The Biochemical and Molecular Regulation of Photosynthesis.

A thesis

submitted to

University of Poona

for the degree of

Doctor of Philosophy

in

Chemistry

By

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May 1996

CERTIFICATE

This is to certify that the work incorporated in the thesis entitled '**The Biochemical and Molecular Regulation of Photosynthesis**' submitted by Mr. Jawahar H. Mehra was carried out by the candidate under my supervision at the Division of Plant Tissue Culture, National Chemical Laboratory, Pune. Material obtained from other sources has been duly acknowledged in the thesis.

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Acknowledgement

I am deeply indebted to my research guide, Dr. A.F. Mascarenhas for accepting me as a research student and inducting me on a research carreer. I wish to express my deep sense of gratitude to Dr. S.K. Rawal for his kind and timely guidance in the present endeavour. His invaluable suggestions and constructive criticisms have enabled me to bring this work to a successful culmination.

I also wish to acknowledge Dr. K.N. Ganesh, for allowing me to use his facility to prepare oligos, and Dr. L. Bogorad, for the all important maize *ppc* clone.

My colleagues and friends in the PTC group have been a great help throughout. I want to especially acknowledge Dr. B.M. Khan for all the helpful suggestions and fruitful discussions, Dr. D.K. Kulkarni for helping me with the tissue culture aspect of my work, Ms. Sunita Mahajan for preparing the cDNA library, Mr. D. Vijay Kumar for helping me with the immunoprecipitation experiments, and Tomal, Gyanendra Tripathi and Adin for prooving friends in need.

Words failed me when I want to express my gratitude to Mr. M.M. Jana, Sunny, Agnello, and Balu Shirke without whose help this work would have been extremely difficult to culminate.

I am also greatful to the Council of Scientific and Industrial Research for awarding me the Research Fellowship.

Finally, I would like to express my gratitude to Dr. Paul Ratnasamy, Director NCL, for permission to submit this work in the form of a thesis.

Jawahar H. Mehra

CONTENTS

Abstract

Introduction :

Plants, by the virtue of their ability to generate energy through the process of photosynthesis, occupy the top rung of ecological triangle. Photosynthesis is a process by which the energy of sunlight is trapped by the chlorophyll of green plants and used to build up complex materials from carbon dioxide and water. This energy conversion reaction annually results in the world-wide storage of ~$3x10^{21}$ J of energy. More than 90% of the dry weight of plants is derived from carbon dioxide assimilation through photosynthesis.

There are basically three different kinds of photosynthetic reactions, viz, C_3, C_4 and CAM. In the case of C_3 photosynthesis, carbon dioxide diffuses passively, across cell wall, cell membrane and chloroplastic envelope before it is utilized by rubilosebisphosphate carboxylase (RuBISCO : E.C. 4.1.1.39), which is the first enzyme in the photosynthetic reaction. Carbon dioxide, in this case competes with oxygen, which also binds RuBISCO, to lead to photorespiration. Whether the plant photosynthesizes or photorespires is dependent upon the individual partial pressures of carbon dioxide and oxygen respectively, in the chloroplast. Due to photorespiration, the C_3 species, for e.g. rice, wheat, etc., loses as much as 40% of the net carbon dioxide assimilated. Photorespiration consumes light-generated ATP and NADH. Photorespiration thus can be considered wasteful because the carbon dioxide released must be fixed again within the leaf. Net assimilation of carbon dioxide from outside air is reduced.

The C_3 photosynthetic plants thus have a photosynthetic efficiency of ~60%, which results in less water use efficiency and lower biomass yields.

C_4 photosynthetic plants, e.g., sugarcane, maize, sorghum, etc., through a mechanism of carbon dioxide enrichment in the bundle sheath cells, circumvent wasteful photorespiration. The C_4 photosynthetic machinery is compartmentalized into the outer mesophyll cells and the inner bundle sheath strands (Kranz anatomy). Carbon dioxide, which diffuses passively across mesophyll cell wall and cell membrane, is trapped by Phospho*enol*pyruvate carboxylase (PEPC : E.C. 4.1.1.31) in the mesophyll cytoplasm, and converted to oxaloacetic acid by combining it with phospho*enol*pyruvate. Oxaloacetic acid is converted to malic acid in the mesophyll chloroplast with the aid of NADP-dependent malate dehydrogenase (NADP-MDH : E.C. 1.1.1.82) and transported across to the bundle sheath chloroplast. Malic acid is broken up into carbon dioxide and pyruvic acid by NADP-dependent malic enzyme (NADP-ME : E.C. 1.1.1.40). The carbon dioxide released is then utilized by RuBISCO, while the pyruvic acid is transported back into mesophyll choroplast where it is converted back to phosphoenolpyruvate by the enzyme pyruvate orthophosphate dikinase (PPDK : E.C. 2.7.9.1). The phosphoenolpyruvate thus generated is recycled back for further trapping of carbon dioxide.

The C_4 photosynthetic plants thus have a photosynthetic efficiency of ~90%, which in turn means a better water use efficiency and average biomass yields are approximately twice that of C_3 photosynthetic plants.

Hypothetically then, if one introduces C_4 PEPC into a C_3 photosynthetic plant, then at any given time, carbon dioxide partial pressure in the C_3 plant would be greater and this would result in the increase of C_3 biomass yield. Yet for this to be true, regulation of PEPC has to be studied in the native C_4 photosynthetic plant.

The present thesis is the study of regulation of PEPC in sugarcane, an NADP-ME type of C_4 photosynthetic plant.

The first chapter of the thesis is **Introduction.** The chapter presents a literature review and the present state of knowledge.

The second chapter is **Materials and Methods**, and gives an in-depth description of the experiments performed and the materials used therein.

The third chapter is **Effect of 5-AzaC on Sugarcane Tissue Culture In Vitro.** In this chapter are described the results of experiments to regenerate sugarcane plantlets in the presence and absence of 5-AzaC, a C-residue methylation inhibitor.

The fourth chapter, **Biochemical Study of the Effect of 5-AzaC on Phosphoenolpyruvate Carboxylase on Day 15 of Subculture,** discusses the influence of 5-AzaC on PEPC enzyme activity.

The fifth chapter is **Time Course Analysis of the Influence of 5-AzaC on the Emzyme Activities of PEPC (Mesophyll Cytoplasmic Enzyme), PPDK (Mesophyll Chloroplastic Enzyme), and NADP-ME (Bundle Sheath Chloroplastic Enzyme).** The effect of short- as well as long-term exposure of 5-AzaC on the activities of the three enzymes from 12th to 18th day of subculture is discussed.

The sixth chapter is **Sequence analyses of PEPC cDNA from Sugarcane**. This chapter deals with the cDNA library preparation, screening of PEPC cDNAs and their partial sequencing and characterization.

Introduction

Photosynthesis is the process whereby green plants, algae and certain bacteria utilize a portion of the photon energy from the sun to drive endergonic reactions that store chemical energy. This energy conversion reaction annually results in the world-wide storage of ~$3x10^{21}$J of energy (Bolton and Hall, 1991). More than 90% of the dry weight of plants is derived from CO_2 assimilation through photosynthesis (Zelitch, 1982). This ability of plants to generate energy on their own, makes them primary providers and hence places them on the top of the ecological pyramid. Since photosynthesis is very closely associated with plant biomass yield (Porter and Grodinzki, 1985; Idso and Kimball, 1991; Long and Drake, 1991), it would be beneficial to increase photosynthetic efficiency to increase plant productivity.

Experiments carried out by Porter and Grodinzki (1985), in green house, showed that CO_2 enrichment increases net photosynthesis and yields of C_3 species such as cereals, legumes, vegetables and woody plants, which have rapid rates of photorespiration. The same was the result obtained with long-term outdoor experiments (Idso and Kimball, 1991; Long and Drake, 1991).

Various routes for achieving the goal of increasing photosynthesis in plants include; (a) induction of polyploidy, (b) decreasing the rate of photorespiration and/or (c) increasing photosynthetic efficiency by increasing the CO_2 partial pressure inside the chloroplast.

In polyploid plants the photosynthetic rate per cell is correlated with the amount of DNA per cell (Warner and Edwards, 1993). The photosynthetic rate per unit leaf area is the product of the rate per cell times the number of photosynthetic cells per unit area (Warner and Edwards, 1993). Therefore the photosynthetic rate per area will increase if there is a less than proportional increase in the cell volume at higher ploidy levels, or if the cell packing is altered to allow more cells per unit leaf area (Warner and Edwards, 1993). Although photosynthesis per cell increases with ploidy, photosynthesis per leaf area decreases. Alternatively, doubling of photosynthetic rate per cell with the doubling of DNA, with apparent natural selection for decreased cell volume per unit DNA, would result in higher rates of photosynthesis per unit leaf area.

Photosynthetic rate in *Medicago sativa* polyploids is correlated with the amount of DNA and chloroplast number, which increase with ploidy, and the rate per chloroplast is similar for all ploidy levels. However, with higher ploidy, there is an apparent increase in cells per unit leaf area such that photosynthesis per leaf is increased (Meyers *et al.*, 1982; Mollin *et al.*, 1982).

