9.416
6.557
4.361
2.322
2.027

5.3.2. Isolation of ACC synthase cDNA clones

The cDNA libraries of alpine and prairie ecotypes were screened using exon III of SACS-1 as probe. Several ACC synthase cDNA clones were obtained and sequenced. Inserts of two clones named ALAS-1 (isolated from the cDNA library of alpine ecotype) and PLAS-2 (screened from the cDNA library of prairie ecotype) were taken for further analyses. The nucleotide sequence of ALAS-1 is 1832 bp long and consists of 96 bp 5'-UTR, 1497 bp coding region, and 3'-UTR of 239 bp (Fig. 5.5). PLAS-2 is 1757 bp long that includes 87 bp 5'- UTR, 1497 bp coding region and 173 bp 3'-UTR (Fig. 5.6). Although the 5' and 3' UTRs of ALAS-1 and PLAS-2 are not identical, the coding regions of these two clones exhibit 78% identity at the nucleotide level. Conceptual translation of ALAS-1 and PLAS-2 revealed 70% over all identity between each other. Fig. 5.7 shows the homology of the predicted amino acid sequence of these two clones with three other closely related ACC synthases from potato, *Dianthus caryophyllus* and *A. thaliana*. Interestingly these two clones show significant divergence in their C-terminal regions (Fig. 5.8) with 34.3% identity.

5.3.3. Cloning 3' ends of ACC synthase mRNA

To determine if ACC synthase family members are differentially expressed under SDC and LDW, the 3' ends of ACC synthase mRNAs that are accumulated under SDC conditions were cloned using the RACE technique. A PCR was carried out using a reverse-transcribed single stranded cDNA pool as template, and AL and the degenerate WF as primers (Fig. 5.2). The

Fig. 5.5. Nucleotide sequence of an ACC synthase cDNA (ALAS-1) isolated from the cDNA library of alpine ecotype. The twelve amino acid residues of ACC synthase active site and putative polyadenylation signal sequences are underlined. Boxed amino acid residues represent invariant amino acids conserved between aminotransferases and ACC synthases (Zarembinski and Theologis, 1994).

```
   1 atcgcatcgggcattaccattgccagctagcgattagcggactatcagttttacgatcccagctacgactaactagggca 80

  81 tgcagcaattaegcat ATG GTA AGT CAG AGC AAG AAC AAT CAG GAT CAG GTC TTG AGT AAG ATC 144
   1               M  V  S  Q  S  K  N  N  Q  D  Q  V  L  S  K  I  16

 145 GCA GTA AAT GAG GAA CAC GGT GAG GAT AGT TCA TAT TTT TTA GGC TGG AAA GCT TAT GAG  204
  17 A  V  N  E  E  H  G  E  D  S  S  Y  F  L  G  W  K  A  Y  E  36

 205 AAG GAT CAA AAC GCT TTT TTC CAC GCT TAC CAT GAA ATC AAC CAG AAT AGT AAT GGC GTC  264
  37 K  D  Q  N  P  F  F  H  P  Y  H  E  I  N  Q  N  S  N  G  V  56

 265 ATC CAG ATG GGC TTG GCA GAG AAC CAA TTA TGT TTT GAC TTA ATC GAG GAG TGG ATC AAA  324
  57 I  Q  M  G  L  A  E  N  Q  L  C  F  D  L  I  E  E  W  I  K  76

 325 AGG AAC CCT GAC GCT GCT ATA TGC ACT AAT GAT GGC ATC AAG AGC ATA TTT AGG GCA TTA  384
  77 R  N  P  D  A  A  I  C  T  N  D  G  I  K  S  I  F  R  A  L  96

 385 TTC CAG GAC TAC GAT GGC TTA CAA GAA TTT AGA CAG GCA GTC GCA CGA TTT ATG GAA AAG  444
  97 F  Q  D [Y] H  G  L  Q  E  F  R  Q  A  V  A  R  F  M  E  K  116

 445 ACG CGA GGA GGT AGG GTT AGG TTT GAT TGG AAT TTG GTA TTA ACG GCT GGA GCT ACT AGC  504
 117 T  R  G  G  R  V  R  F  D  S  N  L  V  L  T  A  G  A  T  S  136

 505 GCT AAC GAA ACT ATA ATC TTT TGC ACT GCA AAT CCT GGA GAT GCA TTT TTG GTC CCA ACT  564
 137 A  N  E  T  I  I  F  C  T  A  N  P  G  D  A  F  L  V  P  T  156

 565 GCT TAC TAT GCA GCT TTC GAT AGG GAT TTA AAA TGG AGA ACT GGA GCA GAG ATT TTG CCC  624
 157[F] Y  Y  A  A  F  D  R  D  L  K  W  R  T  G  A  E  I  L  P  176

 625 ATA TGC CAT GAA AGC AGC AAT AAC TTC AAG ATT ACG AGC AAA GCT AAG GAA GCA TAT GAA  684
 177 I  C  H  E  S  S  N  N  F  K  I  T  S  K  A  K  E  A  Y  E  196

 685 AAT GCA CAA AGC AAA TAC AAG GCT AAA AAG TTA GAT TTG AAT GTC AAA GGC TTA TTG ATG  744
 197 N  A  Q  S  K  Y  K  A  K  K  L  D  L  N  V  K  G  L  L  M  216

 745 ACG AAC CCC AGT AAT GGG TTA GGC ACT ACG ACG TTG GAC AGG GAC ACG TTA AAA AAG ATC  804
 217 T  N  P  S [N][P] L [G] T  T  T  L  D  R  D  T  L  K  K  I  236

 805 AGT ACG TTT ACG AAC CAA AAG GGC ATT CAT TTA GTC TGT GAC GAG ATT TAC GCA GCA ACC  864
 237 S  T  F  T  N  Q  K  G  I  H  L  V  C [D] E  I [Y] A  A  T  256

 865 GTC TTG TCA GCT AAA TTT GTC TCA ATA GCA GAC ATA TTA TTG GAT GAT GAC GTA AAC CAA  924
 257 V  F  S  P  K  F  V  S  I  A  D  I  L  L  D  D  H  V  N  Q  276

 925 GAC TTA GTC CAT ATC GTC TAC AGC TTA TCG AAA GAT TTG GGC TTA CCA GGC TTC AGG GTC  984
 277 D  L  V  H  I  V  Y· S  L  S [K] D  L  G  L  P  G  F [R] V  296

 985 GGA ATC GTA TAC TCG AAA GAC GAA ATG GTC GTC AGC GCA GCA ACT AAA ATG AGT AGC TTT 1044
 297 [G] I  V  Y  S  K  D  E  M  V  V  S  A  A  T  K  M  S  S  F  316

1045 GGC TTA GTC TGC TTT GCA AAC ATG GAC GAC AGC AGC CAA ACG CAA TTA ATG ATA GCA GCT 1104
 317 G  L  V  C  F  A  N  M  D  D  S  S  Q  T  Q  L  M  I  A  A  336

1105 TTA CTG AGC GAC AAA AAC TTC ACG GAA GAA TTT TTG ATC GAG TCC AGC GAC AGG TTA AGT 1164
 337 L  L  S  D  K  N  F  T  E  E  F  L  I  E  S  S  D  R  L  R  356

1165 AAG TAT GAG ATG ATT ACG GAA GGC TTA AAA AGC GCT GGA ATA AAT TGT TTA CAA GGC AAT 1224
 357 K  Y  E  M  I  T  E  G  L  K  S  A  G  I  N  C  L  Q  G  N  376

1225 GCA GGC TTA TTC GTC TGG ATG GAC TTA AGA CAG TTG TTG AAA GAC AAT ACG TTT GAT AGC 1284
 377 A  G  L  F  V  W  M  D  L  R  Q  L  L  K  D  N  T  F  D  S  396

1285 GAA ACT GAG TTA TGG AAC GCT ATC TTA CAC GAG GTA AAG TTA AAC ATC AGC CCA GGA AGC 1344
 397 E  T  E  L  W  N  A  I  L  H  E  V  K  L  N  I  S  P  G  S  416

1345 AGC TGC CAC AGT GAA TGC AGC GAA CCG GGA TGG TTC GGA TGC TTC GCA AAC ATG GAT ATC 1404
 417 S  C  H  S  E  C  S  E  P  G  W  F [R] C  F  A  N  M  D  I  436

1405 GAC GAA ACT TTA GAT TTA GAC TTG GGA GTC AAA AGG TTG AAA GAT TTT GCA CAG AGG GGC 1464
 437 D  E  T  L  D  L  D  L  G  V  K  R  L  K  D  F  A  Q  R  G  456

1465 AGA GTA GAT AAC AGT ACG ATG ACG ACG ACT AAA CAG CCG TTT AAG TTA CCG TTG AAG AAG 1524
 457 R  V  D  N  S  T  M  T  T  T  K  Q  P  F  K  L  P  L  K  K  476

1525 ACT GTC AGG AGT AAC TTG CGG GCA GCT GAC AGT GCT ATG AAC AGC GCA TTA GTC AGG GCA 1584
 477 T  V  R  S  N  L  R  P  A  H  S  P  M  N  S  P  L  V  R  A  496

1585 AGG ACT TAA gattacggagtatgcaggcaggatccagacgatcacgtcatcatgcatgagcttagattcatttaaat 1661
 497 R  T  *                                                                      499

1662 acgatctattatttagtatttatcatgactattcgctggcacggcatatctaaacgacgtaattactgatgataaactag 1741

1742 ccgacgcgattcagcttaccttaatttaataacgacgactatcaataacagataataacgacgactaaaaaaaaaaaaaa 1821

1822 aaataaaaaaa                                                                    1832
```

Fig. 5.6. Nucleotide sequence of PLAS-2, an ACC synthase cDNA isolated from prairie ecotype. The 10 amino acid residues conserved between known ACC synthases and aminotransferases are boxed. The dodecapeptide part of ACC synthase active site and polyadenylation signal sequence are underlined.

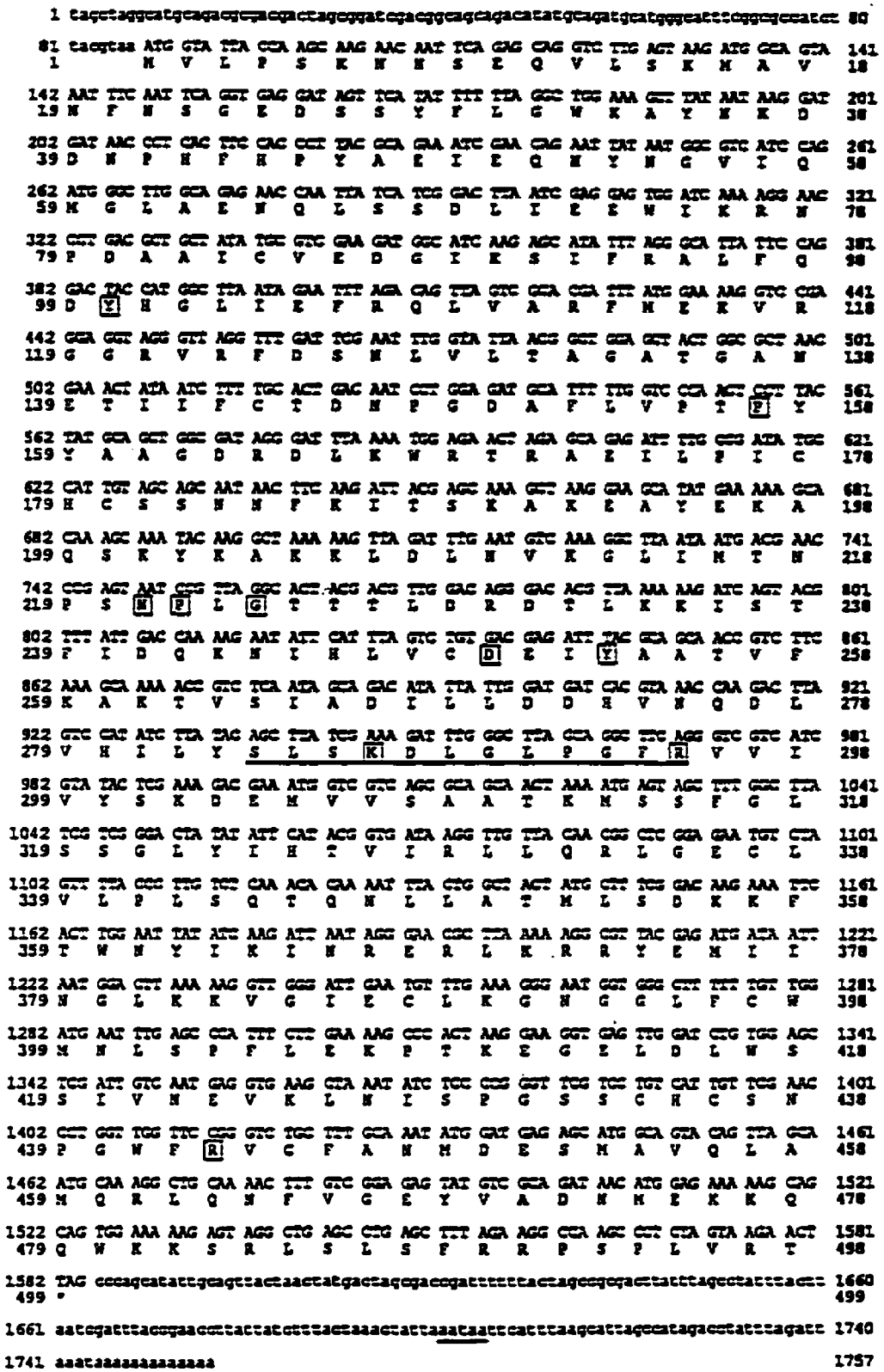
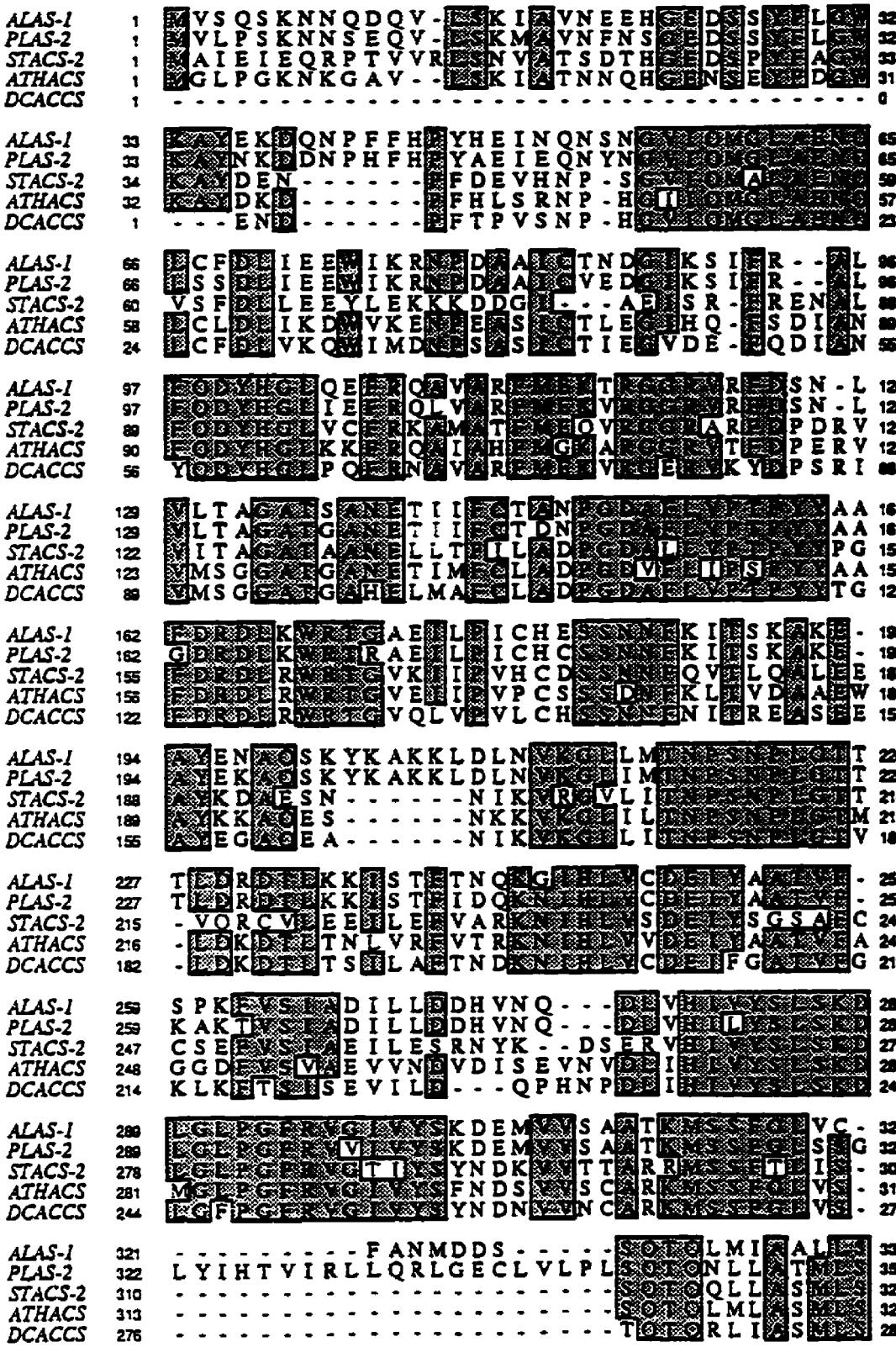


Fig. 5.7. Amino acid sequence alignments of ACC synthase from alpine (ALAS-1) and prairie (PLAS-2) ecotype of *S. longipes*, tomato (STACS-2), *A. thaliana* (ATHACS), and *Dianthus caryophyllus* (DCACCS). Identical amino acid residues are presented in shaded boxes.


ALAS-1
PLAS-2
STACS-2
ATHACS
DCACCS

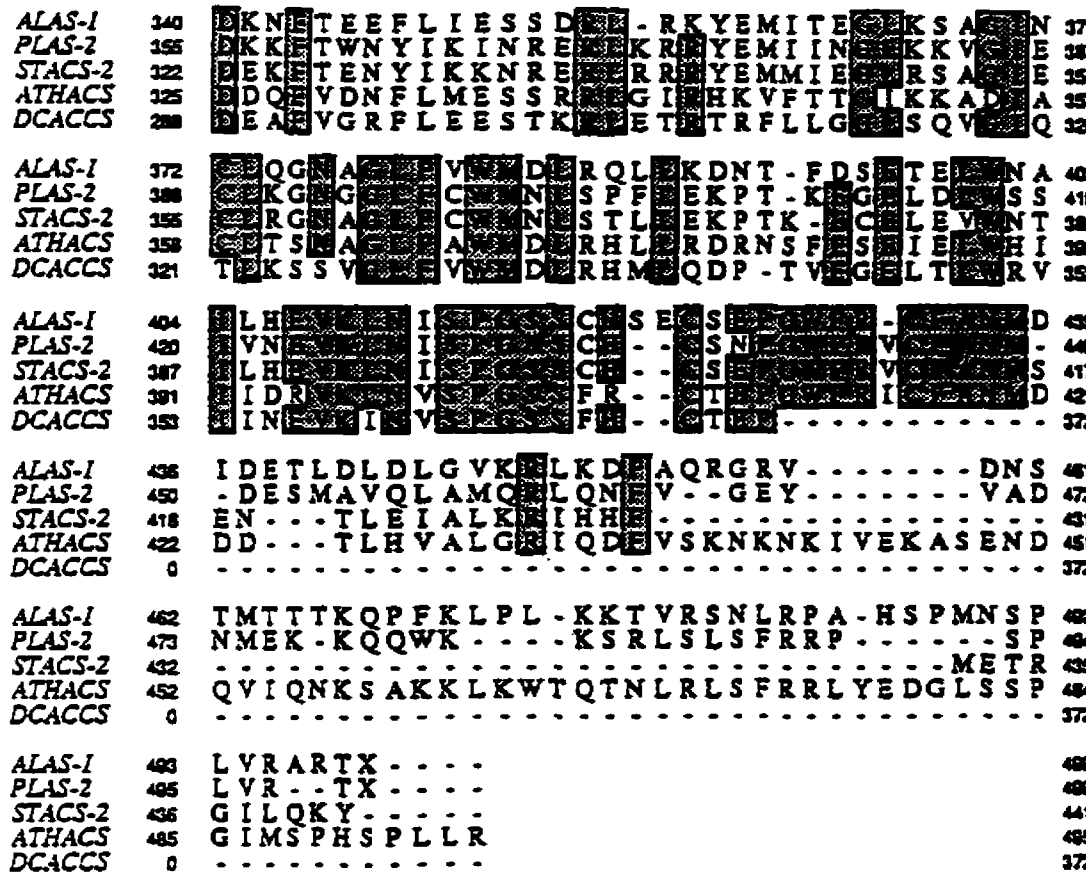
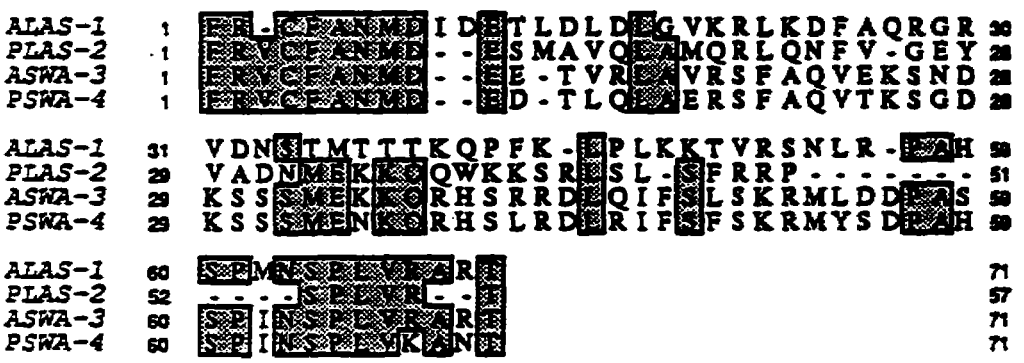

ALAS-1
PLAS-2
STACS-2
ATHACS
DCACCS

Fig. 5.8. Comparison of the hypervariable C-termini (last 71 amino acid residues) encoded by the four ACC synthase cDNA clones obtained from LDW grown alpine (ALAS-1) and prairie (PLAS-2) ecotypes, and SDC grown alpine (ASWA-3) and prairie (PSWA-4) ecotypes. The identical amino acids encoded by these four clones upon conceptual translation, are shown in shaded boxes.

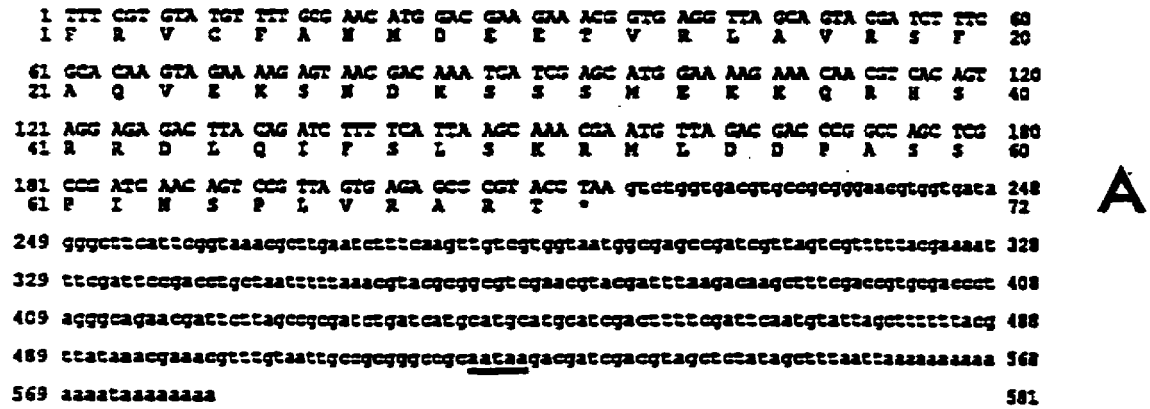

ALAS-1
PLAS-2
ASWA-3
PSWA-4

3'RACE products obtained from alpine and prairie ecotypes were cloned and sequenced. The nucleotide sequences of two 3'RACE products, namely ASWA-3 (alpine ecotype) and PSWA-3 (prairie ecotype), were homologous (62 to 83 %) to C-terminal regions of ACC synthase from other species. ASWA-3 is 581 bp long and consists of 216 bp coding region and 365 bp 3'-UTR (Fig. 5.9A). PSWA-4 is 599 bp with a 216 bp coding region and 383 bp putative 3'-UTR (Fig. 5.9B). Despite their divergent 3'-UTRs, the regions of ASWA-3 and PSWA-4 encoding the last 71 amino acid residues of ACC synthase show 79.0% identity at the amino acid level. Thus the predicted amino acid sequences of the ACC synthase clones obtained from SDC exhibit more homology between alpine and prairie ecotypes than those from LDW. Nevertheless the nucleotide sequences of 3' UTRs of all these four clones are divergent and likely represent different members of the ACC synthase gene family. When the C-termini (last 71 residues) of clones obtained from SDC and LDW are compared, they exhibit only 31.0% identity in alpine ecotype and 26.4% identity in prairie ecotype (Fig. 5.8). This indicates that the gene expressed under SDC is different from the one under LDW in both ecotypes.

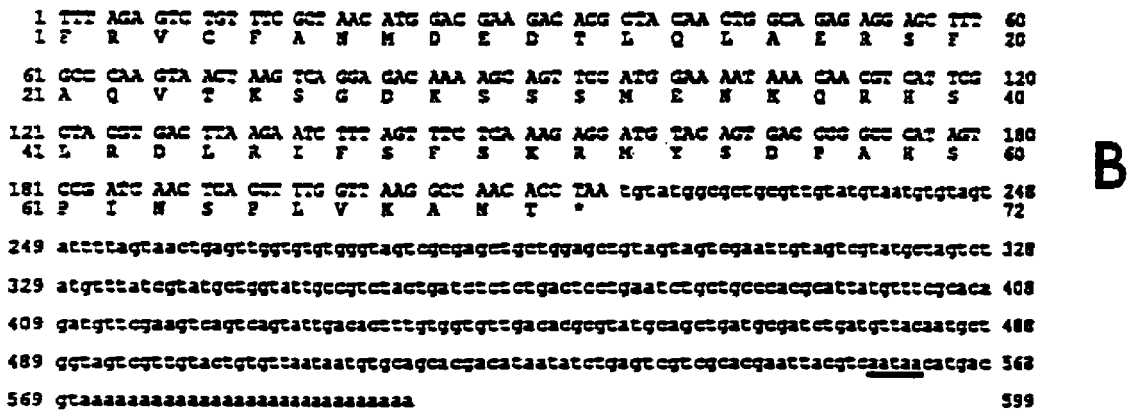
5.3.4. Differential expression pattern

The differential regulation of ACC synthase mRNA in alpine and prairie ecotypes was further examined under different photoperiod and temperature conditions. Poly(A)$^+$ RNA extracted from SDC and LDW grown plants was hybridized with unique 3'-UTRs of different ACC synthase cDNA clones obtained in the present study. As seen in figs. 5.10A and 5.11A, in both ecotypes the transcripts that are accumulated under SDC

Fig. 5.9 (A-B). The nucleotide sequence of partial ACC synthase cDNA clone isolated from SDC grown alpine ecotype (ASWA-3) (A) and prairie ecotype (PSWA-4) (B). The putative polyadenylation signal sequences are underlined.



A



B

Fig. 5.10 (A-C). Expression characteristics of ACC synthase genes in alpine ecotype. The filter containing 10 μg of poly(A)$^+$ per lane from plants grown under SDC and LDW was probed with 3'UTR of ASWA-3 (A). After stripping ASWA-3 probe, the same filter was probed with 3'UTR of ALAS-1 (B) or H2Av clone of *A. thaliana* (C). The size of ACC synthase transcript in A and B is 1.8 Kb, and that of H2Av is 1.0 Kb. Age of the plants at the time of collection is indicated below the blots.

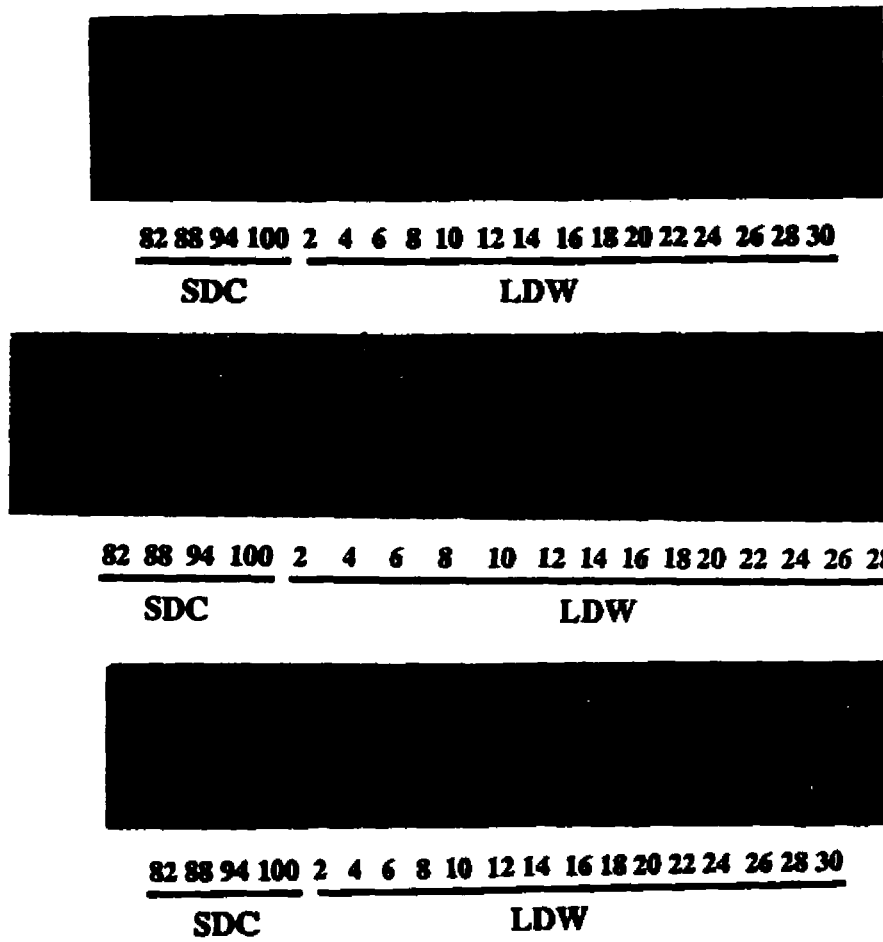
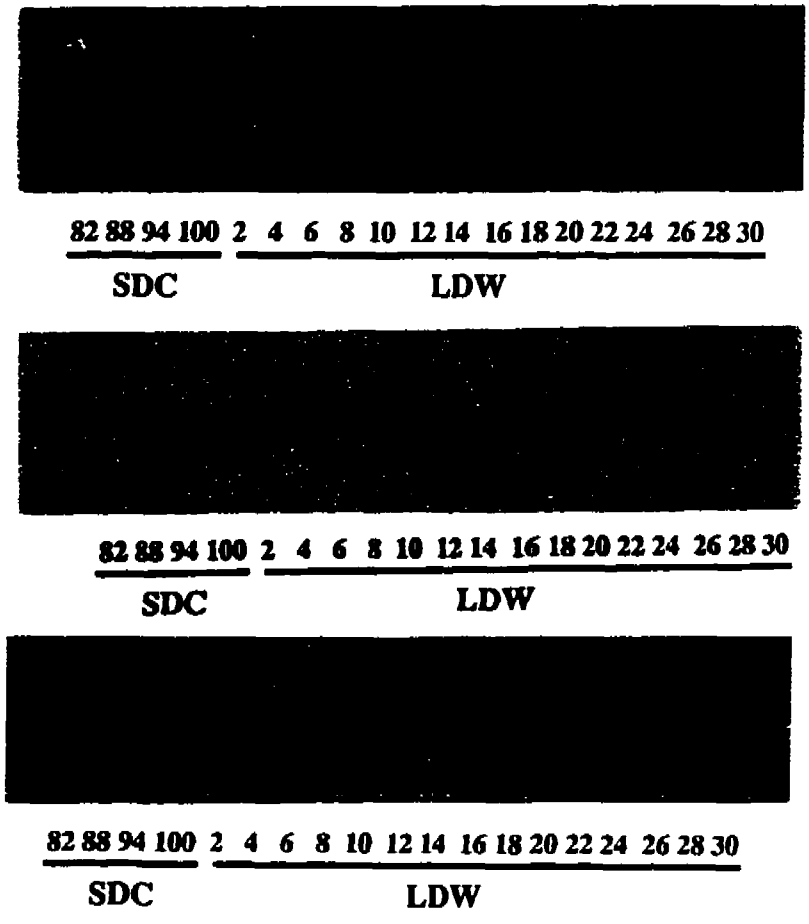

A
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW
B
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW
C
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW

Fig. 5.11 (A-C). Differential ACC synthase gene expression in prairie ecotype. Northern blot containing 10 μg of poly(A)$^+$ per lane from SDC and LDW grown plants was individually hybridized with the following gene specific probes; (A) (PSWA-4), (B) (PLAS-2), and H2Av clone of *A. thaliana* (C). The size of ACC synthase transcript in A and B is 1.8 Kb, and that of H2Av is 1.0 Kb.

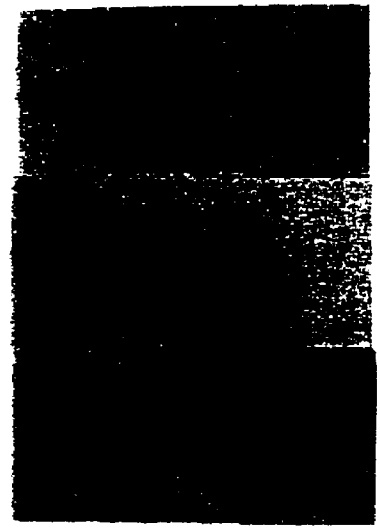

A
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW
B
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW
C
82 88 94 100 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
SDC
LDW

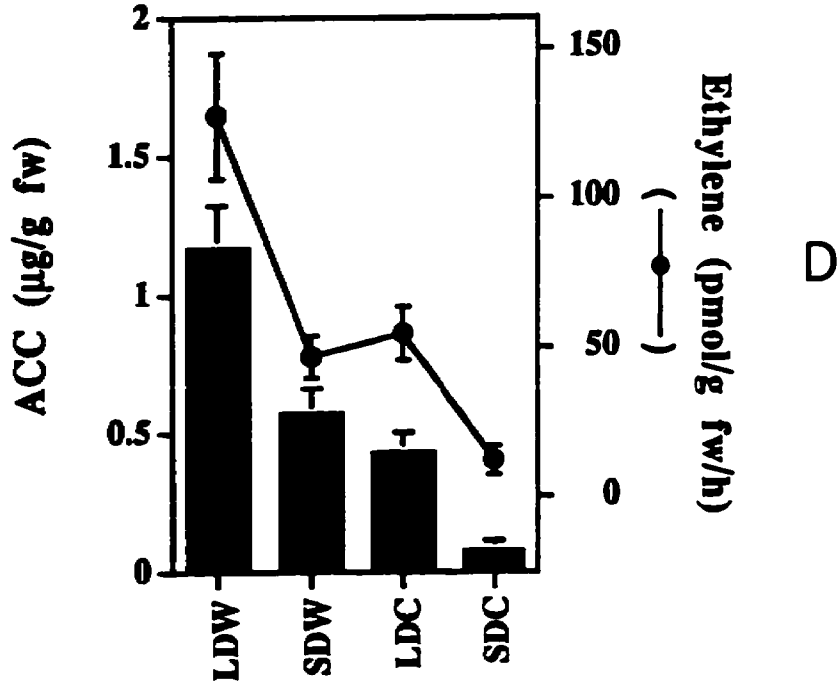
conditions are not found under LDW. Similarly the transcripts that are observed under LDW do not appear under SDC in both ecotypes (Figs. 5.10B, 5.11B). In contrast, the transcript levels of the control histone 2A variant (H2Av) are constant under both conditions (Figs. 5.10C, 5.11C). Thus the expression of distinct members of ACC synthase gene family appears to be regulated by different growth conditions. Interestingly the accumulation of LDW specific transcripts of alpine and prairie ecotypes show differences in their temporal regulation. While the LDW specific transcript of alpine ecotype disappeared after about 14 days (Fig. 5.10B), accumulation of LDW specific ACC synthase mRNA of prairie ecotype disappeared only after about 24 days (Fig. 5.11B).