Warner and Edwards (1988), found much the same result with C_4 plant pearl millet (*Pannisetum americanum*). They found that the photosynthetic rate per unit DNA is the same, and is doubled per cell in the tetraploid compared with the diploid. Since there are half as many cells per unit leaf area in the tetraploid, the photosynthetic rate is the same per unit area at both ploidy levels (Warner and Edwards, 1988).

Poskuta and Nelson (1986), in a study of hexaploid, octaploid and decaploid *Festuca arundinaceae* (C_3) found that the rate of photosynthesis per leaf area increased with increase in ploidy. However, growth decreased with an increase in ploidy. Thus, growth is negatively correlated with increase in photosynthesis per unit leaf area, but is positively correlated with area of leaves per plant in *Festuca arundinaceae* (Postuka and Nelson, 1986).

Photosynthesis per unit leaf area is 40% higher in the octaploid of switch grass (C_4, *Panicum virgatum*), as compared to its tetraploid (Warner *et al.*, 1987). Cell volumes increase only 15 to 20% when ploidy level increases from tetraploid to octaploid, but contents of DNA, chlorophyll and soluble proteins, as also photosynthetic rate per cell, all double (Warner *et al.*, 1987).

Hence, effect of ploidy on photosynthesis varies in both C_3 and C_4 plants. However, photosynthesis has been shown to be dependent upon the amount of DNA present.

Almost 33 to 55% of the net CO_2 assimilated by C_3 species is lost by them under normal atmospheric conditions (Bolton and Hall, 1991; Zelitch, 1992). In fact photorespiration also consumes light generated ATP and NADPH. Photorespiration is considered wasteful because the CO_2 released must be fixed again within the leaf. Net assimilation of CO_2 from the outside air is thus reduced.

Although the high level (21%) of oxygen in the atmosphere is needed to sustain animal life, this high oxygen content limits photosynthesis in plants. Compared with 1 to 2% oxygen, the oxygen level in normal air inhibits net CO_2 uptake in C_3 plants by 33 to 55%. The inhibition is fully reversible; the same photosynthetic

rates appear in experiments shifting back and forth between 1 to 2% oxygen and 21% oxygen (Zelitch, 1992; Hanson and Peterson, 1986). The extent of this reversible oxygen inhibition is determined by the CO_2/O_2 ratio, and there is no inhibition of photosynthesis in 21% oxygen if CO_2 levels are raised sufficiently (Hanson and Peterson, 1986).

At higher temperatures and oxygen levels, photorespiration increases greatly relative to net photosynthesis in C_3 plants. Measured photorespiration under controlled conditions was 28% of net photosynthesis at 29°C and 44% at 34°C (Hanson and Peterson, 1986). Thus CO_2 losses by photorespiration increase appreciably as temperature increases.

One mechanism for regulating photorespiration would be to change the characteristics of the enzyme so that the affinity of ribulosebisphosphate carboxylase (RuBISCO), for CO_2 is greater and there is relatively less oxygenation. Jordan and Ogren (1988), showed that the specificity of the enzyme for reaction with the two gaseous substrates under standard conditions varies among widely divergent types of plants. The CO_2/O_2 specificity ratio under 25°C, 320 µl CO_2/l. and 21% oxygen was highest in C_3 plants (and relative photorespiration was lowest) with a ratio of 3.1-3.3. The ratio was 2.4-3.0 in C_4 plants, 2.6 in green algae, and 1.9-2.2 in cyanobacteria (Jordan and Ogren, 1983; Zelitch, 1992). This finding indicates that it might be possible to increase the CO_2/O_2 specificity by appropriate selection for genetic changes or by use of site-directed mutagenesis to alter the properties of the enzyme in transgenic plants (Zelitch, 1992).

Chen *et al* (1990), described a mutant of the alga *Chlamydomonas* which has RuBISCO specificity ratio decreased compared to wild-type. Although the specificity was not altered in the direction needed to reduce photorespiration, these results demonstrate that CO_2/O_2 specificity is not necessarily fixed within a species (Chen *et al.*, 1990; Zelitch, 1992).

On the basis of photosynthesis plants are generally classified into three types, *viz*, C_3-photosynthetic plants, C_4-photosynthetic plants and CAM plants. The initial reaction of CO_2 fixation in C_3-photosynthetic plants is catalyzed by RuBISCO, wherein the enzyme catalyzes the joining of CO_2 with ribulose*bis*phosphate (RuBP), to form the three carbon compound, 3-phosphoglyceric acid (PGA). C_3-photosynthetic plants (for example rice, wheat, etc.) suffer from a lack of effective CO_2 concentrating mechanism, since here CO_2 has to first diffuse through the cell-wall, then through cell membrane, cell cytoplasm and finally through chloroplast envelope before it can be utilized by RuBISCO. Besides, CO_2 has to compete with oxygen for binding to RuBISCO active site. Hence, the overall yield of C_3-photosynthetic plants in terms of biomass are much lower than that of other photosynthetically advanced plants (Zelitch, 1982).

Through a mechanism of CO_2 enrichment in the bundle sheath (BS) cells, the C_4 species (maize, sorghum, sugarcane, etc.), circumvent wasteful photorespiration. These species usually have higher rates of photosynthesis than do C_3 species (Edwards and Walker, 1983) and maximal average yields are approximately twice as great (Zelitch, 1982).

In the case of C_4-photosynthetic plants, CO_2 is fixed into a 4-carbon compound, oxaloacetic acid (OAA), through the action of phospho*enol*pyruvate carboxylase (PEPC), present in the mesophyll (MC) cytoplasm. OAA is subsequently converted to malate or aspartate, depending on the type of C_4-photosynthetic species. These 4-carbon compounds are then transported to the neighboring BS cells, where they are decarboxylated by one of the three decarboxylating enzymes - NADP-dependent malic enzyme (NADP-ME), NAD-dependent malic enzyme (NAD-ME) or phospho*enol*pyruvate carboxykinase (PEP-CK).

Carbon dioxide released by this decarboxylation reaction *in situ* is fixed by RuBISCO, as in C_3-photosynthetic plants (Hatch *et al.*, 1976; Edwards and Huber, 1981; Edwards and Walker, 1983; Hatch, 1987; Furbank and Foyer, 1988).

The C_4 pathway, thus eliminates photorespiration by splitting photosynthetic reactions between the two morphologically distinct cell-types, MC and BS, wherein RuBISCO is physically separated from atmospheric oxygen by compartmentalization in the inner BS cells (Nelson and Langdale, 1992). Due to its complex anatomy and compartmentalization of C_4-photosynthetic enzymes, photosynthesis is not inhibited at 21% or 42% oxygen levels, even if accompanied by low CO_2 levels (Edwards and Walker, 1983; Zelitch, 1992). Photorespiration is suppressed here, rather than being concealed by an efficient CO_2 refixation. Measurements of the carbon flux through the glycolytic pathway of photorespiration of maize leaves shows that the pathway accounts for less than 2% of the CO_2 fixed (Hanson and Peterson, 1983).

C_3-C_4 intermediates (*Panicum*, *Mollugo*, *Moricanda*, *Neurachne* and *Flaveria*) are another example of compartmentalization in plants that partially regulate photorespiration. These plants have as a common feature, a rudimentary BS system in their leaves (Brown and Hattersley, 1989) and show less inhibition of photosynthesis by oxygen than do C_3-photosynthetic plants and more than C_4-photosynthetic plants. Their CO_2 compensation points (steady-state level of CO_2 when leaves are placed in light in a closed system) is also intermediate between C_3 and C_4 values (Monson *et al.*, 1984). Many of the photosynthetic features of the C_3-C_4 intermediates, including a decreased rate of photorespiration, can be explained by the finding that the glycine decarboxylase reaction of the photorespiratory pathway that produces CO_2 is confined to mitochondria of the BS cells. There RuBISCO carboxylase reaction can more efficiently recapture photorespiratory CO_2 (Hylton *et al.*, 1988). Thus in the case of C_3-C_4 intermediate species, photorespiration is suppressed by an efficient CO_2 refixation system in the BS cells.

In C_4-photosynthetic plants, PEPC, NADP-dependent malate dehydrogenase (NADP-MDH), pyruvate *ortho*phosphate dikinase (PPDK), NADP-ME and RuBISCO are differentially expressed in the two cell-types MC and BS of green leaves. With the exception of PPDK, the other photosynthetic enzymes accumulate only in the cell-type where they normally function, i.e., PEPC and NADP-MDH in MC cells, and NADP-ME and RuBISCO in BS cells. This has been established by cell separation experiments (Huber *et al.*, 1976; Kirchauski and Park, 1976; Broglie *et al.*, 1984; Aoyagi and Nakamoto, 1985; Sheen and Bogorad, 1987a,b ; Sheen, 1991) and immunoprecipitation experiments

(Hattersley et al., 1975; Matsumoto *et al.*, 1977; Perrot-Rechemann *et al.*, 1982; Perrot-Rechemann *et al.*, 1983; Langdale *et al.*, 1987; Sheen and Bogorad, 1987a,b).

Differential Expression :

In the development of most C_4-photosynthetic plants, the expression of C_4-photosynthetic genes does not occur until kranz anatomy has been established (Nelson and Langdale, 1992). Crespo *et al* (1979), compared maize leaves 1 to 5 with the appearance of C_4-enzymatic activities. They found that the 1^{st} leaf was more C_3 in character, while the 5^{th} leaf was fully C_4. Other workers have shown in a variety of C_4 species, that relative use of C_4 and C_3 scheme varies from young to mature to senescent leaves of the same plant, showing that C_4 system is a continuously regulated one (Kennedy and Laetsch, 1973; Khanna and Sinha, 1973; Imai, 1979; Thangarajah *et al.*, 1981; Moore *et al.*, 1986). Combined with studies on the appearance of kranz anatomy in developing leaves (Miranda *et al.*, 1981; Dengler *et al.*, 1986; Dengler *et al.*, 1990), these whole leaf physiological and biochemical studies indicate that the *system* appears at a time consistent with the maturity of kranz anatomy and suggest that exclusive use of C_3-photosynthetic pathway may occur prior to full differentiation of kranz anatomy (Nelson and Langdale, 1992).

A second approach has been to exploit the developmental age gradients in maize leaves. Such studies have characterized the time of appearance of C_4 activities (Williams and Kennedy, 1978; Perchorowicz and Gibbo, 1980; Miranda *et al.*, 1981), proteins (Mayfield and Taylor, 1984; Martineau and Taylor, 1985; Langdale *et*

al., 1987), and mRNAs (Martineau and Taylor, 1985; Langdale *et al.*, 1987). The major findings of these studies are that C_4 function appears in the region of leaf blade expansion and is present to a lesser extent in the sheath region, which is less vascularized. Further, levels of C_4 proteins increase towards the tip of the leaf, while levels of the corresponding mRNAs peak near the base of the blade and decrease towards the tip. The regions displaying detectable C_4 function and C_4 enzymes in the developing leaf are well vascularized. In combination with studies of anatomical differentiation along monocot leaves (Miranda *et al.*, 1981; Langdale *et al.*, 1987), these age gradient studies suggest that the onset of C_4 gene expression is coordinated with differentiation of the bundle sheath. Aoyagi and Bassham (1986), found that C_4-photosynthetic enzymes do not make their appearance in green regenerating callus until some vascularization has occurred. However, green callus and suspension cultures from other C_4-photosynthetic plants were found to contain C_4-enzymatic activities (Laetsch and Kartschak, 1972), and to fix CO_2 into C_4 primary products (Usada *et al.*, 1971; Seeni and Gnanam, 1983), but without full function of the C_4 pathway. These studies suggest that kranz anatomy is an essential element for the accumulation of C_4-photosynthetic enzymes (Nelson and Langdale, 1992).

Immunolocalization methods have been used to visualize the cellular distribution of C_4-photosynthetic enzymes in leaf sections of varying developmental age (Langdale *et al.*, 1987). These studies have confirmed that C_4-protein accumulation is always associated with a mature vein and BS anatomy, even in relatively young (basal) regions of a developing maize leaf. In immunochemical studies on

mutants with clonal sectors of defective cells adjacent to normal cells, Langdale *et al* (1987), found that a MC cell can accumulate C_4-enzymes adjacent to a nonphotosynthetic BS cell. This suggests that the signal to accumulate C_4-proteins is either positional or involves a nonphotosynthetic metabolic interaction with the BS cell neighbour. Interestingly, expression in maize protoplast of genes for RuBISCO, PEPC, PPDK and NADP-ME is repressed by sucrose, glucose and acetate (Sheen, 1990).

In situ hybridization experiments have permitted the visualization of cellular patterns of C_4 mRNA accumulation during development (Langdale *et al.*, 1988). Certain C_4 genes are expressed concurrent with provascular cell divisions, but before extensive vascular differentiation. For example, RuBISCO large- and small-subunit genes are expressed in a ring of cells tightly surrounding the region of provascular cell divisions (Sheen and Bogorad, 1986; Schaffner and Sheen, 1991). The NADP-MDH gene is expressed locally in the same region, but at a greater distance from the vein. This position-specific expression occurs before the MC and BS are formed, and chloroplasts become distinguishable (Langdale *et al.*, 1988; Nelson and Langdale, 1992). Further only when the veins are fully differentiated, does the expression of genes for PEPC and NADP-ME reach equivalent local levels (Langdale *et al.*, 1988; Nelson and Langdale, 1992).

These studies show that positional control of BS and MC photosynthetic development must begin very early in the leaf primordium, concurrent with or just after the initiation of veins (Nelson and Langdale, 1992).

Light Induced Expression :

Light is essential to normal plant growth, both because it provides energy for photosynthesis and because it provides many of the environmental signals that regulate plant development. From germination through growth, flowering and fruiting, light is essential for the initiation and regulation of all these functions. During the process, the expressions of many genes is affected in many different ways. Light interacts with endogenous developmental programmes to modulate these gene responses, often by acting through two or more different photoreceptors (Simpson and Harrere-Estrella, 1990; Thompson and White, 1991).

The best characterized photoregulated genes are the RuBISCO small-subunit gene (*rbcl*) and the gene for chlorophyll a/b binding protein (*cab*) (Tobin and Suttie, 1980; Golmer and Apel, 1983; Jenkins *et al.*, 1983; Sasaki *et al.*, 1983; Stiekema *et al.*, 1983; Batschauer and Apel, 1984; Kaufman *et al.*, 1984; Nelson *et al.*, 1984; Pichersky *et al.*, 1987; Otto *et al.*, 1988; Pichersky *et al.*, 1988; Marrs and Kaufman, 1989; Oemuller *et al.*, 1989; Pichersky *et al.*, 1989; Stayton *et al.*, 1989; Warpeha *et al.*, 1989; Simpson and Herrera-Estrella, 1990; Wehmeyer *et al.*, 1990; Thompson and White, 1991). Most of the changes in gene expression in response to light is mediated through phytochromes (Gilmartin *et al.*, 1990; Simpson and Herrera-Estrella, 1990; Thompson and White, 1991), and is regulated at the transcriptional level (Gallagher and Ellis, 1982; Silverthorne and Tobin, 1984; Berry-Lowe and Meagher, 1985; Masinger *et al.*, 1985).

Phytochrome is the best characterized of the three known photoreceptors. The other two photoreceptors are Cryptochrome and UV-B photoreceptor (Kendrick and Kronenberg, 1986; Gilmartin *et al.*, 1990; Simpson and Herrera-Estrella, 1990; Thompson and White, 1991). Collectively, these receptors can absorb photons over a wide range of wavelengths, ranging from the far-red to ultraviolet.

Analyses of the kinetics of light-responsive gene induction shows that the rate of mRNA accumulation is variable among genes and can be dependent on the developmental state of the plant (Gallagher *et al.*, 1985; Fluhr and Chua, 1986). Hence, whereas expression of genes like *cab*, *rbcs* etc. increases in the presence of light, expression for genes coding for phytochrome (Lissemore and Quail, 1988; Kay *et al.*, 1989), *NADPH-protochlorophyllide reductase* (Batshauer and Apel, 1984; Darrah *et al.*, 1990) and asparagine synthetase (Tsai and Coruzzi, 1990), are down-regulated by light.

The complexity of light regulated gene expression is accentuated by many other factors which come into play, for example, the *cis*-acting elements. These elements are usually found at the 5'-upstream region of the gene (Kuhlemeier *et al.*, 1987; Silverthorne and Tobin, 1987; Jenkins, 1988; Benfey and Chua, 1989; Dean *et al.*, 1989; Stockhaus *et al.*, 1989). However, there is evidence that other regions of the gene can mediate changes in transcript abundance in response to light. For example, in the case of a pea gene encoding feredoxin, sequences within the transcribed region modulate mRNA levels by affecting transcript stability (Elliot et al., 1989). However, interpretation of the role played by individual elements is complicated by the distinct responses seen in different tissues at

various stages of development (Gallagher *et al.*, 1985; Fluhr and Chua, 1986).

One of the reasons for localizing the *cis*-acting elements mediating light regulation is to use them as a tool to identify *trans*-acting transcription factors (Kuhlemeier *et al.*, 1987). It has been suggested that in addition to nuclear regulatory factors, at least one plastid-derived factor contributes to light-regulated transcription of specific nuclear genes (Batschauer *et al.*, 1986; Simpson *et al.*, 1986). Elimination of functional chloroplasts, as a result of mutation or herbicide treatment precludes light induction of *cab* transcription (Mayfield and Taylor, 1984; Batschauer *et al.*, 1986; Simpson *et al.*, 1986). The *ppc* gene does not contain any L-, I- or G-box motifs, which are found in genes encoding photosynthetic enzymes such as RuBISCO (Nelson and Langdale, 1992). On the other hand, it contains RS1-3 motifs in the 5'-flanking regions, which interacts with nuclear trans-acting factors (Yanagisawa and Izui, 1990,1992).