5.3.5. Role of photoperiod and temperature

Since photoperiod and temperature are the two important external cues that govern stem elongation in *S. longipes* (Macdonald *et al.*, 1984), the individual effect of photoperiod and temperature on the differential expression of the ACC synthase gene family was investigated. Alpine plants that were grown under SDC for 3 months were transferred to LDW or LDC or SDW conditions. The steady-state levels of ACC synthase mRNA were monitored in 7 day old plants using 3'-UTRs of ACC synthase clones obtained from SDC and LDW grown plants. Northern analyses show that transcripts that are accumulated under SDC did not appear under SDW or LDC or LDW (Fig. 5.12A). The transcripts that are accumulated under LDW however, were also found under SDW and LDC although the observed levels of accumulation under these conditions are less than that under LDW (Fig. 5.12B). The abundance of the control H2Av mRNA did

Fig. 5.12 (A-D). Effect of photoperiod and temperature on expression of ACC synthase genes (A and B), ACC and ethylene levels (D) in alpine ecotype. Stem and leaf tissues were collected from alpine plants grown under LDW, LDC, SDW and SDC conditions after 7 days. Each lane in the Northern blot contain 10 μg of poly(A)$^+$ RNA. The same blot was hybridized individually with ASWA-3 (A), ALAS-1 (B), and H2Av (C). The size of ACC synthase transcript in A and B is 1.8 Kb, and that of H2Av is 1.0 Kb. Endogenous ACC levels (bars) and ethylene production (line) of plants are shown in D.


A
B
C


ACC (µg/g fw)
Ethylene (pmol/g fw/h)
2
1.5
1
0.5
0
150
100
50
0
LDW
SDW
LDC
SDC
D

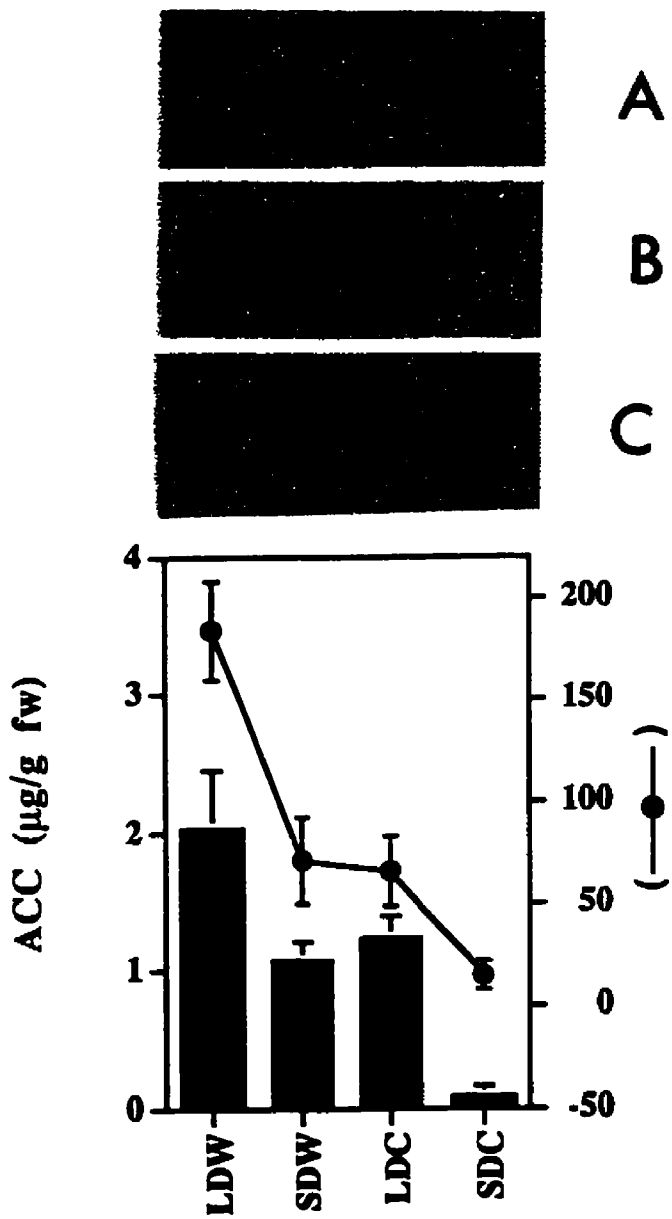
not show any such noticeable changes under the tested conditions (Fig. 5.12C). In a parallel experiment, a similar set of results was obtained in prairie plants (Figs. 5.13A through C).

The endogenous ACC levels in SDC, SDW, LDC and LDW grown plants reflected the changes in expression pattern of ACC synthase members. For example, the ramets of SDC grown alpine plants contained 75 ng/g fw of ACC (Fig. 5.12D). When the plants were transferred to LDW conditions, ACC levels increased dramatically by 16 fold (1.17 μg/g fw). A change in either the temperature or day length also modified the endogenous ACC levels. While the plants grown under SDW showed an 8 fold increase (570 ng/g fw), LDC grown plants showed a 6 fold increase (430 ng/g fw) above the ACC levels observed under SDC (Fig. 5.12D). In parallel with the ACC levels, their ethylene production also showed significant changes. Ethylene production increased from 12.1 pmol/h/g fw in SDC grown plants to 127.4 pmol/h/g fw in LDW grown plants (Fig. 5.12D). Plants grown under SDW and LDC conditions produced intermediate amounts of ethylene 48.9 pmol/h/g fw and 54.6 pmol/h/g fw respectively (Fig. 5.12D). In a parallel experiment, the pattern of changes in ACC levels in prairie plants was also found to be the same (Fig. 5.13D).

5.3.6. ACC synthase mRNA accumulation across a day

Since the activity and transcript accumulation of ACC oxidase in *S. longipes* have been shown to be under the control of a circadian clock (Kathiresan *et al.*, 1996), ACC synthase mRNA abundance was also monitored across a day. Poly(A)$^+$ RNA was extracted from stem and leaf

Fig. 5.13 (A-D). Effect of photoperiod and temperature on expression of ACC synthase genes (A and B), ACC and ethylene levels (D) in prairie ecotype. Stem and leaf tissues were collected from prairie plants grown under LDW, LDC, SDW and SDC conditions after 7 days. Each lane in the Northern blot contain 10 μg of poly(A)$^+$ RNA. The same blot was hybridized individually with ASWA-3 (A), ALAS-1 (B), and H2Av (C). The size of ACC synthase transcript in A and B is 1.8 Kb, and that of H2Av is 1.0 Kb. Endogenous ACC levels (bars) and ethylene production (line) of plants are shown in D.


A
B
C
4
3
2
1
0
200
150
100
50
0
-50
ACC (µg/g fw)
Ethylene (pmol/g fw/h)
LDW
SDW
LDC
SDC
D

tissues that were collected at 6 h intervals over a period of 24 h. The Northern blot was hybridized with a Bgl II/Sal I fragment of SACS-1 that contains coding regions of the ACC synthase active site. The results show no oscillation in ACC synthase transcript levels (Figs. 5.14A, C). On the other hand, the abundance of ACC oxidase mRNA exhibited a typical rhythm as expected (Figs. 5.14B, C). These results were consistent in both alpine and prairie ecotypes (Figs. 5.15A through C).

5.4. Discussion

ACC synthase is a cytosolic enzyme encoded by a divergent gene family in many plant species (Zarembinski and Theologis, 1994). Amino acid sequence analyses of ACC synthase from various plant species reveal only a few conserved domains, their C-termini being the most divergent (Fluhr and Mattoo, 1996). In this study, an ACC synthase genomic fragment was amplified using primers that corresponded to two conserved domains of ACC synthase. Southern hybridizations performed using the exon III of SACS-1, which encodes amino acid residues that were previously shown to constitute the active site in tomato (Yip *et al.*, 1990) and apple (White *et al.*, 1994), as a probe suggests that the genome of *S. longipes* contains approximately 4 gene copies. Despite a relatively high genetic variance between alpine and prairie ecotypes (Cai *et al.*, 1990; Emery *et al.*, 1994a), no polymorphism was observed in Bgl II, Sal I and Bgl II/Sal I digestions.

In each ecotype, an ACC synthase cDNA clone from LDW grown plants and a partial ACC synthase cDNA clone that encodes C- terminal regions and 3'-UTRs from SDC grown plants were obtained.

Fig. 5.14 (A-C). (A) Changes in ACC synthase and ACC oxidase transcript abundance during a period of 24 h in alpine plants. Northern blot containing 10 μg of poly(A)$^+$ RNA per lane was probed with a Bgl II/Sal I fragment of SACS-1. After stripping the SACS-1 probe, the same blot was hybridized with ACC oxidase probe (SlACO-1). (B) The size of ACC synthase transcript in A is 1.8 Kb and that of ACC oxidase in B is 1.2 Kb. (C) Quantification of Northern blot. The filled and open circles represent ACC oxidase and ACC synthase respectively. The maximum value of relative abundance of each mRNA was adjusted to 1.0. The open and dark bars at the bottom indicate light and dark phases respectively.

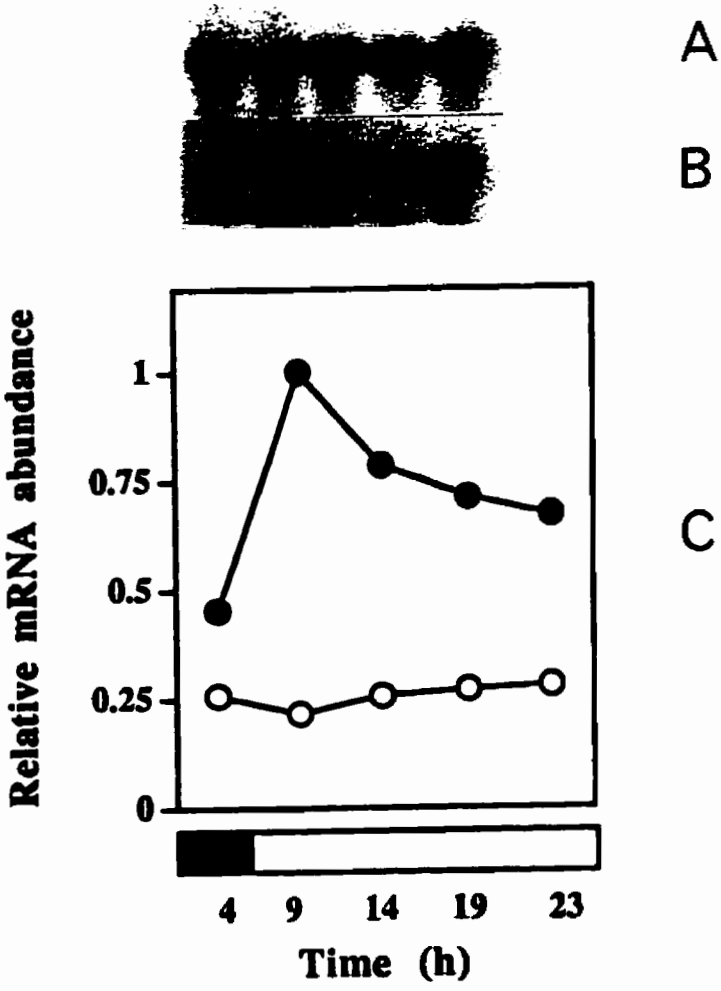
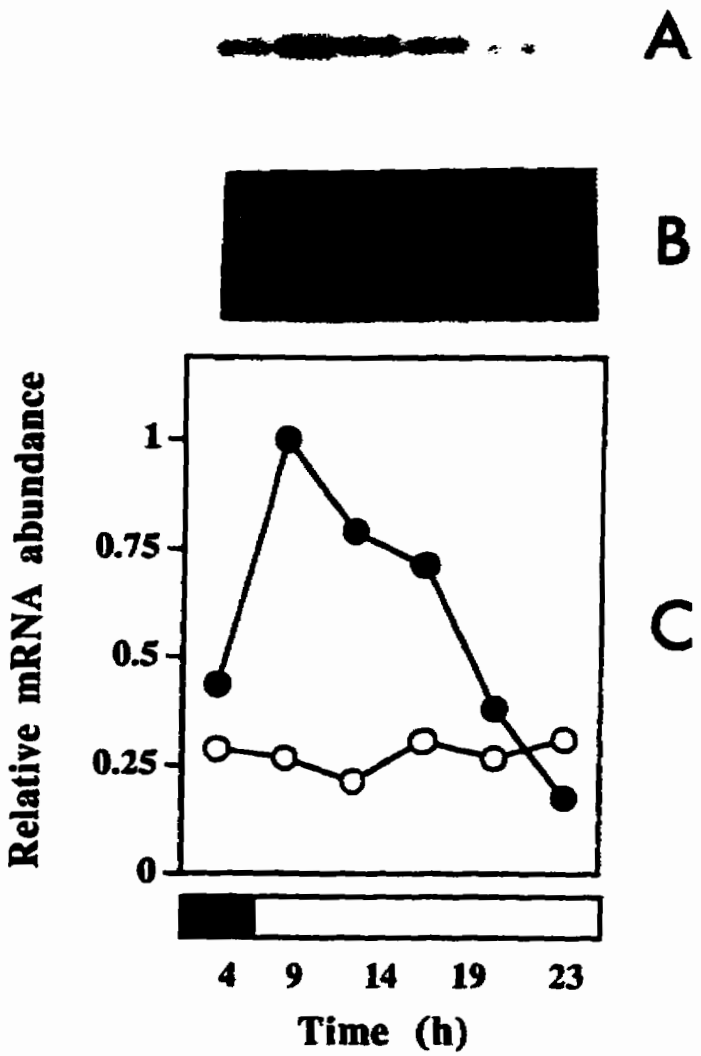

A
B
C
Relative mRNA abundance
1
0.75
0.5
0.25
0
4
9
14
19
23
Time (h)

Fig. 5.15 (A-B). (A) Changes in ACC synthase and ACC oxidase transcript abundance during a period of 24 h in prairie plants. Northern blot containing 10 μg of poly(A)$^+$ RNA per lane was probed with a Bgl II/Sal I fragment of SACS-1. After stripping the SACS-1 probe, the same blot was hybridized with ACC oxidase probe (SlACO-1). (B) The size of ACC synthase transcript in A is 1.8 Kb and that of ACC oxidase in B is 1.2 Kb. Quantification of Northern blot is shown in C. The filled and open circles represent ACC oxidase and ACC synthase respectively. The maximum value of relative abundance of each mRNA was adjusted to 1.0. The open and dark bars at the bottom indicate light and dark phases respectively.


A
B
C
Relative mRNA abundance
1
0.75
0.5
0.25
0
4
9
14
19
23
Time (h)

Although these four clones have similar coding sequences, they show sufficient divergence in their 3'-UTRs to prepare gene specific probes. Northern analysis performed using these gene specific probes indicated that in both ecotypes the gene that is expressed under SDC is not expressed under LDW and vice-versa. These results suggest that photoperiod and temperature are two important external signals that regulate the expression of ACC synthase genes.

Transfer of SDC grown plants to either SDW or LDC abolishes the accumulation of ACC synthase transcripts that are normally found under SDC, suggesting that either the warm temperature or long photoperiod used in the present study is sufficient enough to down regulate the abundance of ACC synthase mRNA that normally accumulates under SDC. Interestingly however, ACC synthase mRNA that accumulated under LDW also appeared under SDW or LDC. Nevertheless, the transcript levels were reduced under SDW and LDC in comparison to that under LDW. These results indicate that long day and warm temperature can independently and synergistically upregulate the steady state levels of ACC synthase mRNA that is accumulated under LDW. Such an upregulation of ACC synthase transcript levels might be responsible for the corresponding increase in tissue ACC levels and ethylene production observed under these conditions. These results also positively correlate with the stem elongation pattern observed under SDC, SDW, LDC and LDW by Macdonald *et al.* (1984).

Besides the length of stem, the period of stem elongation is also longer in prairie ecotype (over 4 weeks) than in alpine ecotype (2-3 weeks) under LDW (Emery *et al.*, 1984). The prolonged duration of stem

elongation in prairie ecotype is an adaptive strategy to avoid shading by the other competing plants in the habitat. In contrast, at higher altitude, the alpine plants are dwarf as they are faced with lesser density of competing plants and greater wind stress (Emery *et al.*, 1994a). Other workers have shown that adaptive plasticity in stem elongation improves the fitness of survival in *Nicotiana tabacum* and *Brassica rapa* (Schmitt *et al.*, 1995), and *Impatiens capensis* (Dudley and Schmitt, 1996). In *S. longipes*, Emery *et al.* (1994b) observed significant increases in ethylene production and ACC levels at the early stages of their stem elongation. The increased ACC and ethylene levels were maintained throughout the period of stem elongation as well as flowering in both ecotypes (Emery, 1994; Emery *et al.*, 1994b). Consistent with this observation, the temporal pattern of ACC synthase mRNA abundance under LDW is relatively stable and prolonged in prairie ecotype. However, newly accumulated ACC synthase transcripts of the alpine ecotype disappeared after 14 days indicating that upregulation of a different ACC synthase gene and/or release of ACC from ACC conjugates (Abeles *et al.*, 1992; Fluhr and Mattoo, 1996) might be responsible for maintenance of ACC levels observed during the late stem elongation and/or flowering phases of alpine plants (Emery, 1994; Emery *et al.*, 1994b; Emery *et al.*, 1997).

Although ACC oxidase is regulated by a circadian clock (Kathiresan *et* al., 1996), Emery *et al.* (1997) recently showed that ACC levels in these tissues do not oscillate and therefore regulation of ACC oxidase might be responsible for fluctuations in ethylene production in *S. longipes*. In contrast, Machackova *et al.* (1997) observed a pronounced endogenous rhythm for ACC levels in *Chenopodium rubrum*. Results from this study

however show no oscillation in ACC synthase transcript levels during a day and thus supports the notion that regulation of ACC oxidase is more likely responsible for rhythmic ethylene production in *S. longipes*.