In the case of C_4-photosynthetic enzymes and mRNAs, steady-state levels increase several fold when dark-grown plants are exposed to light (Nelson *et al.*, 1984; Sheen and Bogorad, 1987a,b). This reflects increase over low levels that are developmentally induced. Sheen and Bogorad (1987a), have shown that though there is some expression of PEPC, PPDK, NADP-ME and RuBISCO in dark, both in MC as well as in BS cells, upon illumination, PEPC, PPDK and NADP-MDH levels shoot up in MC cells whereas NADP-ME and RuBISCO levels increase in BS cells. Subsequently, whatever little enzyme was expressed in the "incorrect" cell type, disappears upon illumination. Hence, in the case of photosynthetic enzymes, light

acts as a positive stimulant for enzymes in one cell-type and as a negative stimulant for the same enzyme in the other cell-type.

The C_4-photosynthetic gene expression is thus regulated by the integration of information on light level, cell localization and type, and metabolic state (Nelson and Langdale, 1992).

DNA Methylation :

Although at present no common elements have been recognized as responsible for the BS- or MC-cell specific gene expression, there is however, a strong body of evidence building up that suggests the differential expression might, in part, be explained by the differential methylation of the C_4 genes in the two cell-types (Ngernprasirtsiri *et al.*, 1989; Langdale *et al.*, 1991; Yanagisawa *et al.*, 1991). Ngernprasirtsiri *et al* (1989), used isoschizomeric pairs of restriction endonucleases to show that certain sites in or near the *ppc* gene and *ppdk* genes were methylated in BS cells but unmethylated in MC cells. Similarly, the RuBISCO large subunit (*rbcl*) and *rbcs* genes show a reverse pattern of methylation, which again is consistent with their expression exclusively in the BS cells (Ngernprasirtsiri *et al.*, 1989).

DNA methylation affects DNA-protein interactions (Arber and Linn, 1969; Boyer, 1971; Meselson *et al.*, 1972; Arber, 1974), protects DNA against restriction endonucleases (Nelson and McClelland, 1991), regulates gene expression in eucaryotes (Holliday and Pugh, 1975; Riggs, 1975; Sager and Kitchin, 1975; Christman *et al.*, 1977; Bird, 1978; McGhee and Ginder, 1979; McGhee and Felsenfeld, 1980), enhances mutation and recombination (Karber and Hays, 1982), can affect the structure of synthetic polypeptides

(Behe and Felsenfeld, 1981; Behe *et al.*, 1981; Klysik *et al.*, 1983), and probably of DNA as well (Doerfler, 1983), and may influence DNA replication (Burden and Adams, 1970; Bird, 1978), virus latency and differentiation (Desrosiers *et al.*, 1979; Yousoufian *et al.*, 1982).

DNA methylation can be considered as a modulator of these interactions and often acts as a long-term signal, since demethylation seems complicated to achieve by the cell (Doerfler, 1983).

Patterns of DNA methylation are inheritable (Holliday and Pugh, 1975; Riggs, 1975; Sager and Kitchin, 1975; Bird, 1978; Wigler, 1981; Wigler *et al.*, 1981; Gruenbaum *et al.*, 1982), and are maintained from cell division to cell division by the action of DNA methyltransferases. DNA methylation is passed on in a semiconservative manner. All biochemical evidence indicates that DNA is methylated in the early post-replicative step (Burden and Adams, 1969). It is only the newly synthesized strand that becomes methylated (Bird, 1978). Thus, DNA replication is a prerequisite for changing patterns of DNA methylation.

Maintenance DNA methyltransferases recognize hemimethylated DNA as their specific substrates (Gruenbaum *et al.*, 1982).

5-methylcytosine, appears to be the only major modified base in DNA of eucaryotes and occurs predominantly in the sequence CpG (Grippo *et al.*, 1968; Gautier *et al.*, 1977; Manes and Menzel, 1981; Naveh-Many and Cedar, 1981), but it has also been found in the sequence CpA, CpT and CpC (Gruenbaum *et al.*, 1981). For some genes or groups of genes it has been recognized that frequency clusters of the dinucleotide CpG can be found close to the promoter/leader

and/or 5'upstream regions (Doerfler *et al.*, 1982; Felsenfeld *et al.*, 1982; Fradin *et al.*, 1982). Thus, decisive element in gene regulation may lie in the strategic positioning of regulatory nucleotide sequences that can be methylated at crucial sites.

The percentage of 5-mC in eucaryotic DNA varies over a wide range. It is about 0.03 mol% in some insects such as mosquito *Aedes albopictus*, 2 to 8 mol% in mammals, and can be as high as 50 mol% in higher plants (Thomas and Sherratt, 1956; Dunn and Smith, 1958; Shapiro and Chargaff, 1960; Doskocil and Sorm, 1962; Vanyushin *et al.*, 1968; Vanyushin *et al.*, 1970; Drozhdenyuk *et al.*, 1977; Adams *et al.*, 1979; Drumling, 1981).

Li *et al* (1992), targeted a mutation into the murine DNA methyltransferase gene by homologous recombination in embryonic stem (ES) cells. The embryos were stunted and failed to develop past midgestation. Of particular interest is the observation that ES cells with both the alleles targeted were viable as undifferentiated stem cells in culture, suggesting that DNA methylation may contribute to the developmental programme, but is not required for cell viability.

In *Pisum sativum*, young seedlings had a lower level of DNA methylation as compared to apical buds, which were heavily methylated (Watson *et al.*, 1987). However, as development progressed, the methylation level of sequences such as rDNA genes decreased (Watson *et al.*, 1987).

There seem to be two ways in which DNA methylation can suppress transcription. One way is by directly preventing the binding of transcription factors to promoters (Tate and Bird, 1993). A number

of other reports point to the fact that transcription factors cannot bind to the promoters efficiently if the promoters are methylated (Lamb *et al.*, 1991; Wein *et al.*, 1991; Hengst-Zhang and Weitzman, 1992). Some other transcription factors, however, are insensitive to the presence of methyl CpG. Another way in which methylation can repress transcription is through proteins that bind to methylated DNA. Methyl CpG-binding protein is a protein of this type and binds specifically to DNA sequences that contain multiple symmetrically methylated CpGs. Methyl CpG-binding protein can repress the transcription of methylated promoters *in vitro* and probably *in vivo* (Boyes and Bird, 1991).

If methylated bases serve as a regulatory or modulatory role in various gene functions, one would expect that, in addition to the DNA methylation, demethylating activities would also exist. Apart from inducible alkylating mechanisms of a highly specialized nature in procaryotes and in mammalian cells (Karran *et al.*, 1979; Olsson and Lindahl, 1980), such demethylases are also recognized in eucaryotic cells (Gjerset and Martin, 1982).

The cytidine analogue 5-Azacytidine (5-AzaC) (Piskala and Sorm, 1964; Raska *et al.*, 1966), which cannot be methylated, inhibits DNA methyltransferases (Christman *et al.*, 1980; Jones and Taylor, 1981; Creusot *et al.*, 1982; Jones *et al.*, 1983). There is convincing evidence that 5-AzaC can be incorporated into the replicating DNA and in this state can inhibit the activity of DNA methyltransferases perhaps by irreversibly binding these enzymes (Christman *et al.*, 1980; Jones and Taylor, 1981; Creusot *et al.*, 1982; Jones *et al.*, 1983). However, the paradox is that if the analogue acts as a general enzyme inhibitor, then a general increase in gene expression

would be expected, rather than the selective increases usually found (Jones, 1985). Experiments using 5-AzaC in plant systems are limited and relate predominantly to induction of T-DNA genes in transformed plants. Hepburn *et al* (1983), found that treatment of a flax tumor line with 5-AzaC resulted in the demethylation of, on average, one copy of the nopaline synthase gene per cell. Amasino *et al* (1984), found that 5-AzaC treatment of a heavily methylated tobacco line in which T-DNA was not expressed resulted in subsequent expression and phytohormone independent growth.

Van Slogteren *et al* (1984), were able to show that the genes for octopine, mannopine and agropine in a non-expressing tobacco line became, on addition of 5-AzaC, active. The key therefore, to the growth inhibition exhibited here may be that genes activated by 5-AzaC are inhibitory to growth. Certainly if tissue culture response in maize is related to the presence of nuclear genes (Hodges *et al.*, 1986), then the fact that there is a differential effect of 5-AzaC, dependent on whether the tissue is green or not, may be relevant. Hodges *et al* (1986) state that these factors are nuclear-encoded but the fact that they are not obviously active in etiolated plants would suggest that chloroplast-encoded factors may also be involved in tissue culture response.

As absence of DNA methylation appears a necessary but not sufficient precondition for gene activation (Van der Ploeg and Flavell, 1980; Kuhlman and Doerfler, 1982), it is not expected that all dormant cellular genes can be turned on by treatment of cells with 5-AzaC. This is more so because DNA methylation at highly specific sites, which could be different for different genes, plays a role in the long-term inactivation of genes.