Several eukaryotic proteins are encoded by gene families. Although the encoded protein isoforms are often functionally similar, they can be distinguished by their characteristic kinetic parameters such as Km, Ki, pI, etc. Such differences are thought to carry some physiological significance for the cell types in which they are expressed (Ohta, 1990). Li and Mattoo (1994) demonstrated that the hypervariable C-terminus of a tomato ACC synthase is critical for regulating the enzymatic activity and dimerization. Similarly, White *et al.* (1994) found a decrease in *kcat/Km* and an increase in Km for SAM in a single amino acid transition mutant (Arg-407 to Lys) of apple ACC synthase *in vitro*.

In many plant systems, it has been difficult to study the biochemical differences between ACC synthase isozymes. The low level of ACC synthase gene expression and the low recovery of active ACC synthase by the available extraction procedures necessitate a large amount of tissues (usually in Kilograms). This is particularly difficult in *S. longipes* as a potful of clonally propagated plants weigh on an average about 15.0 g. Furthermore, quantification of the enzyme byproduct, ACC, is done either using HPLC-GCMS or indirectly by measuring the hypochlorite mediated oxidation of ACC. Given the limited amount of ACC synthase activity in unwounded tissues, it is difficult to differentiate the kinetic parameters through elaborate ACC synthase assays. Heterologous expression studies in *E. coli* (White *et al.*, 1994) also has faced inconsistency in rescuing

enzymatic activity. Crystallographic studies have shown that ACC synthase is a homo dimeric protein (Hohenester *et al.*, 1994). The difficulty in functional expression of ACC synthase is thought to be due to improper configuration and dimerization of expressed protein in heterologous systems (White *et al.*, 1994). Although the present study did not study the biochemical differences between the putative ACC synthase isoforms, the four ACC synthase cDNA clones isolated in the present study show significant divergence at their C-termini and therefore might have different catalytic characteristics, as judged by the ACC and ethylene levels in the tissues in which they are expressed. Since ethylene production partially determines the stem elongation plasticity in *S. longipes*, it is proposed that differential regulation of ACC synthase mRNAs might confer some positive selective advantage to plants in their natural habitats.

In summary, a genomic fragment that encodes ACC synthase was cloned from the prairie ecotype of *S. longipes*. Southern analysis suggests that ACC synthase is encoded by a small gene family comprised of approximately 4 members in both ecotypes. Four unique ACC synthase cDNA clones under different growth conditions from alpine and prairie ecotypes of *S. longipes* were isolated. Northern analyses suggest that ACC synthase genes are differentially and synergistically regulated by photoperiod and temperature. The differential regulation of ACC synthase genes positively correlate with the levels of ACC, ethylene (this study) and stem elongation pattern reported by Macdonald *et al.* (1984). It is proposed that differential regulation of ACC synthase genes may represent one of the underlying molecular mechanisms of phenotypic plasticity in *S. longipes*.

CHAPTER SIX

γ-Aminobutyric Acid Stimulates Ethylene Biosynthesis and Stem Elongation

6.1. Introduction

Photoperiod and temperature are the two important external cues that regulate stem elongation in *S. longipes* (Macdonald *et al.*, 1984). The increase in ethylene production during stem elongation and the dependence of stem elongation on ethylene synthesis (Emery, 1994) might suggest that induction of ethylene biosynthesis is regulated by some as yet unknown cellular intermediates that can signal the changes in photoperiod and temperature. Despite many studies on regulation of ACC synthase and ACC oxidase in various plant systems, our knowledge on the upstream signals that transduce the environmental cues has been lacking.

A large number of studies has shown that plants produce ethylene in response to various biotic and abiotic stress conditions (Abeles *et al.*, 1992). Depending on plant species, type of tissues, and type of stress involved, the time taken by tissues to produce ethylene following the stress stimulus might vary from 15 min to a few hours (Abeles *et al.*, 1992). Plants also accumulate a non-protein amino acid called γ-aminobutyric acid (GABA) under those stress conditions in which ethylene is produced. For example, plants can accumulate considerable amounts of GABA under mechanical stress (Wallace *et al.*, 1984; Ramputh and Bown, 1996), hypoxia (Streeter and Thompson, 1972; Roberts *et al.*, 1992; Shelp *et al.*, 1995), cold shock (Cholewa *et al.*, 1997), darkness (Wallace *et al.*, 1984), heat shock (Mayer

et al., 1990), and water stress (Rhodes *et al.*, 1986). In most instances, the synthesis and accumulation of GABA is very rapid. For instance, GABA levels in mechanically damaged soybean leaves increase by 10 to 25 fold within 1 to 4 min of the start of the stimulus (Ramputh and Bown, 1996). Thus it would appear that GABA accumulation precedes any increase in ethylene production. Interestingly, one conceivable conformation of the GABA molecule could mimic ACC and therefore might share common binding sites on interacting proteins. Furthermore, α-aminoisobutyric acid (AIBA), an analog of GABA, can competitively inhibit the conversion of ACC to ethylene (Satoh and Esashi, 1980a). It is therefore tempting to hypothesize that GABA might influence ethylene production.

In higher plants, as in vertebrates, GABA is commonly synthesized from 2-oxoglutarate via glutamate. 2-oxoglutarate is converted to glutamate by glutamate dehydrogenase (GDH). Glutamate is then decarboxylated to GABA by glutamate decarboxylase (GAD). GAD has recently been identified as a Ca^{2+}/calmodulin dependent enzyme (Baum *et al.*, 1993; Snedden *et al.*, 1996). Regulation of GAD is under developmental control (Chen *et al.*, 1994) and has recently been proposed to play an important role in normal plant growth and development (Baum *et al.*, 1996) and fruit ripening (Gallego *et al.*, 1995). GABA can be shunted back to Kreb's cycle as succinic acid (Bown and Shelp, 1989 and 1997).

Recent studies have demonstrated that transgenic tobacco plants overexpressing active GAD accumulate high levels of free GABA and show severe abnormalities in growth and development. For example, stems of

these transgenic plants are severely stunted and show inhibition of cell elongation (Baum *et al.*, 1996). Such observations indicate that regulation of GABA biosynthesis might be important in controlling stem elongation.

In this study the following possibilities were investigated; (i) an increase in GABA might lead to an increase in ethylene production (ii) GABA might either act itself as a precursor for ethylene, or might indirectly promote ethylene production via the known ethylene biosynthetic pathway and (iii) GABA might influence stem elongation by regulating ethylene production.

6.2. Materials and Methods

6.2.1. Plant materials

Plants of *S. longipes* were grown in soil or *in vitro* as described in section 2.1. Shoot tips grown *in vitro* were subcultured in MS media (section 2.1) supplemented with various concentrations of GABA, L-glutamate, L-alanine or succinate. The concentration of aminoethoxyvinylglycine (AVG) and silver thiosulfate (STS) used were 10 and 25 μM respectively. After 21 days in culture, plants were carefully removed and used for measurements of internodal and stem lengths or immediately frozen in liquid N_2 and stored at -70° C for later GABA extraction.

Seeds of sunflower (*Helianthus annus* L.) var Dahlgren 131 were germinated in terragreen in a growth chamber with 16 h photoperiod of 75 μmol $m^{-2} s^{-1}$ under 22° C day and 18° C night temperature cycle.

Cotyledons, hypocotyls and primary leaves from 6 to 8 day old seedlings were excised and after the specified chemical treatment the tissues were either immediately incubated for ethylene measurements or frozen in liquid nitrogen and stored at -80° C for later extraction of RNA, ACC, and ACC oxidase separately.

6.2.2. Chemicals and incubation for ethylene production

The pH of all the solutions used for incubation of sunflower tissues was adjusted to 7.0. Excised sunflower tissues were incubated under dark with different concentrations of GABA, AIBA, AVG, L-glutamate, L-alanine or succinate in petri plates. Tissues were then collected at different time intervals and incubated in 10-ml syringes with the plungers adjusted to 6 ml. After 40 min of incubation, a 3-ml gas sample was transferred to a second syringe via a three-way valve and analyzed for ethylene content.

Effect of GABA on ethylene production in *S. longipes* was measured on day 2 in plants grown *in vitro*. Plants were gently removed from the culture at 15:00 h. After excising the roots, the shoots were immediately incubated in gas tight 10 ml syringes. After 20 min of incubation, a 3-ml gas sample was transferred to a second syringe through a three-way valve, and the amount of ethylene in the sample was analyzed.

6.2.3. Experiments with [^{14}C] GABA

Approximately 6 g of excised sunflower cotyledons were incubated with 52.5 KBq of G-[^{14}C(U)]- aminobutyric acid (1.85 MBq/μmole; DuPont,

NEN products, Boston, MA, USA) in a gas-tight 50-ml glass syringe with a teflon-faced plunger. At different time intervals, the CO_2 and C_2H_4 evolved from the tissues were trapped in separate vials each containing 10 ml of Carbo-sorb® (Packard Instrument Co., Downers grove, IL, USA) and mercuric perchlorate respectively. The radioactivity in CO_2 and C_2H_4 trapping solutions was determined using a Packard TriCarb 2200CA liquid scintillation analyzer. To quantify the amount of ethylene released from tissues, the ethylene trapped by mercuric perchlorate was released by mixing vigorously with 2M LiCl solution in a 10-ml test tube capped with a rubber septum, and 1-ml of headspace sample was analyzed for ethylene content using gas chromatography as described earlier.

6.2.4. ACC extraction and *in vitro* ACC oxidase assay

About 1 g of sunflower tissue was extracted twice at 70° C in 5 ml of 80% ethanol for 15 min each. The extracts were then dried *in vacuo* at 40° C and dissolved in 2 ml of distilled water. ACC was then determined as described in section 2.2.2.2. ACC oxidase extraction and assay procedures for sunflower has previously been described by Finlayson and Reid (1994). The V_{max} of ACC oxidase was estimated using 3 mM ACC at 5% CO_2.

6.2.5. Cloning of sunflower ACC synthase gene fragment

Genomic DNA was extracted from sunflower cotyledons as described in section 2.2.1.1.1. Using degenerate primers namely N1F, N2F, C1R, and C2R (section 5.2.2), a 1.1 Kb secondary PCR fragment (HACS-1) was obtained. HACS-1 was ligated into pBluescript (Stratagene) and sequenced.

HACS-1 is 1008 bp long and contains an intron (Genbank accession number U54789). Upon conceptual translation, the coding regions of HACS-1 shows a high degree (80 to 95%) of homology to known ACC synthase sequences from other species at the amino acid level.

6.2.6. Northern hybridization

The control 18S rRNA probe was made by labeling a 1.6 Kb Xba I-EcoR I fragment of pGmr-1 (from Dr. E.A. Zimmer, National Museum of Natural History, Smithsonian Institution, Suitland, MD, USA). The probe for ACC oxidase in sunflower was generated from HaACO-1 (Genbank accession number L29405), a 1.0 Kb fragment encoding ACC oxidase in sunflower (obtained from Dr. J. -H. Liu, Lethbridge Research Center, Agriculture and Agri-Food Canada, Lethbridge). For blots containing RNA from *S. longipes*, histone 2A variant (H2Av) of *Arabidopsis thaliana* (Genbank accession no. Z26465) was used as a control probe and exon III (Bgl II/ Sal I fragment) of SACS-1 was used as ACC synthase probe. After hybridization, membranes were washed twice with 2x SSPE plus 0.1% SDS at RT, twice with 1x SSPE plus 0.1% SDS at 65° C for 30 min, and twice with 0.1x SSPE plus 0.1% SDS at 65° C for 30 min.

6.3. Results

The role of GABA on ethylene biosynthesis was studied by treating excised plant tissues with natural and isotopic GABA over a period of time. This was a difficult task with the excised stem and leaf tissues of *S. longipes* as they tend to wilt quickly. Recently Miranda *et al.* (1997) developed a

tissue culture system to raise plantlets from young shoot tips of *S. longipes*. The effect of GABA on ethylene production was studied by supplementing the media with various concentrations. However the subtle differences in the timing of diurnal ACC oxidase and ethylene oscillations in individual ramets imposed some difficulties in analyzing all the details of the effect of GABA on ethylene production. Furthermore, one fully grown ramet of *S. longipes* weighs about 0.10 g which is often less than the amount of tissues needed for incubation. Hence, hardy tissues such as cotyledons, hypocotyls and broad primary leaves of sunflower (*Helianthus annus* L.) were used as a substitute system for *S. longipes* to investigate in depth the effect of GABA on ethylene biosynthesis. Sunflower has long been used as a model system to study ethylene physiology in the laboratory of Dr. David M. Reid, Department of Biological Sciences, University of Calgary. Excised cotyledons, hypocotyls and leaves of sunflower have been shown to withstand long periods of incubation at room temperature (Holbrook *et al.*, 1997). Ethylene production in these tissues can be stimulated by various hormonal and chemical stimuli. Even developmental events such as adventitious rooting have been shown in excised sunflower hypocotyl tissues in response to ethylene and other hormones. The recent cloning of ACC oxidase cDNA (Liu and Reid, 1994; Liu *et al.*, 1997) and ACC synthase gene (Kathiresan *et al.*, 1997a) allow molecular analyses of regulation of ethylene biosynthesis possible in this system. A great deal of information is also available on *in vitro* ACC oxidase assay, ACC and ethylene levels in various tissues of young sunflower seedlings (Finlayson, 1993). All these features make sunflower an attractive substitute system to study the role of GABA on ethylene production.

6.3.1. GABA promotes ethylene production

The ethylene production observed in excised sunflower cotyledons treated with different concentrations of GABA after 16 h of treatment is shown in Fig. 6.1. Ethylene production increased with rising GABA concentration up to 100 mM after which no further increase on ethylene was seen. Parallel treatments done using 100 mM each of glutamate, succinate or alanine however did not stimulate ethylene production (Fig. 6.2).

In separate experiments, a similar response was also observed for excised hypocotyl segments and primary leaves of sunflower. The magnitude of the response to GABA was more pronounced in cotyledons and leaves than in hypocotyls. The increase in the rate of ethylene production in cotyledons and leaves was not seen until approximately 12 h after the addition of GABA (Fig. 6.3). By 16 h, the ethylene synthesis rate raised by about 14 fold in these two tissues, and by only about 5 fold in hypocotyls (Fig. 6.3). The ethylene response of hypocotyls begins to diminish after 16 h of treatment, while that of cotyledons and leaves was maintained.

GABA also promoted ethylene production in *S. longipes*. Plants grown at different GABA concentrations *in vitro* were measured for their ethylene production on day 2. As seen in Fig. 6.4, alpine plants grown at 100, 250, and 500 μM GABA concentrations did not show any significant

Fig. 6.1. Ethylene production in sunflower cotyledons treated with different concentrations of GABA. Excised sunflower cotyledons were incubated under dark with various concentrations of GABA for 16 h, and analyzed for their rate of ethylene production. Data are mean of at least 8 observed values. Vertical bars indicate ± S.E. where larger than symbols.

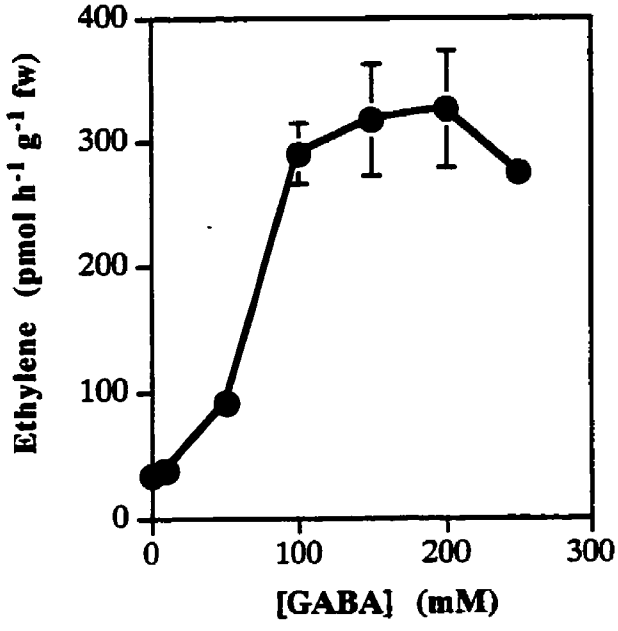

400
300
200
100
0
Ethylene (pmol h-1 g-1 fw)
0
100
200
300
[GABA] (mM)

Fig. 6.2. Effect of L-glutamate, L-alanine, GABA, and succinate on ethylene production in sunflower cotyledons. Cotyledons of 7 day old seedlings were excised and incubated with 100 mM each of L-glutamate (A), L-alanine (B), GABA (C), succinate (D) or water (E), and ethylene production was measured after 16 h. Data are mean of 8 observed values and representative of 3 individual experiments. Vertical bars represent ± S.E.

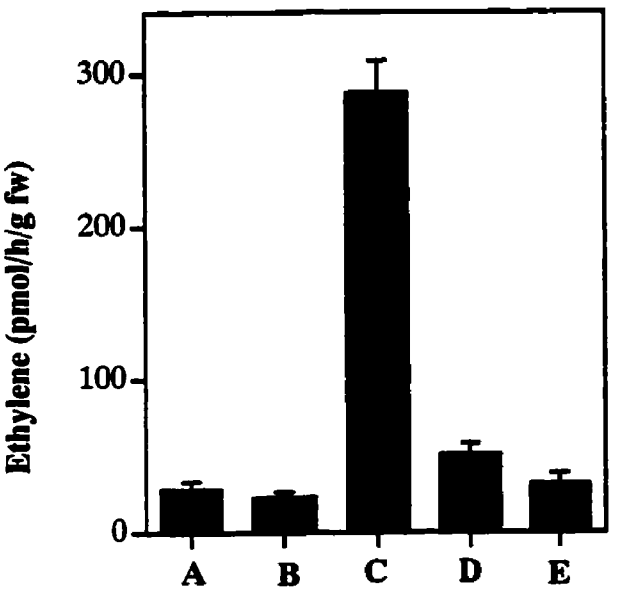

Ethylene (pmol/h/g fw)
300
200
100
0
A
B
C
D
E

Fig. 6.4. Response of sunflower hypocotyls (O), cotyledons (●), and primary leaves (□) to 100 mM GABA over a period of time. The excised tissues were incubated under dark, collected at different time intervals and their ethylene production rates were monitored. Data are mean of at least 8 observed values. Vertical bars indicate ± S.E. where larger than symbols.

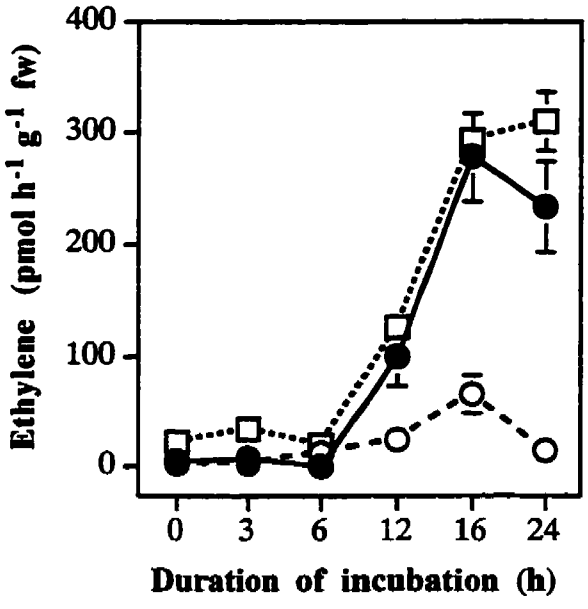
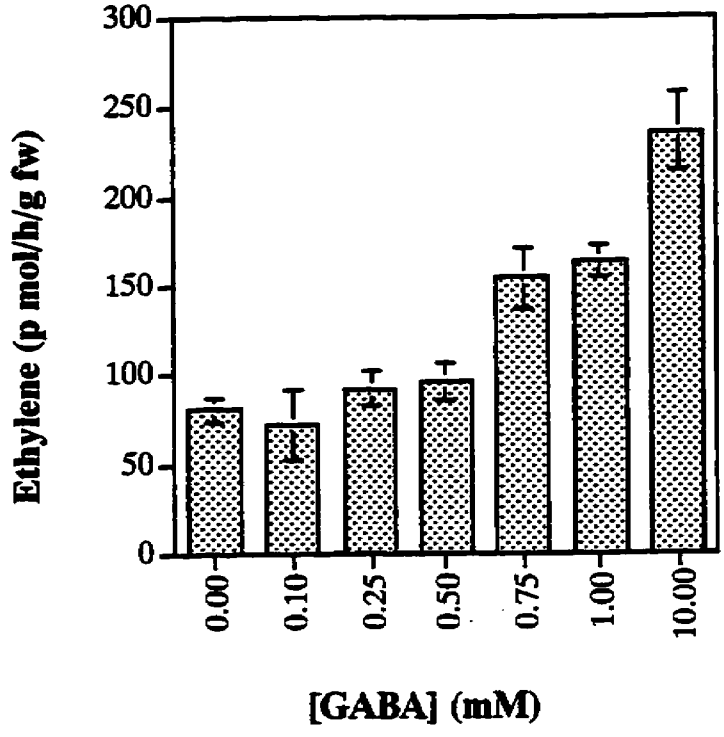

400
300
200
100
0
Ethylene (pmol h-1 g-1 fw)
0
3
6
12
16
24
Duration of incubation (h)

Fig. 6.4. Effect of GABA on ethylene production in *S. longipes*. Shoot tips of alpine plants cultured in MS media supplemented with GABA were monitored for ethylene production on day 2. Each datum is a mean of at least six observed values.


300
250
200
150
100
50
0
Ethylene (p mol/h/g fw)
0.00
0.10
0.25
0.50
0.75
1.00
10.00
[GABA] (mM)

increase in ethylene production over control plants which produced about 83.16 pmol/g/fw. However plants grown at 0.75, 1.0 and 10.0 mM GABA concentrations produced more ethylene than the control plants (Fig. 6.4). A maximum of six fold increase in ethylene production (235.68 pmol/g fw/h) over control plants was observed in plants that were grown at 10.0 mM GABA concentration (Fig. 6.4).

6.3.2. GABA accumulation in treated tissues

Endogenous GABA levels were measured in 100 mM GABA treated sunflower tissues over a period of time. As seen in Fig. 6.5, the GABA level in these tissues increases gradually and reaches 7 fold by about 12 h, when ethylene production begins to rise. The endogenous GABA level in control tissues remained fairly constant. Similarly, the free endogenous levels of GABA in stem and leaf tissues of *S. longipes* grown at different concentrations of GABA *in vitro* are shown in Fig. 6.6. There is a linear relationship between the endogenous GABA levels and the concentration of GABA in the media. The plants that produced the maximum ethylene production contained upto a six fold increase in endogenous GABA (0.16 μ mole/g fw) over the control plants (0.047 μ mole/g fw) (Fig. 6.6).

6.3.3. GABA is not an alternate precursor for ethylene

To determine whether GABA drives ethylene biosynthesis by somehow enriching the pool of ethylene precursors and/or itself serving as an alternate precursor for ethylene, [^{14}C]GABA was fed to excised sunflower cotyledons and the induced ethylene was tested for radioactivity. No

Fig. 6.6. Quantitative determination of endogenous GABA. Excised sunflower cotyledons incubated with either 100 mM GABA (●) or distilled water (○), and the endogenous GABA levels were estimated by GABAse assay or HPLC as described in the materials and methods over a period of time. Vertical bars represent ± S.E where larger than symbol.

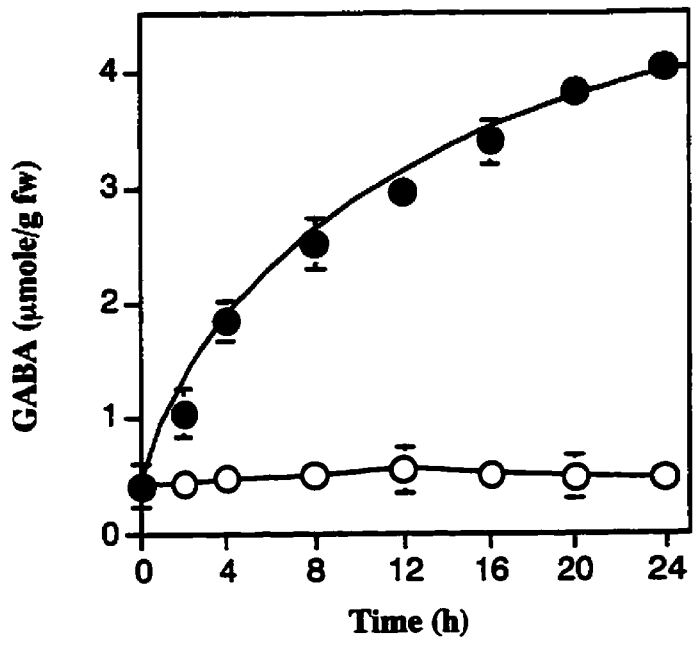
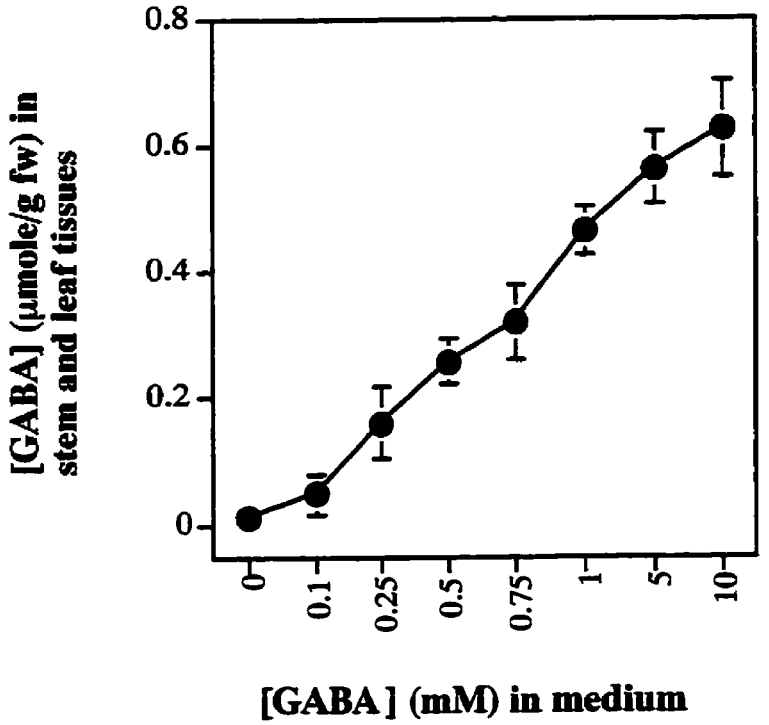

GABA (μmole/g fw)
0
1
2
3
4
0
4
8
12
16
20
24
Time (h)

Fig. 6.6. Endogenous GABA levels in GABA treated plantlets of *S. longipes*. Shoot tips raised in media supplemented with various GABA concentrations were measured for GABA content using GABAse assay on day 2. The data are mean of at least five observed values and are representative of three separate assays.


0.8
0.6
0.4
0.2
0
0
0.1
0.25
0.5
0.75
1
5
10
[GABA] (μmole/g fw) in stem and leaf tissues
[GABA] (mM) in medium

substantial amount of radioactive ethylene was detected in the head space above the cotyledons treated with [^{14}C]GABA (Table 6.1). However a much larger amount of $^{14}CO_2$ was detected, indicating that the tissues actively metabolized [^{14}C]GABA (Table 6.1). These results thus indicated that GABA might have increased ethylene production by influencing the known ethylene biosynthetic pathway i.e. SAM → ACC → Ethylene.

6.3.4. AVG and AIBA inhibit GABA induced ethylene production

In the presence of AIBA (1.0 mM), a structural analog of ACC, GABA was unable to stimulate ethylene production in sunflower cotyledons (Fig. 6.7). Similarly 10.0 mM AVG (an inhibitor for ACC synthase) treatment eliminates the effect of GABA on ethylene production (Fig. 6.7). This indicates that GABA is promoting the flux through ACC, the usual precursor for ethylene.

6.3.5. Changes in ACC levels and *in vitro* ACC oxidase activity

As shown in Table. 6.2, the relative ACC levels in GABA-treated primary leaves were approximately 16 fold higher than in the corresponding control tissues, and only about 7 fold higher in GABA-treated cotyledons and hypocotyls.

Enzyme extracts of sunflower cotyledons were assayed for ACC oxidase activity under *in vitro* conditions. While the enzyme could actively convert 5.0 mM ACC to ethylene, no ethylene was detected when GABA alone was added to the assay tubes (Fig. 6.8). In the presence of saturating

Table. 6.1. Incorporation of ^{14}C from [^{14}C]GABA into $^{14}CO_2$ and $^{14}C_2H_4$. Sunflower cotyledons were fed with G-[^{14}C(U)]- aminobutyric acid in a gas tight syringe. The gases were collected at different time intervals and monitored for their radioactivity.

Time incubated (h)	$^{14}C_2H_4$ (% incorporation)	$^{14}CO_2$ (% incorporation)
0	0.02	0.3
0.5	0.05	1.1
1	0.06	8.1
5	0.005	8.6
24	0.04	9.9
30	0.001	12.7

Fig. 6.7. Effect of GABA in the presence and absence of inhibitors of ethylene synthesis. Excised sunflower cotyledons were incubated without or with 100 mM GABA, 100 mM GABA plus 1.0 mM AIBA, and 100 mM GABA plus 10 mM AVG. Data are mean of at least 8 observed values. Vertical bars indicate ± S.E. where larger than symbols.

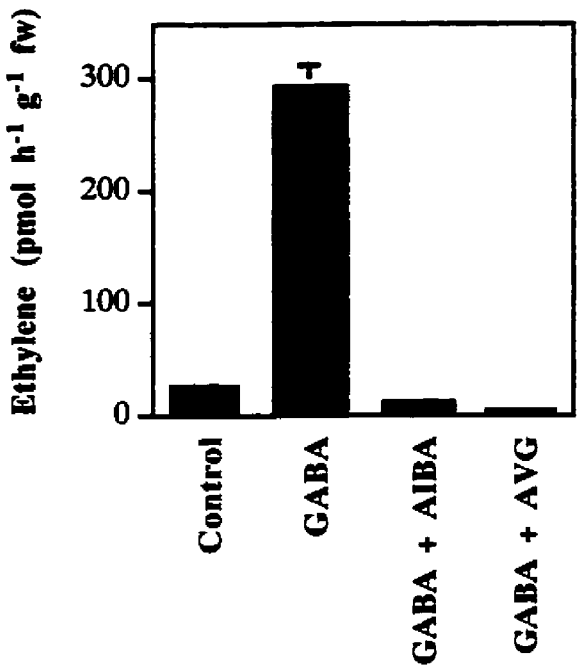

Ethylene (pmol h-1 g-1 fw)
300
200
100
0
Control
GABA
GABA + AIBA
GABA + AVG

Table. 6.2. Effect of GABA on ACC levels in GABA-treated tissues. Excised primary leaves, cotyledons, and hypocotyls of sunflower were incubated in 100 mM GABA or distilled water (control) for 24 h under dark, and their ACC contents were estimated. Data indicate the means of 6 observed values in ng of ACC per g of fresh tissues ± SE.

Tissues	Control	GABA (100 mM) treated
Leaves	20.34 ± 1.97	311 ± 37.51
Cotyledons	8.65 ± 1.61	60.22 ± 20.71
Hypocotyls	2.87 ± 0.98	22.67 ± 4.99

ACC concentration (5 mM), AIBA inhibited ACC oxidase activity where as GABA did not show any effect on ACC oxidase activity *in vitro* (Fig. 6.8). *In vitro* ACC oxidase activity was monitored in GABA-treated sunflower cotyledons. The V_{max} of ACC oxidase in GABA-treated tissues was approximately 3 fold higher than that observed in control tissues (Fig. 6.9A). This result may reflect the amount of ACC oxidase present in these tissues. The effect of GABA on ACC oxidase activity was less clear in *S. longipes*. As seen in fig. 6.9B, there is a slight increase in V_{max} after 4 h of GABA treatment. The cycling of ACC oxidase activity however, was not affected by the GABA treatment (Fig. 6.9B).

6.3.6. ACC synthase and ACC oxidase transcript abundance

To study the effect of GABA on transcript accumulation for ACC synthase and ACC oxidase, total RNA was isolated from sunflower cotyledons, primary leaves and hypocotyls after 16 h of GABA treatment, when their ethylene production rates reached the maximum (Fig. 6.3). Northern blot analyses clearly show a substantial increase in both ACC synthase (Fig. 6.10A) and ACC oxidase (Fig. 6.10B) transcript accumulation. In contrast, 18S rDNA show fairly constant transcript levels (Fig. 6.10C). Thus these results indicate that GABA stimulates the transcript abundance of both ACC synthase and ACC oxidase. Increase in ACC synthase transcript levels was also observed in *S. longipes* grown at different GABA concentrations *in vitro*. The results show that in parallel to their ethylene production, there is an increase in ACC synthase mRNA accumulation in GABA treated alpine (Fig. 6.11) and prairie plants (Fig. 6.12).

Fig. 6.8. The effect of GABA on ACC oxidase activity *in vitro*. ACC oxidase was extracted from sunflower cotyledons, and assayed under *in vitro* conditions for their capacity to produce ethylene in the presence of 5.0 mM ACC alone, 100.0 mM GABA alone, 5.0 mM ACC plus 1.0 mM AIBA, and 5.0 mM ACC plus 100.0 mM GABA. Data are mean of at least 8 observed values. Absence of symbol for GABA treatment indicates no ethylene production. Vertical bars indicate ± S.E. where larger than symbols.

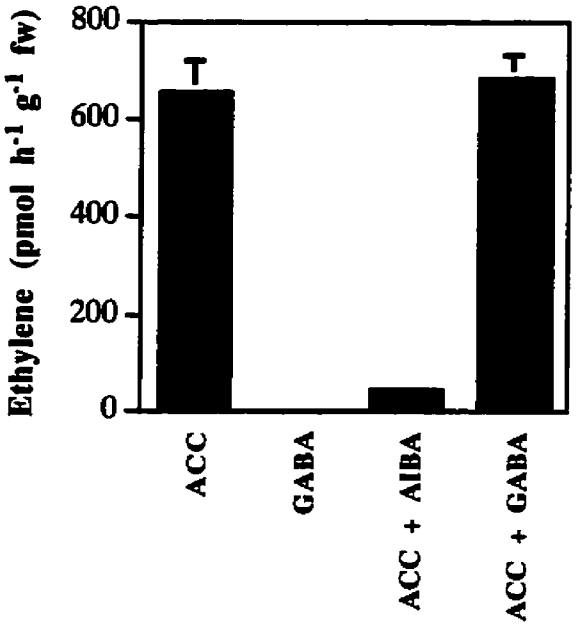
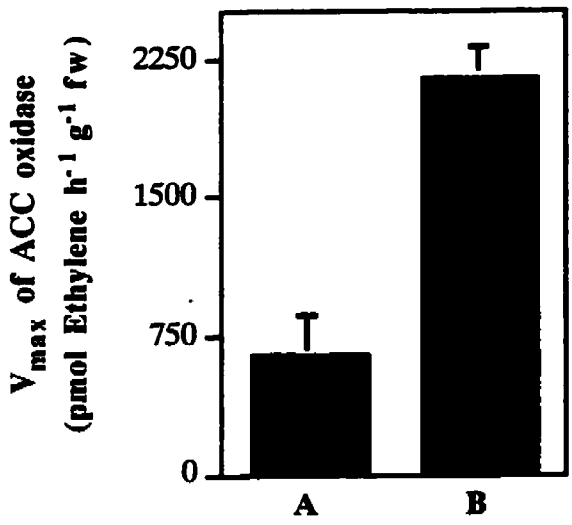

Ethylene (pmol h-1 g-1 fw)
800
600
400
200
0
ACC
GABA
ACC + AIBA
ACC + GABA

Fig. 6.9. Changes in V_{max} of ACC oxidase in GABA-treated tissues. *Upper panel*: The V_{max} of ACC oxidase extracts from sunflower cotyledonary tissues treated with distilled water (a) or with 100 mM GABA (b) was estimated using 5.0 mM ACC. The data are the mean of six replicates, and are representative of three separate assays.
Lower panel: The V_{max} of ACC oxidase extracted from 2 days old GABA treated plantlets collected at various time intervals during light period. Vertical bars indicate ± S.E.