A crucial mechanism like gene activity is probably subject to multifaceted regulatory mechanisms, DNA methylation constituting only one important parameter. Thus, depending on the stringency of inactivation for a given gene, 5-AzaC treatment may or may not lead to the inactivation of a certain gene or set of genes (Doerfler, 1983).

It may affect cellular metabolism in several different ways and there is no evidence ascertaining that the inhibition of DNA methyltransferases is in fact the only, or the major influence on gene activity that this analogue exert.

Methylated sites associated with maize *ppc* genes (Langdale *et al.*, 1991; Yanagisawa *et al.*, 1991), and *rbcs* gene (Langdale *et al.*, 1991), were mapped in both BS and MC cells. Langdale *et al* (1991), showed that methylation in the *ppc* genes were present as far as 3.5 kb upstream of the transcription start site in the BS cells but not in the MC cells. CpG islands have been identified thus far in sequences flanking maize and sorghum *ppc* genes (Hudspeth and Grula, 1989; Matsuoka and Minami, 1989; Cretin *et al.*, 1991; Langdale *et al.*, 1991), and in sorghum NADP-MDH (*nmdh*) (Luchetta *et al.*, 1990) genes, although no functions has yet been ascribed to these islands in higher plants. These are however, not differentially methylated in the case of *ppc* genes (Langdale *et al.*, 1991), and the methylation status of *nmdh* genes is not known. The correlation of differential methylation with the expression pattern of C_4 genes is striking, although its functional significance remains to be determined.

Hypothetically then, if one is able to introduce the CO_2-concentrating enzyme - PEPC from a C_4-photosynthetic plant, into a C_3-photosynthetic plant, then the yield and biomass production of these C_3-photosynthetic plants could be enhanced. But for this to be true, one has to fully understand the regulatory mechanism of CO_2-concentrating enzyme, PEPC, in its native C_4-photosynthetic plant. The present thesis is aimed at understanding some of these factors.

The present thesis is divided into the following sections:

The general section which includes the first chapter, **Introduction**, and the second chapter, **Materials and Methods.**

The second section is on Tissue Culture and includes the third chapter, **Effect of 5-Azacytidine on Sugarcane Tissue Culture *In Vitro*.**

The third section is on biochemical aspects and include the fourth chapter, **Biochemical Study of the Effect of 5-Azacytidine on Phospho*enol*pyruvate Carboxylase on Day 15 of Subculture**, and the fifth chapter, **Time Course Analysis of the Influence of 5-Azacytidine on the Enzyme Activities of PEPC (Mesophyll Cytoplasmic); NADP-ME (Bundle Sheath Chloroplastic) and PPDK (Mesophyll Chloroplastic Enzyme).**

The last section is on the molecular biology aspect and includes the sixth chapter, **Sequence Analyses of PEPC cDNA from Sugarcane.**

Materials and Methods

Plant Material :

Sugarcane, *Saccharum officinarum* cv. CO 740, plants were regenerated and maintained *in vitro* as reported earlier (Rawal *et al*, 1985). The terminal part of the shoot of sugarcane (*Saccharum officinarum*, cv. CO 740), just below the first visible node was cut, the mature leaves removed and the bases of inner immature leaves were used as explant. These were rinsed with double distilled water (dH_2O), followed by wash in 0.1% Nonidet P-40. These were then rinsed again in sterile dH_2O and surface sterilized with 0.1% $HgCl_2$ for 10 mins. under aseptic conditions. The basal 5 cm portion were cut into 2-3 cm thick slices and inoculated on to induction medium of Murashige and Skoog, (1962) medium, supplemented with 2,4-D (3 mg/L), 10% coconut milk and 3% sucrose. The explants were incubated on the induction medium for 12 days under constant light at 26 °C. The cultures were next transferred to the differentiation medium, which was the same as the induction medium, except that 2,4-D was omitted. Plants differentiated in 8-12 days and were designated as the control.

Plant regeneration was affected both in presence and absence of 5-AzaC (15 μM). Plants were designated as :

CL - control plants grown in continuous light,

AL - plants cultured– in presence of 5-AzaC and in continuous light,

CD - control plants grown in dark and

AD - plants cultured in presence of 5-AzaC and incubated in dark.

On the 12th day of culture a further 15 µM 5-AzaC was added to different cultures listed above. These were designated as :

CLA - control plants grown in light and 5-AzaC added on day 12,

CDA - control plants grown in dark with 5-AzaC added on day 12.

Seventy two hours after further addition of 5-AzaC, the plants were harvested for further analysis.

Fully differentiated plants were transferred to MS medium supplemented with benzyl amino purine (BAP, 0.1 mg/L), kinetin (0.2 mg/L), coconut milk (10%), sucrose (1.5%), glucose (1.0%) and without or with 5-AzaC (15 µM), for further growth and development and multiplication and subculture.

Cell Separation :

The cells were separated by gentle maceration of the leaves in an ice cold mortar and pestle, in one volume of the extraction buffer containing, 50 mM Tris-HCl (pH 8.0), 350 mM mannitol, 7 mM EDTA, 1 mM $MgCl_2$, 0.1% BSA and 5 mM β-ME. The extract which served as the mesophyll lysate was passed through 4 layers of Mera cloth and centrifuged at 12,000 x g for 10 min at 4 °C and used for PEPC, PPDK and NADP-ME assay. The residue on Mera cloth was washed thoroughly with dH_2O to remove any adhering mesophyll cells. The pure bundle sheath strands were then ground in liquid N_2 and suspended in 1/2 volume of the extraction buffer. The extract was centrifuged at 12,000 x g for 10 min at 4 °C and was used for PEPC, PPDK and NADP-ME assay in the bundle sheath cells.

Protein Estimation :

Proteins were estimated by the sensitive dye binding method of Bradford (1976). Crystalline bovine serum albumin was used as standard.

A portion of 10 µl of the cell extracts was mixed with 1.0 ml Bradford's reagent (Coomassie Brilliant Blue G-250 10 mg dissolved in 5 ml, 95% ethyl alcohol and 9.66 ml phosphoric acid. Deionised water was added to a final volume of 100 ml). After 2.0 min, absorbance at 595 nm was recorded. BSA (0.5 mg/ml, dissolved in 50 mM Tris-HCl, pH 7.8), was used as standard in the range of 0-10µg.

Chlorophyll Estimation :

Chlorophyll was estimated essentially as described by Arnon (1949).

Cell extract (250µl), was mixed with 80% acetone to a final volume of 2.5 ml. Absorbance at 663 and 645 nm was recorded. Chlorophyll A and B was measured using the following equations :

Chl A (mg/ml) = (12.7 x A663 - 2.69 x A645) x 250 / 1000 x 25

Chl B (mg/ml) = (22.9 x A645 - 4.68 x A663) x 250 / 1000 x 25

Phospho*enol*pyruvate Carboxylase Assay :

PEPC assay was carried out according to Jiao and Chollet (1988), with minor modifications. The assay buffer of 1.0 ml contained 50 mM Hepes-KOH (pH 7.3), 2.5 mM PEP, 10 mM $MgCl_2$, 1 mM $NaHCO_3$, 0.2 mM NADH and 10 U of malate dehydrogenase. Reaction was

initiated by the addition of crude enzyme extract, and was linear upto 2 mins. The reaction was followed by drop in OD at 340 nm.

Pyruvate *Ortho*phosphate Dikinase Assay :

Pyruvate *ortho*phosphate dikinase (PPDK) assay was performed according to Andrews and Hatch (1969). The PPDK reaction was measured in the reverse direction by coupling it with lactate dehydrogenase. The reaction, in addition to the crude enzyme extract, contained 50 mM Tris-HCl (pH 8.3), 6 mM $MgSO_4$, 10 mM DTT, 0.15 mM NADH, 1 mM PEP, 1 mM AMP, 1 mM PPi and approximately 6 U lactate dehydrogenase, in a final volume of 1 ml. The rate of decrease in A340, due to NADH oxidation, dependent on the addition of PPi was measured for 2 mins.

NADP-Dependent Malic Enzyme Assay :

NADP-dependent malic enzyme (NADP-ME) assay was performed according to Kobayashi *et al* (1980), with slight modifications. The reaction, in addition to the crude enzyme extract contained, 0.1 M Tris-HCl (pH 8.0), 0.2 mM EDTA, 5 mM DTT, 0.2 mM $NADP^+$, 10 mM L-malate and 10 mM $MgCl_2$ in a total volume of 1 ml. Mg^{2+}-dependent formation of NADPH was measured by increase in A340 over a period of 2 min.

Immunoblot analysis :

The mesophyll cell and bundle sheath lysates were centrifuged at 12,000 x g for 10 min at 4 °C and stored in aliquots of 100 µl at -70 °C. 15 µl of this was slot-blotted on to nitrocellulose and probed with rabbit polyclonal antibodies raised against corn PEPC.