A


V_{max} of ACC oxidase
(pmol Ethylene h^{-1} g^{-1} fw)
2250
1500
750
0
A
B

B

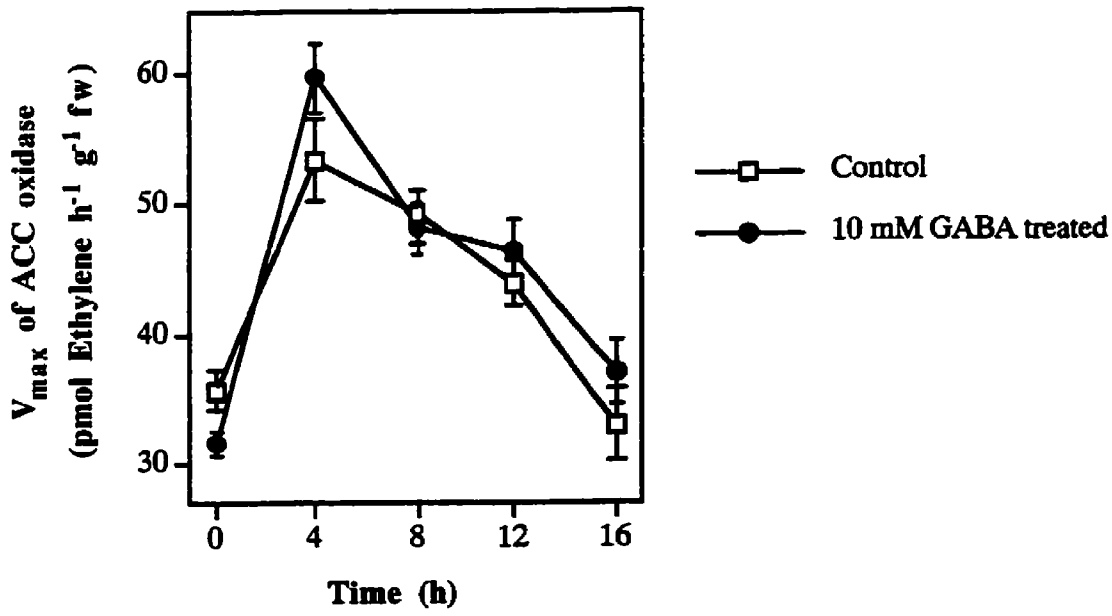

V_{max} of ACC oxidase
(pmol Ethylene h^{-1} g^{-1} fw)
60
50
40
30
0
4
8
12
16
Time (h)
Control
10 mM GABA treated

Fig. 6.10 (A-C). Effect of GABA on ACC synthase and ACC oxidase transcript abundance in various sunflower tissues. The Northern blot containing total RNA (15 μg per lane) from tissues treated without (-) and with (+) GABA was probed with HACS-1 (A). After stripping the probe, the blot was hybridized with either HaACO-1 (B) or 18S rDNA probe prepared from pGmr-1 (C). The size of ACC synthase transcript in A is about 2.0 Kb, ACC oxidase transcript in B is about 1.3 Kb, and 18S rRNA in C is about 1.8 Kb.

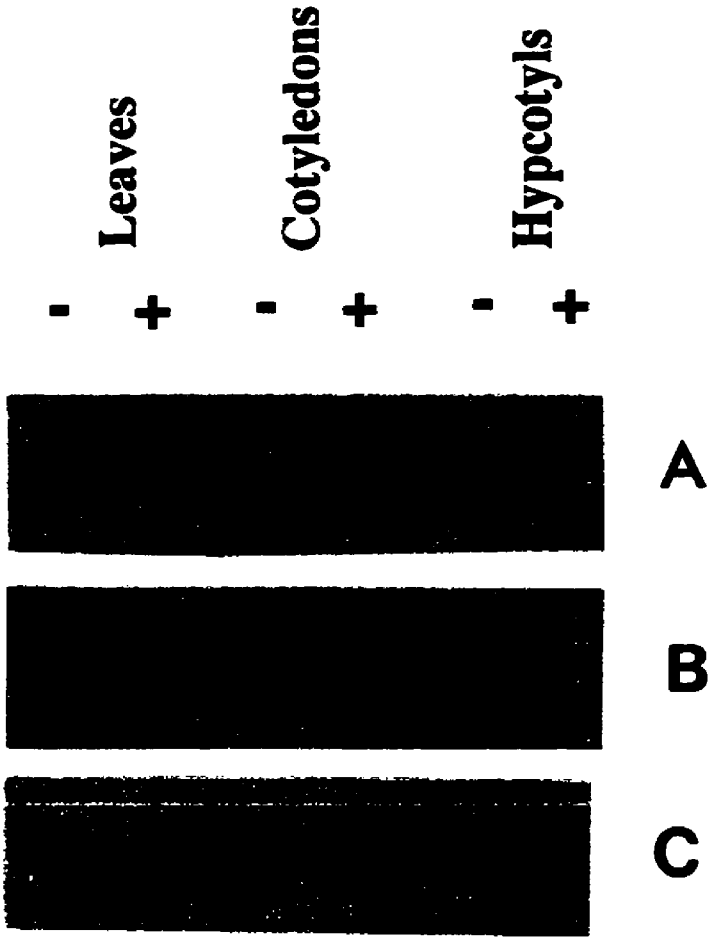

Leaves
Cotyledons
Hypcotyls
- + - + - +
A
B
C

Fig. 6.11 (A-C). Effect of GABA on ACC synthase transcript abundance in alpine plants. The Northern blot containing 10 μg of poly(A)$^+$ per lane from tissues treated with various concentrations of GABA was probed with exon III of SACS-1 (C). After stripping the probe, the blot was hybridized with H2Av (B). The ethylene production observed in the corresponding treatments are shown in A.

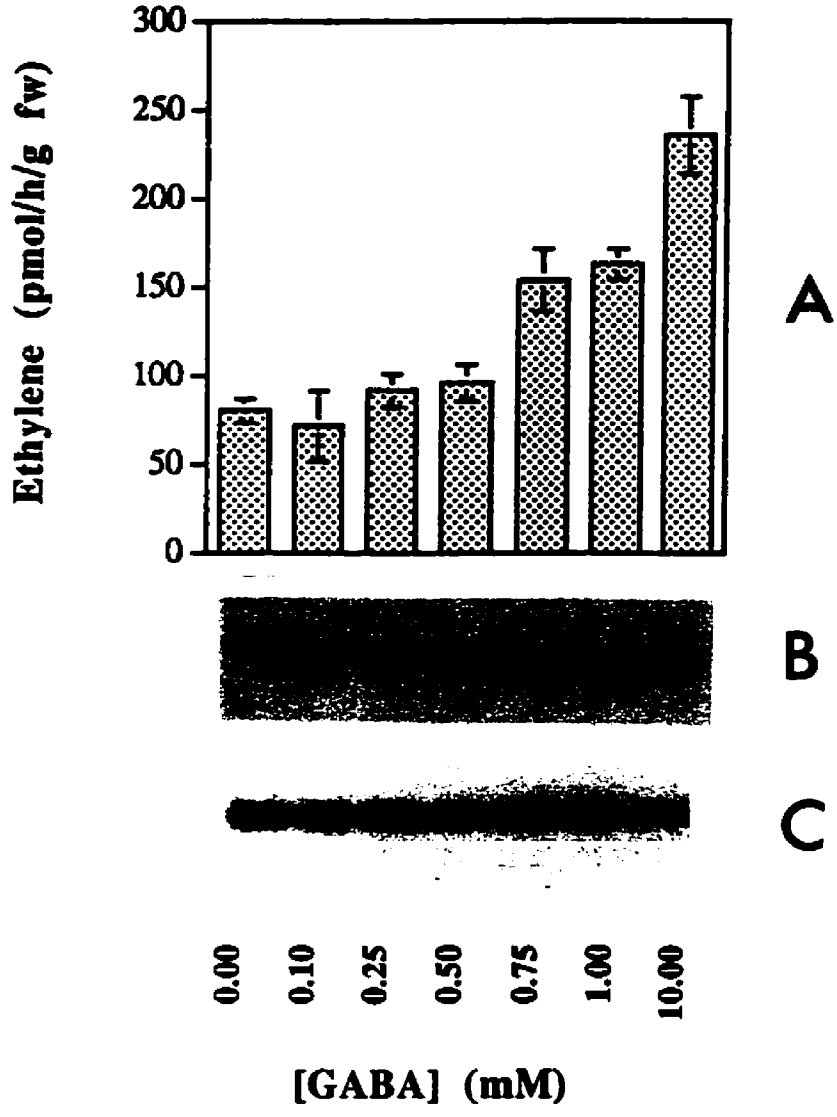
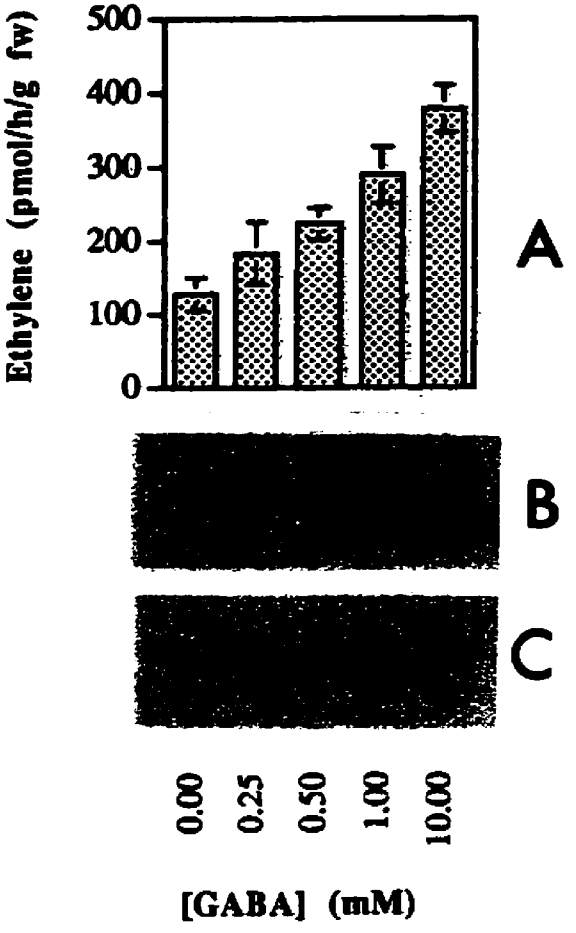

Ethylene (pmol/h/g fw)
300
250
200
150
100
50
0
A
B
C
0.00
0.10
0.25
0.50
0.75
1.00
10.00
[GABA] (mM)

Fig. 6.12 (A-C). Effect of GABA on ACC synthase transcript abundance in prairie plants. The Northern blot containing 10 μg of poly(A)$^+$ per lane from tissues treated with various concentrations of GABA was probed with exon III of SACS-1 (C). After stripping the probe, the blot was hybridized with H2Av (B). The ethylene production observed in the corresponding treatments are shown in A.


Ethylene (pmol/h/g fw)
500
400
300
200
100
0
A
B
C
0.00
0.25
0.50
1.00
10.00
[GABA] (mM)

6.3.7. Effect of GABA on stem elongation

Since GABA can promote ethylene production in *S. longipes*, the effect of GABA on the ethylene-dependent stem elongation was also investigated in this study. Cultured *in vitro* shoot tips of *S. longipes* generally produced at least three new internodes and primary roots after 21 days. The total stem length in alpine control plants was about 3.8 cm (Fig. 6.13). With an increase in GABA concentration in the culture, there was an increase in stem length until a concentration of 250 μM was reached, after which there was a decrease in stem length (Fig. 6.13). A stem length of about 5.72 cm was observed at the optimal GABA concentration of 250 μM (Fig. 6.13). Unlike GABA, 250 μM of alanine or glutamate or succinate could not significantly promote stem elongation *in vitro* (Fig. 6.13). A similar effect of GABA on stem elongation was also found in prairie plants, with an optimal response at a GABA concentration of 250 μM (Fig. 6.14). Among the three newly produced internodes, the maximum elongation response to GABA was observed in the first internode that is immediately below the shoot meristem in both ecotypes. For example, in alpine ecotype, while the elongation responses of the first internode were the maximum, the elongation responses were the least in the third internode, an intermediate response was observed in the second internode (Fig. 6.15).

These *in vitro* results suggest that GABA may be a limiting factor for stem elongation in control plants. The correlation between stem elongation and endogenous GABA levels was studied by estimating GABA levels in pot grown alpine and prairie plants under SDC and LDW conditions. As seen in Fig. 6.16, alpine and prairie plants grown under SDC contained higher

Fig. 6.13. Effect of GABA on stem elongation in alpine plants *in vitro*. Young shoot tips of alpine plants were cultured in MS media supplemented without or with various GABA concentrations, alanine (250 μM), glutamate (250 μM) or succinate (250 μM), and their stem length was measured on day 21.

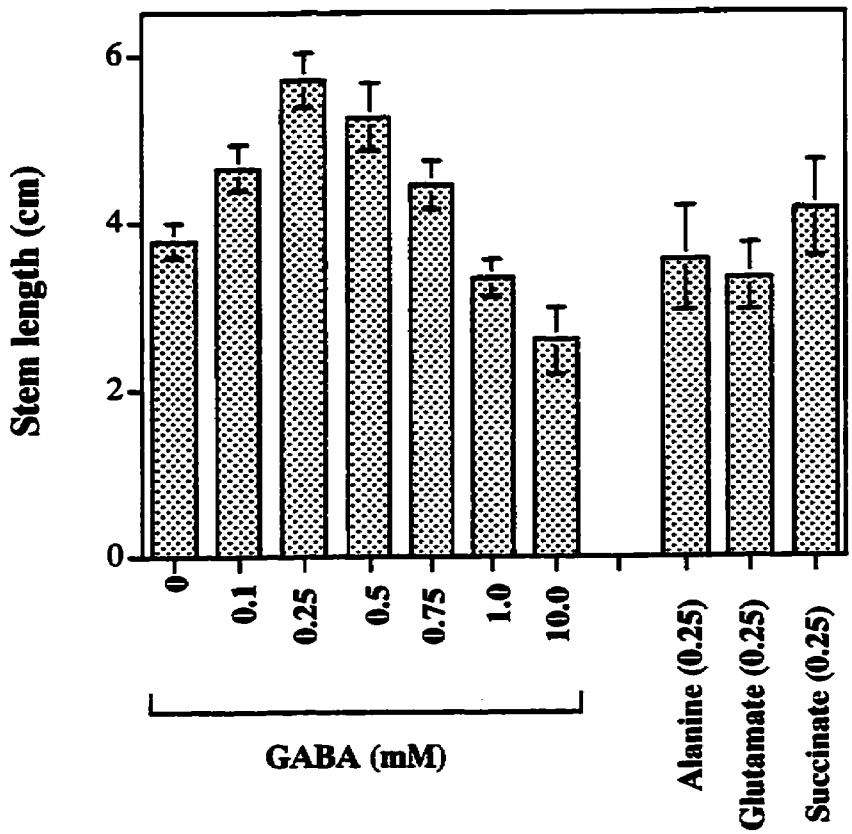

Stem length (cm)
6
4
2
0
0
0.1
0.25
0.5
0.75
1.0
10.0
GABA (mM)
Alanine (0.25)
Glutamate (0.25)
Succinate (0.25)

Fig. 6.14. Effect of GABA on stem elongation in prairie plants *in vitro*. Young shoot tips of prairie plants were cultured in MS media supplemented without or with various GABA concentrations, alanine (250 μM), glutamate (250 μM) or succinate (250 μM), and their stem length was measured on day 21.

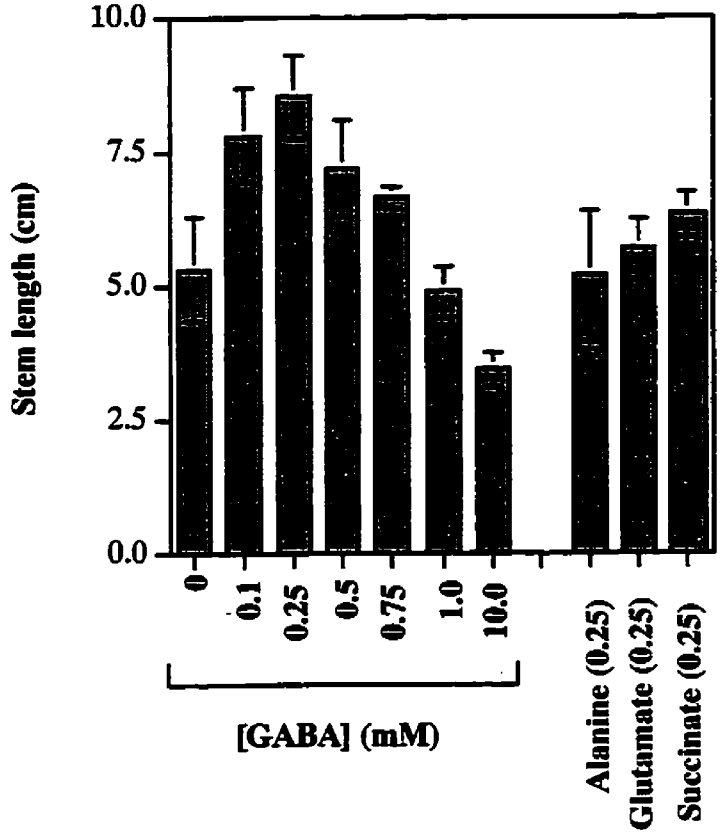

10.0
7.5
5.0
2.5
0.0
Stem length (cm)
0
0.1
0.25
0.5
0.75
1.0
10.0
[GABA] (mM)
Alanine (0.25)
Glutamate (0.25)
Succinate (0.25)

Fig. 6.15. Effect of GABA on various internodes of alpine plants *in vitro*. The internodal lengths of plantlets cultured in MS media supplemented with various GABA concentrations were measured on day 21. The internodes were numbered based on their order of appearance below the shoot meristem.

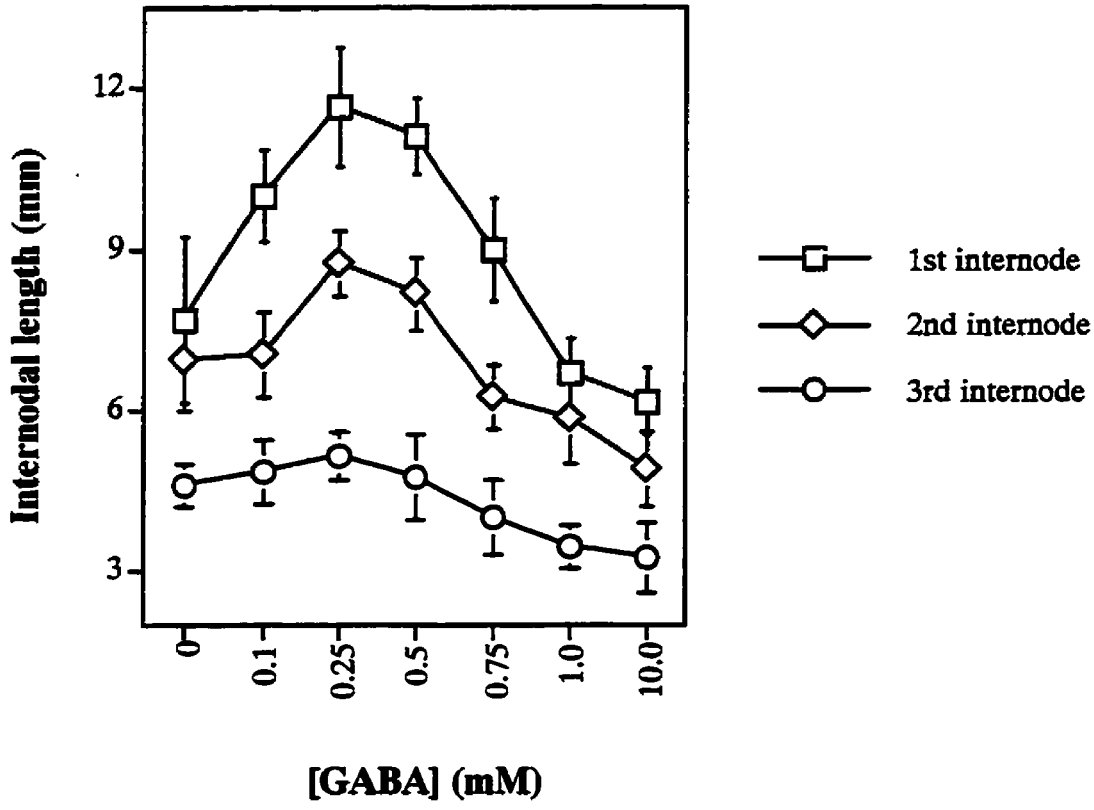
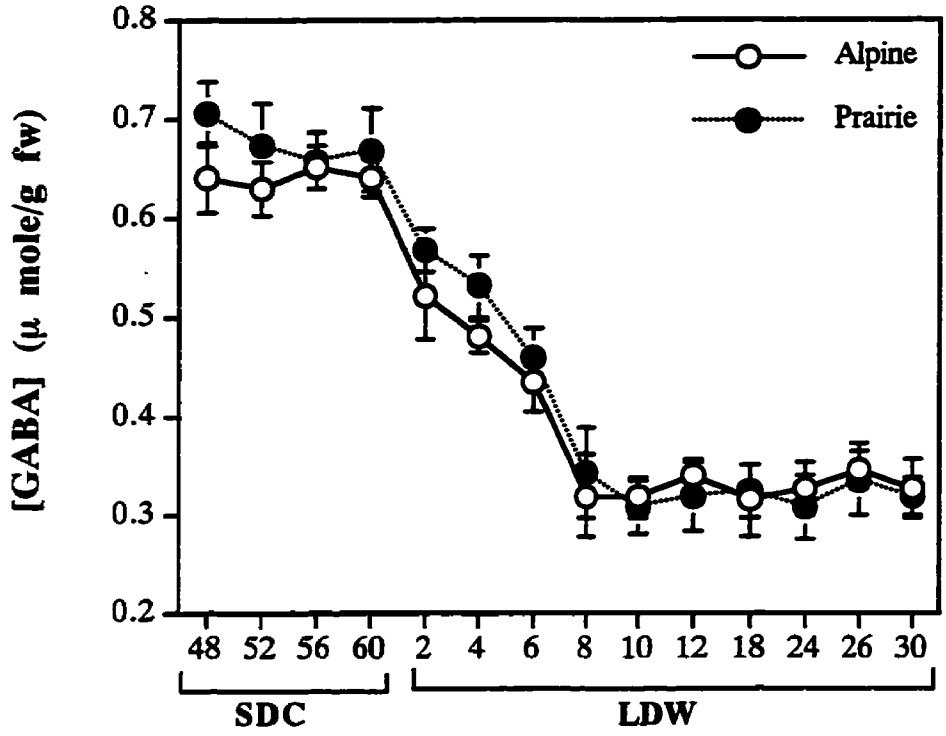

Internodal length (mm)
12
9
6
3
0
0.1
0.25
0.5
0.75
1.0
10.0
[GABA] (mM)
1st internode
2nd internode
3rd internode

Fig. 6.16. Endogenous GABA accumulation in plants grown under various conditions. The plants were initially grown under SDC for 60 days and transferred to LDW conditions. Stem and leaf tissues were collected at various time intervals and their endogenous GABA content was measured using GABAse assay.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
[GABA] (μ mole/g fw)
Alpine
Prairie
48 52 56 60 2 4 6 8 10 12 18 24 26 30
SDC
LDW

GABA levels than under LDW conditions. Upon transfer of SDC grown plants to LDW, the endogenous GABA levels in alpine and prairie plants gradually decreased to 50% by day 8 and were maintained even after the plants completed their stem elongation phase (3 weeks) (Fig. 6.16).

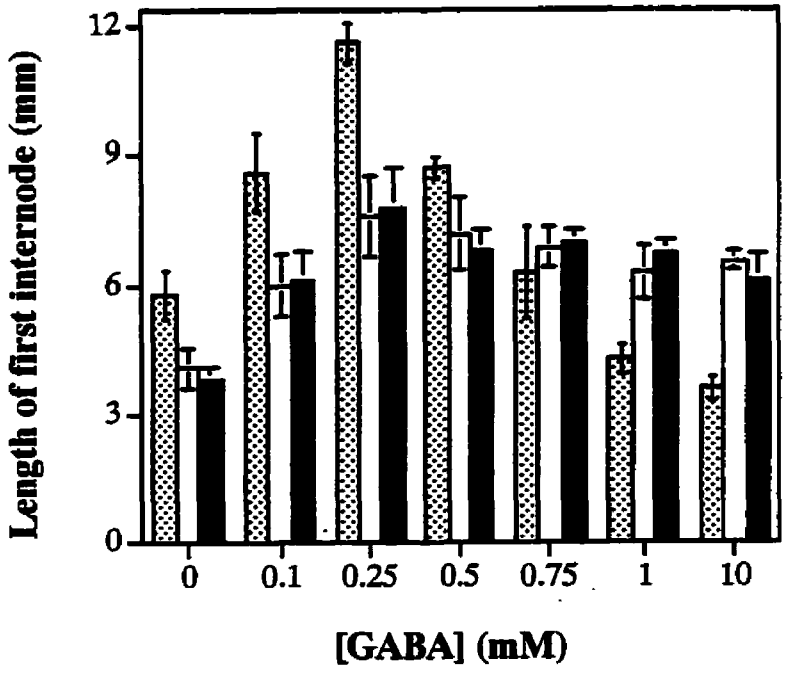
6.3.8. Role of ethylene in GABA induced stem elongation

The role of GABA induced ethylene production on the elongation of first internode was studied using silver thiosulfate (STS), an ethylene action inhibitor (Veen, 1983) or aminoethoxyvinylglycine (AVG), an ethylene synthesis inhibitor (Amrhein and Wenker, 1979) in alpine plants *in vitro*. Fig. 6.17 shows that lower concentrations of GABA ($\leq$ 500 μM) promoted the internodal elongation even in the presence of STS or AVG, although the observed values of internodal length in these treatments were lower than the corresponding control plants. Interestingly however, the inhibition of internodal elongation by higher GABA concentrations ($\geq$ 750 μM) is reduced in the presence of STS or AVG (Fig. 6.17).

6.4. Discussion

The natural occurrence of GABA was first demonstrated in potato tubers in 1949 (Steward *et al.*, 1949). Since then, the presence of GABA has been reported in many higher plants (reviewed by Satyanarayan and Nair, 1990). Despite the widespread occurrence of GABA in higher plants, its biological role(s) is still not clearly elucidated. Several possible roles of GABA have been suggested. GABA may be involved in cytoplasmic pH regulation (Snedden *et al.*, 1992; Roberts *et al.*, 1992; Crawford *et al.*, 1994),

Fig. 6.17. Role of ethylene in GABA induced internodal elongation. The plants were grown in MS media supplemented without (stippled bars) or with 10 μM AVG (open bars) or with 25 μM STS (filled bars) in the presence of various GABA concentrations. The length of the first internode was measured on day 21. Data are mean of at least 6 observed values. Vertical bars indicate ± S.E.


Length of first internode (mm)
12
9
6
3
0
0
0.1
0.25
0.5
0.75
1
10
[GABA] (mM)

temporary nitrogen storage, defense mechanisms against pest and disease (Satyanarayan and Nair, 1990; Ramputh and Bown, 1996), and nitrogen transport (Bown and Shelp, 1989). This study demonstrates that GABA can promote synthesis of the plant hormone ethylene and stem elongation, and thus widens the potential scope of GABA's role in plant growth and development.

Since [^{14}C]GABA-treated tissues did not substantially incorporate [^{14}C] into ethylene, it is very unlikely that GABA could act as an alternate precursor for ethylene. Also when GABA was added to ACC oxidase extracts, there was no detectable production of ethylene. Therefore GABA is probably not acting as a direct ethylene biosynthetic precursor.

ACC synthase is often considered as the rate limiting step in ethylene biosynthesis (Kende, 1993). When ACC synthase was inhibited by AVG, GABA failed to promote ethylene synthesis, indicating that GABA stimulates ethylene by acting on ACC synthase. Consistent with this idea, endogenous ACC levels were several fold higher in GABA-treated tissues. This result also suggests that there is a higher ACC synthase activity *in vivo* in GABA-treated tissues. Such an increase in ACC synthase activity could be due to *de novo* protein synthesis, as judged by the abundance of ACC synthase transcripts in GABA-treated tissues. When the tissues were supplied with both AIBA and GABA, the effect of GABA on ethylene is virtually eliminated, suggesting that GABA could have enhanced ethylene biosynthesis only via ACC.

Even though ACC oxidase is usually considered as a constitutive enzyme (Kende, 1993), a growing number of recent studies indicate that ACC oxidase can also be regulated (Kim and Yang, 1994; Barry *et al.*, 1996; Kathiresan *et al.*, 1996) and thus be involved in regulation of ethylene biosynthesis. *In vitro* ACC oxidase assays done in this study, show an increase in ACC oxidase mRNA abundance and ACC oxidase activity in GABA-treated sunflower tissues. The increase in ACC oxidase mRNA abundance and enzyme activity could be due to GABA itself and/or a possible positive feedback by the GABA induced ethylene.

It seems unlikely that GABA imposes a chemical stress since the GABA treated tissues do not show large increases in endogenous GABA levels indicating that either less GABA is taken up than might be expected or GABA is taken up and is quickly utilized by tissues. Similar to these observations, Aurisano *et al.* (1995) reported that rice seedlings accumulate as high as 8 μmol of GABA/g fw under anoxic conditions. The authors however, observed only about 1 μmol of GABA/g fw after soaking the tissues in 1 mM GABA. This may also partly explain why large GABA doses are required to increase ethylene production in our study. Yet one can not completely rule out the possibility that GABA induced the ethylene production by exerting a chemical stress. For instance, GABA could have indirectly altered the cytoplasmic pH status (Snedden *et al.*, 1992) or incorporated itself into proteins by mimicking protein amino acids, which could have been perceived by plant cells as stress. Stimulation of ethylene production by D-isomers of several amino acids has been reported by Satoh and Esashi (1980b) in various cocklebur seed tissues. Similarly Cohen *et*

al.(1994) reported that α- and β-aminobutyric acid but not GABA can stimulate ethylene production in tomato plants.

Recently it has been shown that over expression of active GAD causes severe abnormalities in growth and development of tobacco plants (Baum *et al.*, 1996). In the present study, the observation that ethylene production is stimulated only by GABA but not by glutamate, the precursor of GABA, indicates the importance of regulation of GAD activity in plants. GABA is usually shunted back to Kreb's cycle in the form of succinate. Since succinate could not increase ethylene production, it is unlikely that GABA enhanced ethylene synthesis by increasing the flux through Kreb's cycle. Rather, GABA may act as a signaling regulatory molecule and cause ACC synthase transcript accumulation at transcriptional and/or post transcriptional levels. For instance, the rapid 10 to 25 fold increase in endogenous GABA levels following mechanical damage (Ramputh and Bown, 1996) can stimulate ethylene production in a manner shown in the present study.

The present study also shows that GABA, up to a concentration of 500 μM can promote stem elongation in *S. longipes*. However a further increase in GABA concentration inhibited stem elongation. Unlike GABA, glutamate, the precursor of GABA, could not promote stem elongation. This result suggests that regulation of GAD is important for stem elongation. Plants cultured in optimal GABA concentration (250 μM) showed a six fold increase in their endogenous GABA levels over control plants indicating that endogenous GABA accumulation could be a limiting factor in stem elongation of control plants. Plants that possessed more than

a 12 fold increase in internal GABA levels however showed inhibition of stem elongation. Baum *et al* (1996) recently showed that over expression of active GAD in tobacco plants caused severe stunting of internodes. The endogenous GABA levels in the stems of these transgenic plants were only 7 fold higher than that of wild type plants (Baum *et al.*, 1996). It is important to note that such transgenic tobacco also suffered 17 fold reduction in glutamate levels due to the over expression of active GAD (Baum *et al.*, 1996). Nevertheless the present results suggest that the nature of response of stems to GABA might vary in different plant species.

While GABA concentrations that promoted stem elongation ($\leq$ 500 μM) failed to promote ethylene production, GABA concentrations that were inhibitory for stem elongation ($\geq$ 750 μM) significantly induced ethylene evolution. These results may suggest that GABA induced ethylene production is a stress response in *S. longipes*. When the synthesis or action of ethylene was inhibited using AVG or STS respectively, a reduction in the internodal length of control plants was observed. This observation supports the notion that ethylene is important for stem elongation in *S. longipes*. Interestingly, lower concentrations of GABA ($\leq$ 500 μM) could still promote internodal elongation in the presence of AVG or STS suggesting that GABA mediates stem elongation via an ethylene independent pathway. Yet one can not ignore the possibility that a very low level of ethylene induced by lower concentrations of GABA may not have been detected by the gas chromatographic equipments used in this study. However, since the inhibition of stem elongation caused by higher GABA concentrations ($\geq$ 750 μM) is significantly reduced in the presence of AVG or STS, it is concluded

that the inhibitory effect of GABA on stem elongation is mediated at least in part by ethylene.

A large amount of endogenous GABA accumulation in plants that were grown in soil mix under SDC. When these plants were transferred to LDW, the GABA levels dropped by 50%. Monroy and Dhindsa (1995) showed that a decline in temperature from 15° C to 4° C caused a 15 fold increase in extracellular Ca^{2+} influx, whereas a change in temperature from 15° C to 25° C did not affect Ca^{2+} influx in alfalfa plants. Recently Cholewa *et al* (1997) showed that the rapid increase in GABA following a cold stress is due to increased levels of cytosolic Ca^{2+} in mesophyll cells of *Asparagus sprengeri.* It is therefore possible that the high steady state level of GABA accumulation observed under SDC in the present study is due to the activation of Ca^{2+}/calmodulin dependent GAD by cold-induced Ca^{2+} influx.

Previously it has been shown that while stem elongation in *S. longipes* occurs rapidly under LDW, very little or no stem elongation takes place under SDC (Macdonald *et al.*, 1984). Thus there is a negative correlation between the amount of free GABA and stem elongation of soil grown plants. It is possible that the high GABA levels found under SDC inhibit stem elongation and such an inhibition is relieved upon transfer of plants from SDC to LDW due to the reduction in GABA accumulation.

In summary, the results show that (i) GABA can promote ethylene production by mainly upregulating the steady state levels of ACC synthase transcripts, (ii) GABA is not an alternate precursor for ethylene, (iii) GABA (up to a concentration of 500 μM) can promote stem elongation in *S.*

longipes, but inhibit stem elongation at higher concentrations and (iv) the inhibitory effect of GABA on stem elongation is partly mediated by GABA induced ethylene production.

CHAPTER SEVEN

General Discussion and Future Perspectives

Given their sessile nature, plants are constantly challenged by various environmental factors. An inflexible genetic plan might therefore not be advantageous for survival in habitats where environmental changes are frequent. Flexibility in executing the basic genetic plan however, often leads to changes in growth and developmental program resulting in altered phenotypes. Flexible genomic expression could therefore be considered as an adaptive feature that underlies phenotypic plasticity. Such an adaptive feature likely involves a battery of finely tuned mechanisms by which genes are selectively expressed to initiate a series of physiological events that culminate in a suitable change in phenotype.

In *S. longipes*, one of several physiological events that accompanies its stem elongation, a plastic trait, is an increase in the rate of ethylene production (Emery *et al.*, 1994). Since alpine and prairie ecotypes differ in their degree of stem elongation plasticity, it is generally expected that these two ecotypes might have differences in their physiological strategies. The recent finding that alpine and prairie ecotypes indeed differ in their ability to produce ethylene under a given set of environmental cues (e.g. LDW; Emery, 1994) has opened up new opportunities to learn more about phenotypic plasticity at the molecular level. The long term goal of such research is to use thus gained knowledge as 'building blocks' to understand the complex biological mechanisms of phenotypic plasticity. This study was set out to determine the differences in regulation of ethylene biosynthesis in alpine and prairie ecotypes. The results obtained in this study gives several

insights into not only phenotypic plasticity but also ethylene biosynthesis in general.

ACC oxidase cDNAs that are expressed under LDW in alpine and prairie ecotypes exhibit very high similarity (97.11%) at the amino acid level. Such high level of conservation of ACC oxidase protein might reflect the intense selection pressure on the evolution of protein primary structure because ACC oxidase may have severe structural restraints for its function. Since Southern analysis suggests that ACC oxidase may be encoded by a single gene copy, it is likely that plants that suffer loss-of-function mutation in ACC oxidase locus may become less fit for survival and face threats of elimination from the rest of the population. The importance of ACC oxidase in phenotypic plasticity of the two ecotypes under different habitats and stress conditions needs to be studied using antisense technique or targeted mutagenesis.