Promega Protoblot® Immunoscreening System was used to detect the amounts of PEPC protein, according to the manufacturers protocol. The blots were scanned with Mitsubishi densitometer scan and PEPC protein in each slot blot quantified.

Total Genomic DNA Isolation :

Total genomic DNA was isolated from 2.5 g sugarcane leaf material according to a modification of the protocols by Sacco De Vries *et al* (1988) and Blin and Stafford (1976). Sugarcane leaves were crushed under liquid nitrogen in a precooled mortar and pestle. The powder was transferred to a beaker and 5 ml of grinding buffer (50 mM Tris-HCl, pH 8.0; 7 mM EDTA; 10 mM $MgCl_2$; 350 mM mannitol; 1 mM ATA), was added, which was incubated at 55 °C. 5 mM β-ME, 0.5 % BSA and SDS to a final concentration of 0.5 % was added and the mixture cooled slowly to 50 °C. Finally, 5 ml of Tris-equilibriated phenol (pH 8.0), was added and the mixture filtered through 4 layers of cheese cloth. The filtrate was spun in a Sorvall SS 24 rotor at 5,000 x g at 20 °C for 10 min. To the resultant aqueous phase, 1 vol. phenol:chloroform:isoamyl alcohol (25:24:1) was added, mixed and centrifuged as above. The aqueous phase was finally resuspended in chloroform:isoamyl alcohol (24:1), mixed and centrifuged as above. To the final aqueous phase, 1/10 vol. 3 M sodium acetate (pH 7.0) was added and the total genomic DNA precipitated with addition of 2.5 vol. absolute ethanol and incubating at RT. for 10 min. The DNA was spooled out with the help of a looped glass capillary. The spooled DNA was washed in 70% ethanol, dried and dissolved in 2-5 ml of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

Southern Hybridization :

Total genomic DNA and the λDNA/plasmids carrying putative PEPC cDNAs from sugarcane leaves, were digested with appropriate restriction enzymes and run on 1% TBE (45 mM Tris-base, 45 mM boric acid and 2 mM EDTA, pH 8.0) gel. The fractionated DNA was visualized by staining with 0.5 µg/ml ethidium bromide and photographed. The DNA was subsequently transferred to a S&S NYTRAN nylon membrane using the Southern (1975) capillary transfer method in 10X SSPE (20X SSPE, 3.6 M NaCl, 0.2 M $NaPO_4$ (pH 7.7), 20 mM EDTA). The transfer was carried out overnight. After completion of the transfer, the NYTRAN membrane was washed briefly in 10X SSPE and baked in an oven for 2 hrs. at 80 °C.

The blot was then prehybridized for 4-6 hrs. in prehybridization buffer (6X SSPE, 0.2% ficoll, 0.2% polyvinylpyrrolidone, 0.2% BSA, 0.5% SDS and 50 µg/ml denatured buffalo liver DNA), at 55 °C. The blot was subsequently hybridized overnight in hybridization buffer (prehybridization buffer plus 20 ng/ml denatured ^{32}P labeled DNA probe), at 55 °C. After hybridization, the blot was washed once with 1X SSPE, 1% SDS at room temperature for 15 min, once with 1X SSPE, 1% SDS at hybridization temperature for 15 min, and finally with 0.1X SSPE, 0.1% SDS at 65 °C for 15 min for high-stringency wash. After completion of the washings, the blot was wrapped in saran wrap and autoradiographed for 24-36 hrs. using Kodak XAR-5 or X-OMAT RP film.

Total RNA isolation :

Total RNA was isolated according to modification of the protocols of Hoge *et al* (1982), De Vries *et al* (1983) and Govers *et al* (1985).

In a precooled mortar and pestle, 2.5 g sugarcane leaves were taken and kept under liquid nitrogen. A 1:1 mixture of RNA extraction buffer (Tris-NaOH, pH 9.0, 100 mM LiCl, 1% SDS, 10 mM EDTA and 1 mM ATA) and equilibrated phenol (pH 8.0), was made and heated to 90 °C in a water bath under a fume hood. The leaves were ground to a powder in liquid nitrogen and transferred to a beaker kept at 90 °C. 5 ml of RNA extraction buffer and phenol mixture was added and mixed till a milky suspension, devoid of clumps resulted. 2 ml of chloroform was added and the suspension was thoroughly mixed. The suspension was transferred to a SS 24 centrifuge tube and centrifuged at 20,000 rpm at 25 °C for 30 min. To the upper aqueous phase was added 2 ml of chloroform:isoamyl alcohol (24:1), mixed and centrifuged at 20,000 rpm for 10 min. The resultant aqueous phase was collected and RNA precipitated with 1/3 vol. 8 M LiCl and incubated at 4 °C for 48 hrs. The precipitated RNA was washed once with 2 M LiCl and twice with 80% ethanol. Finally, the RNA precipitate was dried, resuspended in 80% ethanol and stored at -70 °C.

Isolation of Poly A^+ RNA :

Poly A^+ RNA was prepared according to Aviv and Leder (1972) and Sambrook *et al* (1987). Oligo(dT)-cellulose type-7, 0.6 g, was suspended in 2 ml, 1X binding buffer (Tris-HCl, pH 7.5, 0.5 M NaCl,

1 mM EDTA and 0.5% SDS) and 2 ml 0.1 N NaOH. The resin was mixed gently and centrifuged at 1,500 x g for 2 min. The supernatant was discarded. The above procedure was repeated 10 times. Subsequently, the resin was suspended only in 1X binding buffer and treated 10 times as above. Finally, the resin was suspended in 2 ml, 1X binding buffer and 1 ml of the slurry was transferred to a DEPC-treated, sterile blue tip, previously plugged loosely at the narrow end with sterile, DEPC-treated glass wool. The column was allowed to settle under gravity for 15 min.

About 200 µl of total RNA solution was mixed with 200 µl 2X binding buffer and loaded on the oligo(dT)-cellulose column. The column was transferred to a fresh eppendorf tube and centrifuged at 1000 x g for 1 min. The filtrate was reloaded on the same column and the above procedure repeated thrice. Subsequently, the column was washed thrice with 300 µl wash buffer (0.01 M Tris-HCl, pH 7.5, 0.1 M NaCl and 1 mM EDTA). Poly A^+ RNA was eluted in 200 µl elution buffer (Tris-HCl, pH 7.5 and 1 mM EDTA), and the eluate collected by centrifugation. The elution was carried out thrice and the filtrate collected in fresh DEPC-treated, sterile eppendorf tubes. The three eluates were pooled together, and the Poly A^+ RNA precipitated with 0.1 vol. 3 M sodium acetate and 2.5 vol. absolute ethanol and overnight incubation at -20 °C. Subsequently, precipitated Poly A^+ RNA was centrifuged at 12,000 x g for 15 min, washed with 80% ethanol, dried and dissolved in 50 µl TE (pH 8.0).

cDNA Library Preparation :

Sugarcane cDNA library was prepared using Promega's RiboClone® cDNA preparation kit, according to the manufacturer's protocol.

First Strand Synthesis :

The final reaction condition for first strand synthesis was : 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermidine, 10 mM DTT, 4 mM sodium pyrophosphate, 1 mM each of dATP, dGTP, dTTP and dCTP, 1 U RNasin, 0.5 µg Xba I-oligo dT primer/µg RNA and 15 U AMV reverse transcriptase and 1µg Poly A^+ RNA, in a total reaction volume of 25 µl. 5 µl of the above was taken in a separate eppendorf tube, to be used as tracer. The reaction was carried out at 42 °C for 1 Hr.

Second Strand Synthesis :

The final reaction conditions for the second strand synthesis included : 50 mM Tris-HCl, pH 7.6, 100 mM KCl, 5 mM $MgCl_2$, 50 µg/ml BSA, 5 mM DTT, 8 U/ml RNase H, 230 U/ml *E. coli* DNA Pol I and from the first strand reaction : 20 µl vol. containing 0.2 mM each of dATP, dGTP, dTTP and dCTP, 0.1 mM spermidine and 0.8 mM sodium pyrophosphate, in a total volume of 100 µl. 10 µl of this was taken in a separate eppendorf tube, to be used as tracer. The reaction was incubated at 14 °C for 4 hrs. The reaction was stopped by heating at 70 °C for 10 min. After transferring on ice, 2 U T4 DNA polymerase was added and the reaction incubated at 37 °C for 10 min to generate blunt ends. The reaction was finally stopped by adding 10 µl of 200 mM EDTA. cDNA was then phenolyzed and recovered by ethanol precipitation. cDNA was dissolved in 50 µl TE (pH 8.0).

Eco RI Adaptor Ligation :

Eco RI adaptors were prepared from two oligonucleotides kindly gifted by Dr. K.N. Ganesh. The two oligos were 5'-AATTCCGTTGCTGTCG-

3' and 5'-CGACAGCAACGG-3'. Eco RI adaptors were synthesized by heating the two oligos to 70 °C for 10 min, in 1:1 molal ratio, and slowly cooling them to room temperature. The Eco RI adaptors were tested by self-ligation and restriction digestion with Eco RI.

The reaction mixture for adaptor ligation was 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 0.1 mg/ml BSA, 2.5 µl cDNA (100 ng/µl), 1 µl Eco RI adaptors (10 nmoles) and 7.5 Weiss U T4 DNA ligase, in a total volume of 30 µl. Ligation was carried out at 15 °C for 18 hrs. Subsequently, the reaction was stopped by heating at 70 °C for 10 min prior to packaging. Unligated Eco RI adaptors were separated through Sephacryl S-400 column chromatography. The cDNA was phenolyzed and ethanol precipitated and dissolved in 20 µl TE (pH 8.0). It was then digested with Xba I, phenolyzed and ethanol precipitated to yield cDNA carrying staggered ends at either ends, one generated by Xba I digestion, and the other through Eco RI adaptor. Similarly, λGEM4 DNA was digested with Xba I and Eco RI to facilitate directional cloning of cDNA.

cDNA ligation to λGEM4 vector arms was carried out in a total of 5 µl reaction mixture containing 30 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 1 µl cDNA, 1 µl λGEM4 vector arms, 1 Weiss U T4 DNA ligase at 25 °C for 4-6 hrs.

Packaging of cDNA clones :

Packaging was performed using Promega's Packagene® In Vitro packaging system, strictly according to the manufacturers protocol. Briefly, to the 50 µl of packaging extract thawed on ice, 5 µl of the above ligation mixture was added and mixed gently. The mixture

was incubated at 22 °C for 2 hrs. Subsequently, 0.5 ml phage buffer (20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgSO_4$) and 25 µl chloroform was added, and mixed. The resultant mixture was stored at 4 °C.

Titration of Packaged Phage on LB Plates :

A single colony of E. coli LE392 was inoculated in 50 ml LB medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract, 5 g NaCl in 1 lt deionized water), supplemented with 0.5 ml 20% maltose and 0.5 ml 1 M $MgCl_2$ and grown overnight with vigorous shaking at 37 °C. The next day, 500 µl of above was further inoculated in 50 ml of supplemented LB and grown at 37 °C with vigorous shaking till O.D.600 was 0.6. Packaging extract was diluted 1:1000 to 1:10,000 in phage buffer. 100 µl of diluted phage buffer and 100 µl of the prepared E. coli cells were mixed together in a test-tube and incubated at 37 °C, with occasional shaking for 30 min, to allow the phage to adsorb on the LE392. 3 ml of molten (45 °C) TB top agar (1% Bacto-tryptone, 0.5% NaCl, 0.6% agar, 0.01 M $MgSO_4$), was added next and the mixture was vortexed. It was then poured onto LB plates containing 100 µg/ml ampicillin. The top agar was allowed to harden and the plates were incubated overnight in inverted position at 37 °C.

Plaque Hybridization :

Plaque hybridization was performed according to Benton and Davis (1977). Plaques were lifted onto a S&S Nitrocellulose paper (S&S NC^{tm} BA85) by placing the membrane directly on the surface of the plate so that the entire surface was covered. After allowing phage transfer for 5 min, the membrane was peeled off and placed

face up in a puddle of 0.5 N NaOH, 1.5 M NaCl for 3-5 min. This was followed by transfer of the membrane to a puddle of 0.5 M Tris-HCl, pH 8.0, 1.5 M NaCl for 3-5 min. The membrane was next transferred to a puddle of 2X SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0). Finally, the membrane was dried under vacuum at 80 °C for 2 hrs.

Hybridization conditions and washings were the same as described for Southern hybridization earlier in this chapter.

Phage λ DNA Isolation :

Recombinant phage λGEM4 DNA was isolated using the method of Zabarovskii and Turina (1988).

A λ GEM4 plaque from a petridish was placed in 10 ml of LB-medium (10 g Bacto-tryptone, 5 g Bacto-yeast extract and 10 g NaCl in 1 lt deionized water), containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$. A portion of 50-500 µl of an overnight E. coli LE392 culture was added to the test tube. The test tube was incubated at 39 °C with vigorous shaking for 6-8 hrs. Finally, 1 ml chloroform was added and the test tube was further incubated with vigorous shaking at 39 °C for 5 min.

The aqueous phase from above was centrifuged at 8000 rpm for 10 min at 4 °C. The supernatant was collected and centrifuged in a Sorval swingout rotor, TH641 at 35,000 rpm for 30 min at 16 °C. The sediment was suspended in 0.5 ml TE containing 10 mM $MgCl_2$ and centrifuged for 5 min in a microfuge at 12,000 rpm, in order to remove E. coli residue and aggregated cell contents.

To the supernatant, was added 3 µl 20% SDS, 8 µl 0.5M EDTA and 10 µl 10 mg/ml RNase A. The mixture was incubated at 70 °C for 10

min. The mixture was subsequently subjected to phenolization and phage λGEM4 DNA recovered by NaCl/ethanol precipitation. The DNA was dissolved in 200 µl TE (pH 8.0) and stored at -20 °C.

E. coli Transformation :

E. coli transformation was done according to Sambrook *et al* (1989), which is a slight modification of the method originally described by Cohen *et al* (1972). For transformation of E. coli with recombinant plasmids, Rec A^- strains were used, such as JM109 and DH5α.

A single colony of E. coli JM109 or DH5α was inoculated in 50 ml LB medium and grown to stationary phase overnight. Next day, 500 µl of the bacteria was inoculated in fresh 50 ml LB medium and incubated at 37 °C with vigorous shaking, for 2-3 hrs. The cells were then harvested by centrifugation in Sorvall HS-4 rotor at 4,000 rpm for 10 min at 4 °C. The supernatant was discarded and the cell-pellet resuspended in 25 ml ice-cold 0.1 M $MgCl_2$ and kept on ice for 10 min. The cells were centrifuged as before and the supernatant discarded. The cell-pellet was suspended in 25 ml ice-cold 0.1 M $CaCl_2$ and kept on ice for 30 min. The cells were then pelleted as before and finally resuspended in 2.5 ml ice-cold 0.1 M $CaCl_2$. This suspension was stored at 4 °C overnight.

About 200 µl of the competent cells was mixed with 25-50 ng of recombinant plasmid DNA in an eppendorf tube and kept on ice for 30 min. Subsequently, the bacteria was given a heat shock at 42 °C for 90 sec and transferred back on ice. Next, 800 µl LB medium was added and the tube incubated at 37 °C for 45-60 min to enable the bacteria

to express the antibiotic resistance marker. Finally, the bacteria was plated on solid LB plates harboring the appropriate antibiotic.

Plasmid DNA Isolation :

Plasmid DNA was isolated by alkaline lysis method of Sambrook *et al* (1989), which is a modification of the methods of Birnboim and Doly (1979) and Ish-Horowicz and Burke (1981).

Confluent culture of E. coli JM109 or DH5α, harboring recombinant plasmids were sedimented in a Sorval HS-4 rotor by centrifugation at 4,000 rpm for 10 min at 4 °C. The supernatant was discarded and the cell-pellet resuspended in 2 ml solution I (25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 50 mM glucose) , and kept on ice for 5 min. Bacteria were then lysed by using 4 ml, freshly prepared solution II (0.2 N NaOH, 1% SDS). Lysis of bacteria was evident when the mixture turned gluey on thorough mixing of the contents. The tube was kept on ice for 5 min. Subsequently, 3 ml solution III (60 ml 5 M potassium acetate, 11.5 ml Glacial acetic acid and 28.5 ml deionized water), was added and the contents mixed thoroughly and kept on ice for 10 min. A flocculent white precipitate forms which is chromosomal DNA and K^+/SDS/protein complexes. The mixture was centrifuged at 12,000 rpm for 15 min. Recombinant plasmid DNA was precipitated from the supernatant by adding 2 vol. absolute ethanol and incubating at room temperature for 20 min.

DNA Sequencing :

Double-stranded DNA was sequenced using United States Biochemical's Sequenase Ver 2.0 kit, strictly according to the manufacturers protocol. The DNA was sequenced by chain termination

method of Sanger *et al* (1977). 10 µg double-stranded plasmid DNA was denatured by adding 0.1 vol. 2 M NaOH, 2 mM EDTA, and incubating at 37 °C for 30 min. The mixture was neutralized with 0.1 vol. sodium acetate (pH 4.5-5.5) and denatured plasmid DNA rescued by adding 4 vol. absolute ethanol. DNA was precipitated at -70 °C for 15-30 min. After washing the pellet in 70% ethanol, the DNA was dissolved in 6.75 µl deionized water. Subsequently, 2 µl Sequenase 5X reaction buffer (200 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl), and 1 µl primer were added. Annealing was performed at 37 °C for 15-30 min.