In both ecotypes, ACC oxidase is regulated by a 'biological clock'. The self sustaining endogenous rhythm is orchestrated by the changes in light and temperature. It is speculated that such an ACC oxidase rhythm might help overcome the competition for the limited internal CO_2 from photosynthetic apparatus. Interestingly, a dampening ACC oxidase rhythm under continuous dark conditions can be reset by a single red light pulse suggesting the involvement of red light signal transduction pathway. Following our report on circadian regulation of ACC oxidase, a research team led by Page Morgan and Scott Finlayson at Texas A&M University monitored ACC oxidase in a sorghum PHYB mutant which possesses a truncated, non-functional phytochrome B protein (Childs *et al.*, 1997).

While both wild type (WT) and mutant produce cycling of ACC oxidase mRNA, the amplitude in the WT is only a small fraction of the mutant (Finlayson *et al.*, 1997) indicating that a functional phyB may act as a negative regulator of ACC oxidase oscillations. However, other clock regulated genes including CAB (chlorophyll a/b binding protein) exhibit normal cycling in PHYB mutant (S.A. Finlayson, personal communication). These results seem to suggest that phyB is not a component of central clock itself, but rather might couple the clock and clock driven events such as ACC oxidase and ethylene rhythms. Availability of clock mutants in plants might help us understand the role of phytochromes in circadian rhythms more thoroughly. The recent cloning of genes that are essential for circadian function in Neurospora (*frq*), Drosophilla (*tim* and *per*) and mouse (*clock*) have shed some light on the central clock components. The emerging picture is that central clock genes are transcriptional factors that participate in an autoregulatory transcriptional feedback loop that has a time distance of ca. 24h (reviewed by Reppert and Weaver, 1997).

Since both ecotypes exhibit circadian rhythm, coupling of ACC oxidase to central clock might have occurred before their divergence. The physiological significance of circadian regulation of ACC oxidase and ethylene is currently unknown. It is conceivable however, that if ethylene production oscillates then it could be reflected in most of the ethylene mediated responses such as stem elongation. Interestingly circadian rhythm for stem extension rate has been reported in plants such as *Chenopodium rubrum* (Lecharny and Wagner, 1984) and *Dendranthema grandiflorum* (Tutty *et al.*, 1994). The question of whether stem elongation is influenced

genomic libraries of the two ecotypes of *S. longipes* with ACC synthase gene specific probes, one should be able to isolate the regulatory elements of family members. Once such information is available, it is possible to identify and characterize unique DNA elements that are responsive to changes in photoperiod and temperature through deletion analysis of regulatory elements. Using such responsive elements, it is also feasible to identify upstream signaling components that transduce the changes in photoperiod and temperature.

It has long been thought that mere acquisition of novel regulatory controls and duplication of existing responsive genes might enhance the adaptability of a plant in diverse environments (Dover, 1986). It is puzzling however, to note that the two ACC synthase cDNAs that are expressed in alpine and prairie ecotypes under LDW exhibit considerable heterology. We do not know if these two cDNAs represent two separate ACC synthase loci or allelic forms of a single ACC synthase locus. Finding an answer to this question may help us understand some basic aspects of the evolution of phenotypic plasticity. Phenotypic plasticity can be considered as an overall strategy comprised of three broader components viz. (a) 'perception' and (b) 'transduction' of the environmental signal(s), and (c) elicitation of a proper biological 'response'. While it is less likely that alpine and prairie ecotypes differ in their sensing mechanisms for fundamental cues such as light and temperature, it is known that their response mechanisms such as ethylene production levels are different. Such differences in responses could be largely due to divergent transduction mechanisms of external signals.

development of higher plants. Upregulation of the same ACC synthase gene member by photoperiod and temperature in *S. longipes* indicates that this gene might have multiple regulatory elements that can respond to photoperiod and temperature. The synergism between these two signals in upregulating ACC synthase also suggest that both may act on some common downstream transducing elements. Using phototransduction mutants such as *hy5* and *det1* of *A. thaliana* (Koornneef and Kendrick, 1994), one could study the role if any, of light signaling components in mediating temperature induced ACC synthase gene expression.

The present study has identified GABA as a novel stimulus for ACC synthase transcript accumulation and ethylene production. The effect of GABA on ethylene production is specific since the other related compounds in its biosynthetic pathway such as glutamate, alanine, or succinate failed to produce any effect on ethylene biosynthesis. Thus in contrast to what has been surmised over the past few years, this result clearly illustrates that GABA accumulation in plant cells can influence the physiology of plant cells. Since GABA could influence ethylene biosynthesis in sunflower and *S. longipes*, the two evolutionarily divergent plant systems, the response pathway(s) to GABA might have been well conserved in plants. Although GABA could have imposed a chemical stress in this admittedly artificial experimental system involving excised tissues and milli molar GABA concentrations, GABA might promote ethylene production as shown in this study, at least in the following two instances.

Hypoxic conditions cause a sequential increase in GABA (Aurisano *et al.*, 1995) and ethylene levels (Metraux and Kende, 1983). It is generally

believed that the decline in cytosolic pH during hypoxia might trigger the GAD activity leading to GABA accumulation (Bown and Shelp, 1989). The increase in ethylene production under hypoxic conditions is due to enhanced ACC synthase activity (Cohen and Kende, 1987). Low O_2 concentration is considered as a signal for such ACC synthase induction (Kende, 1987). It is possible that this signaling is in part aided by the increased GABA levels.

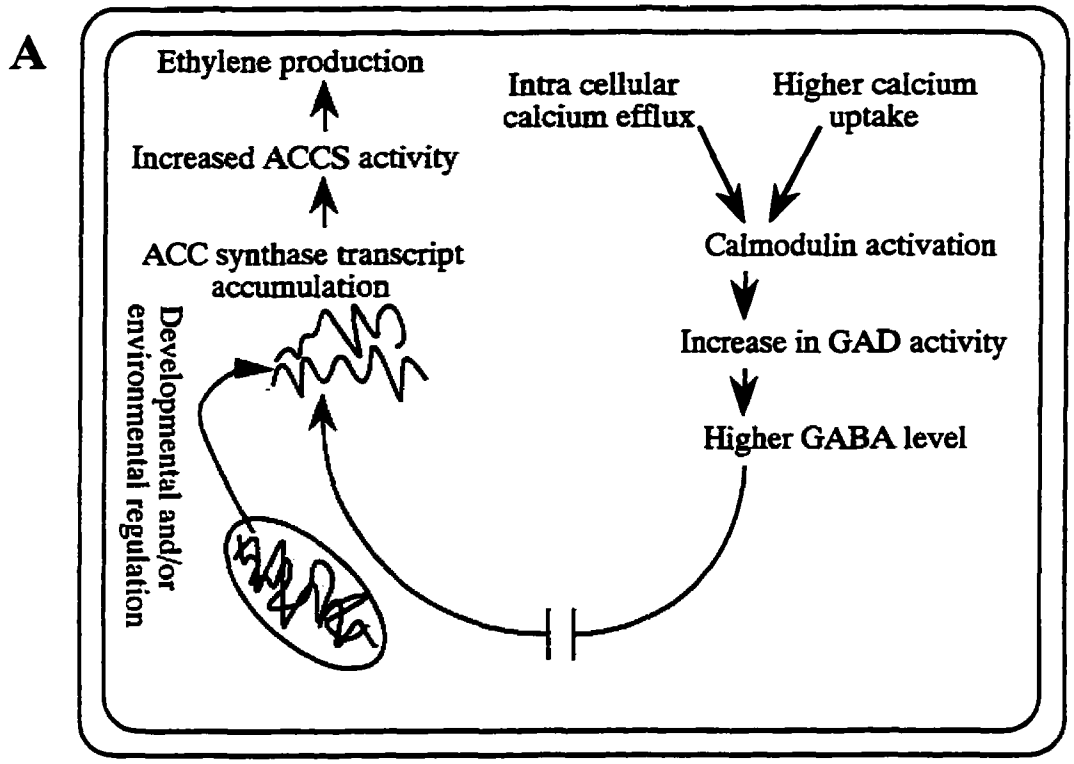
The other instance in which GABA might mediate ethylene biosynthesis is during ripening of climacteric fruits. Inaba *et al.*(1980) reported that GABA and glutamic acid are the two most abundant amino acids in tomato fruits. The GABA levels were higher until the late breaker stage (pink) of ripening tomato fruits. Gallego *et al.*(1995) recently reported a corresponding rise in the accumulation of a calmodulin-binding GAD transcript during the onset of ripening in tomato fruits. It is possible that an increase in ethylene production in these fruits, a vital physiological event during ripening, is mediated by GABA.

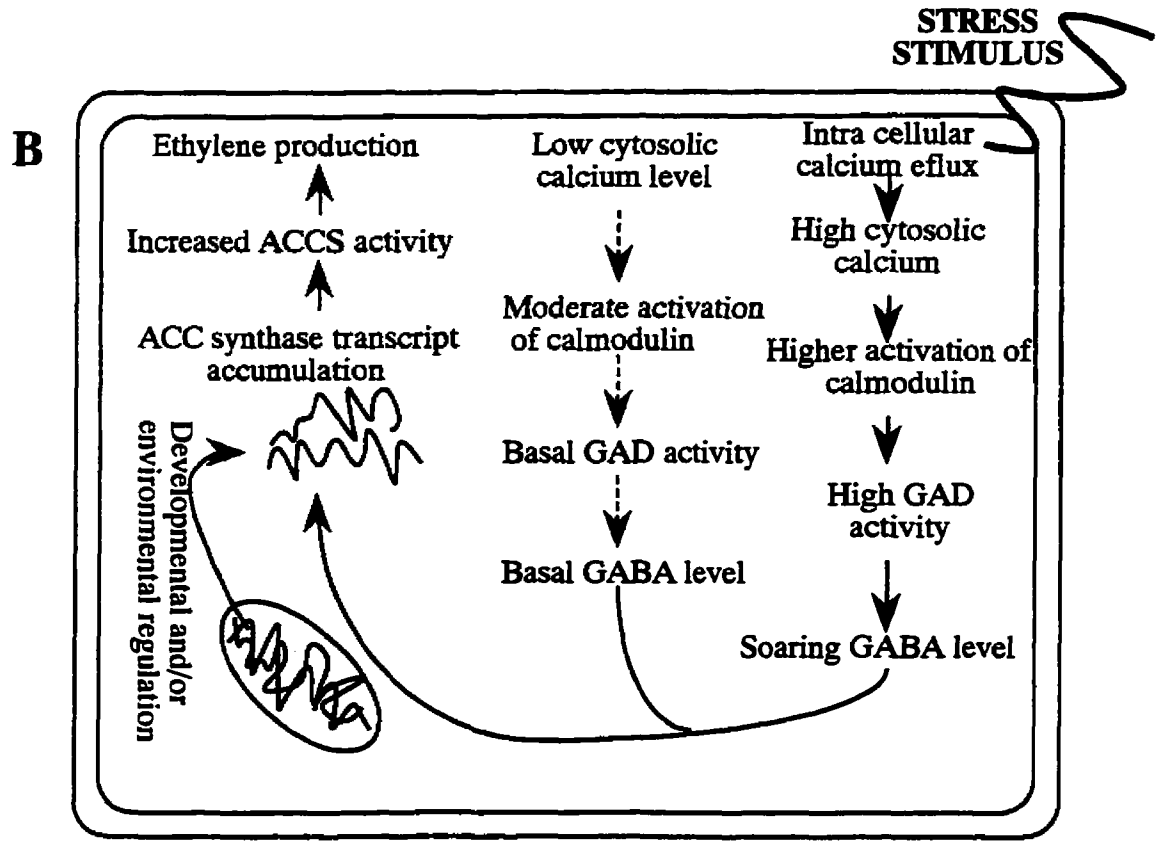
The present investigation also demonstrates that GABA can promote stem elongation *in vitro* at concentrations as low as 100 μM, but inhibit stem elongation at concentrations above 750 μM. Results also suggest that the role of GABA induced ethylene on GABA's effect on stem elongation is limited. While the inhibition of stem elongation by GABA is mediated at least partly by ethylene, it is less clear if the GABA induced stem elongation is completely ethylene independent. The probable reason why there is a high GABA accumulation under SDC conditions is an increase in GAD activity mediated by risen cytosolic Ca^{2+} levels at lower temperatures (Monroy and Dhindsa, 1995). However, in contrast to what one might

expect, the ethylene production in SDC grown plants is low despite of a high endogenous GABA level. Furthermore, the ethylene production increases under LDW conditions when the endogenous GABA accumulation becomes gradually reduced. It is possible that the competence of plant tissues to increase its ethylene production in response to GABA (sensitivity) is negligible or null under SDC conditions. Although the plant tissues are responsive to higher GABA levels under LDW conditions, it is speculated that the general increase in ethylene production under LDW conditions could be due to GABA independent developmental and/or environmental cues. If however there is an increase in internal GABA levels, such as during stress conditions, then the increased GABA levels might cause an increase in ethylene production. Thus one may conclude that (i) GABA induced ethylene production is a stress response and (ii) GABA is a growth factor that can regulate stem elongation in *S. longipes*.

The recent findings that GABA biosynthesis is regulated by Ca^{2+}/calmodulin complexes in plants have heightened the interest on GABA. The present investigation gives us some indication that GABA is acting as a 'secondary messenger' downstream of Ca^{2+} and calmodulin not only under stress conditions but also during normal growth and development. The possible mode of interactions between GABA, ethylene, and stem elongation is illustrated in Figs. 7.1 and 7.2. Since the responses of alpine and prairie ecotypes of *S. longipes* to GABA are similar, the role of GABA does not explain the differences between their plasticity levels. Nevertheless, the dynamic changes in endogenous GABA levels associated with stem elongation and their ability to stimulate ethylene production as well as stem

Fig. 7.1 (A and B). A schematic diagram showing the possible modes of regulation of GABA synthesis and its effect on ethylene production in *S. longipes* under short day and cold (SDC) conditions (A), and long day and warm (LDW) conditions (B). (Key words: ACCS- ACC synthase; GAD- glutamate decarboxylase).


A
Ethylene production
Increased ACCS activity
ACC synthase transcript accumulation
Developmental and/or environmental regulation
Intra cellular calcium efflux
Higher calcium uptake
Calmodulin activation
Increase in GAD activity
Higher GABA level


STRESS STIMULUS
B
Ethylene production
Increased ACCS activity
ACC synthase transcript accumulation
Developmental and/or environmental regulation
Low cytosolic calcium level
Moderate activation of calmodulin
Basal GAD activity
Basal GABA level
Intra cellular calcium eflux
High cytosolic calcium
Higher activation of calmodulin
High GAD activity
Soaring GABA level

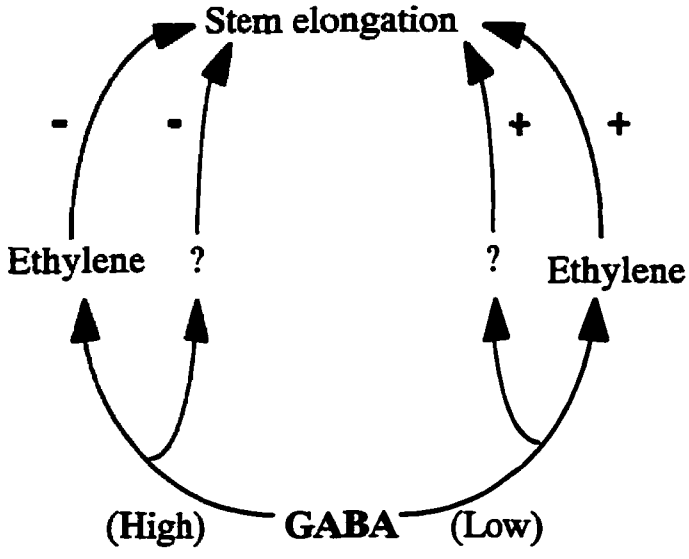


Fig. 7.2. A hypothetical model for GABA response pathways for stem elongation in *S. longipes*. The ? symbol indicates that the signalling intermediate is as yet unknown. Symbols + and - indicate the promotory and inhibitory effects of GABA respectively.

elongation clearly suggest that GABA might represent a general plastic response in *S. longipes*.

Almost all living organisms alter their functions in response to changes in environmental conditions (Stearns, 1989). Such plastic responses are particularly important in plants whose sessile life necessitates them to be flexible in order to survive. Thus research in the area of phenotypic plasticity will certainly enrich our basic understanding of how plants function. The pervasive and intricate nature of plastic responses however is fabricated with several layers of complexity. This work focused on some of the molecular physiological aspects of ethylene - one of the several factors responsible for differences in plasticity in alpine and prairie ecotypes of *S.*

longipes. Several clues about the basic features of phenotypic plasticity have emerged from this study. This work illustrates that differential regulation of gene families encoding crucial proteins might serve as a molecular basis of phenotypic plasticity. Still unknown however, are the underlying factors that are responsible for the differential regulation. The future challenge lies in identification and characterization of such fundamental cellular entities that initiate an array of plastic responses. An unified biochemical, physiological, molecular and (especially) genetic approach will allow us to learn more about the biological machineries of phenotypic plasticity in the years to come.

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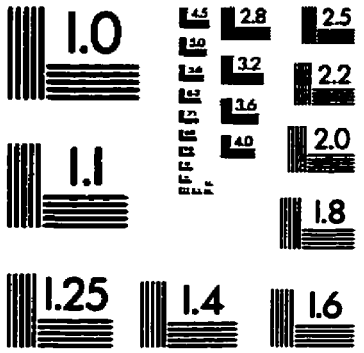

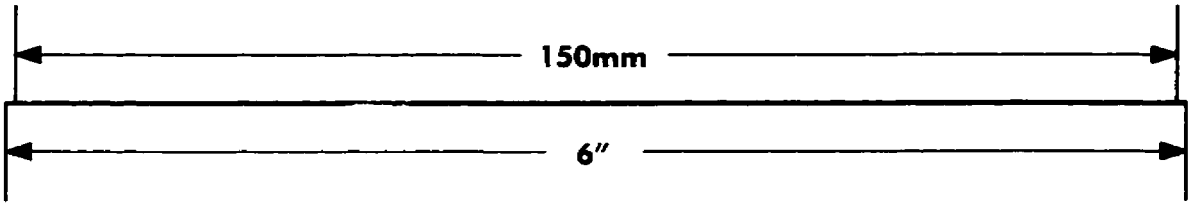

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