While annealing was going on, four tubes were labeled as G, A, T and C and 2.5 µl of corresponding termination mixes (general formulation: 80 µM dNTPs, 8 µM specific ddNTP) were added to each. The termination mixes were incubated at 37 °C. Labeling mix (7.5 µM dGTP, 7.5 µM, dTTP, 7.5 µM dCTP) was diluted four fold. To ice-cold annealed DNA from above, was added 1 µl 0.1 M DTT, 2 µl diluted labeling mix, 0.75 µl ^{35}S dATP and 2 µl diluted Sequenase Ver 2.0 enzyme (3 U). Labeling reaction was carried out at 20 °C for 3 min. The eppendorf tubes were then kept on ice. 3.5 µl of the reaction mixture was then transferred to each of the four termination mixes at 37 °C. Termination was carried out at 37 °C for 3 min. The reactions were stopped by addition of 4 µl of stop dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF). The sequences were separated on 6% polyacrilamide gels. The reactions were heated at 70 °C for 2 min immediately prior to loading the gel.

The gels were pre-run for 30 min. prior to loading to get rid of excess ammonium persulphate. The gels were loaded in four

adjacent lanes in the pattern G A T and C. For each reaction, two loadings were used, one short run of 2 Hrs. and the other long run of 5. Hrs.

Post-electrophoresis, the gels were soaked in about 800 ml of washing buffer (acetic acid 5% and methanol 15%) for 20-30 min. with occasional shaking. This treatment facilitates removal of urea.

Drying was done in an oven at 80 °C for 2-3 Hrs. A thin layer of talcum powder was sprinkled on the dried gel. We found that the use of talcum powder not only ensures that the gel remains dried, it also does not interfere with autoradiography.

The gels were exposed to Kodak XAR-5™ and/or Kodak RP™ films by directly exposing the gel to the emulsion side of the film. Exposure was carried out for 2-3 days.

The autoradiographs were read from base upwards.

Sequence Analysis :

The sequences were keyed in the DNASIS sequence analysis package. Both the 5′ and the 3′ sequences were aligned with all the reported PEPC cDNA/genomic sequences from procaryotic and eucaryotic organisms. Multiple alignments and phylogeny studies were carried out using the CLUSTALV and PHYLIP packages.

Effect of 5-AzaC on Sugarcane Tissue Culture *In Vitro*

Introduction :

Plant tissue culture, over the years has emerged as the single major technology not only for plant propagation, but also for introduction of useful, novel traits in plants, which are stably integrated and inherited, and also as the most important means of germplasm conservation.

The primary aim of a plant tissue culture scientist is to develop a reliable and reproducible regeneration system for a plant species. This can be achieved with the choice of a number of available avenues. For example, plants can be regenerated through a protoplast regeneration system, or through the route of somatic embryogenesis and/or organogenesis etc. This is possible due to the totipotent properties of plant cells. However, even in systems where tissue culture and plant regeneration are regarded as standard techniques (as in many members of Solanaceae), problems such as somaclonal variation and habituation are now regarded as being integral, though often unwanted characteristics (Brown *et al*, 1989). These problems are further compounded when the sources of starting material and media compositions are also taken into consideration.

During plant development, the pattern of DNA methylation changes. Watson *et al* (1987), found that in *Pisum sativum*, while methylation levels were generally low, DNA from apical buds were highly methylated. Similarly, Ngernprasirtsiri *et al* (1989), reported different levels of 5-methyl cytosine and other modified

bases in bundle sheath and mesophyll cells of etiolated, greening and fully green maize leaves.

There are many similarities in DNA methylation between plants and animals. For example, in mammals, 5-methyl cytosine is the sole modified base appearing exclusively in CpG dinucleotides, whereas in plants, not only is the level of 5-methyl cytosine much higher, it is found in CpG as well as CpNpG sequences (Gruenbaum *et al*, 1981).

Since 5-methyl cytosine is the major modified base in plants, comprising about 50 mol% of C-residues (Thomas and Sherratt, 1956; Dunn and Smith, 1958; Shapiro and Chargaff, 1960; Doskocil and Sorm, 1962; Vanyushin *et al*, 1968; Vanyushin *et al*, 1970; Drozhdenyuk *et al*, 1977; Adams *et al*, 1979; Deumling, 1981); C-residue methylation is known to be inversely proportional to gene expression (Doerfler, 1983). Brown *et al* (1989) made attempts at understanding the role of gene methylation in tissue culture. These authors exposed maize and tobacco cultures to the anti-methylation agent, 5-AzaC, to determine whether the exposure induces gene demethylation and, as such, enhances tissue culture response. The results suggested that whilst 5-AzaC may be of use in expanding the leaf areas capable of producing callus as well as increasing the amount of callus produced, in all other aspects it was found to be strongly inhibitory to growth at all but very low concentrations (Brown *et al*, 1989).

Since the aims of the present thesis broadly encompasses the study of role of C-residue methylation in gene expression, sugarcane plants (cv. CO 740) were raised in absence as well as presence of 5-AzaC. To establish the optimal growth condition in presence of 5-

AzaC, sugarcane plants were grown in presence of different concentrations of 5-AzaC in the culture medium.

Results and Discussion :

Sugarcane (*Saccharum officinarum*, cv. CO 740) cultures were initiated from immature leaf base from just below the first visible node. The tissue was cut into 2-3 cm thick slices and inoculated on callus induction medium. Two sets of experiments were performed. In the first set, callus cultures were initiated on the induction medium, allowed to grow for 12 days in continous light, at 26°C, and then transferred to fresh induction medium further supplemented with 5-AzaC (0-50 μM). The cultures were incubated in continous light for another 12 days, at 26°C. These cultures were subsequently, transferred to the differentiation medium supplemented with respective concentrations of 5-AzaC.

In another set of experiments, sugarcane tissue cultures were initiated directly on the induction medium supplemented with 5-AzaC (0-50 μM). After incubation in continous light for 12 days, at 26°C, these were transferred to the same medium and incubated for further 12 days. Subsequently the respective cultures were transferred to the differentiation medium supplemented with respective concentrations of 5-AzaC. This transfer was done to eliminate results from 5-AzaC depletion in the medium, as also with the aim of bringing the two experimental set up at parity.

In the first set were inoculum was initially on 5-AzaC free medium, not much difference was observed between the amounts of callus formed on the control medium as compared with the media

containing different concentrations of 5-AzaC. however, certain differences in the texture of the callus was recorded. The callus at low concentration of 5-AzaC (5 μM), was white and friable (Fig. 3a). In presence of 5-AzaC upto 20 μM callus formed was white, glossy and compact (Fig. 3b), whereas it was brownish in presence of 30-40 μM 5-AzaC (Fig. 3c). At 50 μM 5-AzaC the callus had mostly turned brown.

On transfer of the above calli to the differentiation medium supplemented with respective concentration of 5-AzaC, it was observed that the shoot differentiation response was enhanced as compared with control (Figs. 3d to 3f). However, so far as the number of shoots and the shoot length are concerned, best response was achieved with incorporation of 20 μM 5-AzaC into the differentiation medium (Fig. 3e).

In the second set where inoculation was done directly on initiation medium supplemented with various concentrations of 5-AzaC, higher callus growth ensued as compared to the control. The morphology of the callus changed from white, glossy, and compact, to brownish, glossy, and compact, to brown as 5-AzaC concentration increased from 5-50 μM. Callus growth increased with increasing 5-AzaC concentration upto 15 μM. Callus growth declined in presence of higher 5-AzaC concentration (Figs. 3g to 3i). Shoot differentiation was also observed on initiation medium containing 5-AzaC from 2.5 to 20 μM (Figs. 3g and 3h).

Transfer of these calli to differentiation medium supplemented with respective concentrations of 5-AzaC resulted in shoot

regeneration on 5-AzaC concentration of 2.5 to 30 μM. However, the best response in terms of the number of shoots and length of the shoots are concerned, was obtained with 15 μM 5-AzaC (Figs. 3j to 3l). There was no response in presence of 40 and 50 μM 5-AzaC (Fig. 3l). These cultures turned brown and necrotic.

The fact that in the first experimental set differentiation was achieved even at 5-AzaC concentration as high as 50 μM (Plate II, Fig. 3f), might well be due to large amounts of preformed callus and formation of a low concentration gradient across the callus mass which may not be sufficiently cytotoxic.

Irrespective of the fact that shoot regeneration response from sugarcane callus cultures is 5-AzaC concentration dependent, the undeniable fact is that with the inclusion of the DNA demethylating agent in the medium, the morphogenetic response is enhanced. This suggests that DNA methylation/demethylation has a positive role to play in development. This is contrary to most of the reports available on the influence of 5-AzaC on tissue culture.

Plate I

Sugarcane callus cultures grown on initiation medium. After 12 days transferred to initiation medium supplemented with different concentrations of 5-AzaC, as described in Materials and Methods.

Fig. 3a : From left to right, sugarcane callus in initiation medium supplemented with 0, 2.5 and 5.0 μM 5-AzaC.

Fig. 3b : From left to right, sugarcane callus in initiation medium supplemented with 10, 15 and 20 μM 5-AzaC.

Fig. 3c : From left to right, sugarcane callus in initiation medium supplemented with 30, 40 and 50 μM 5-AzaC.

Fig.3a

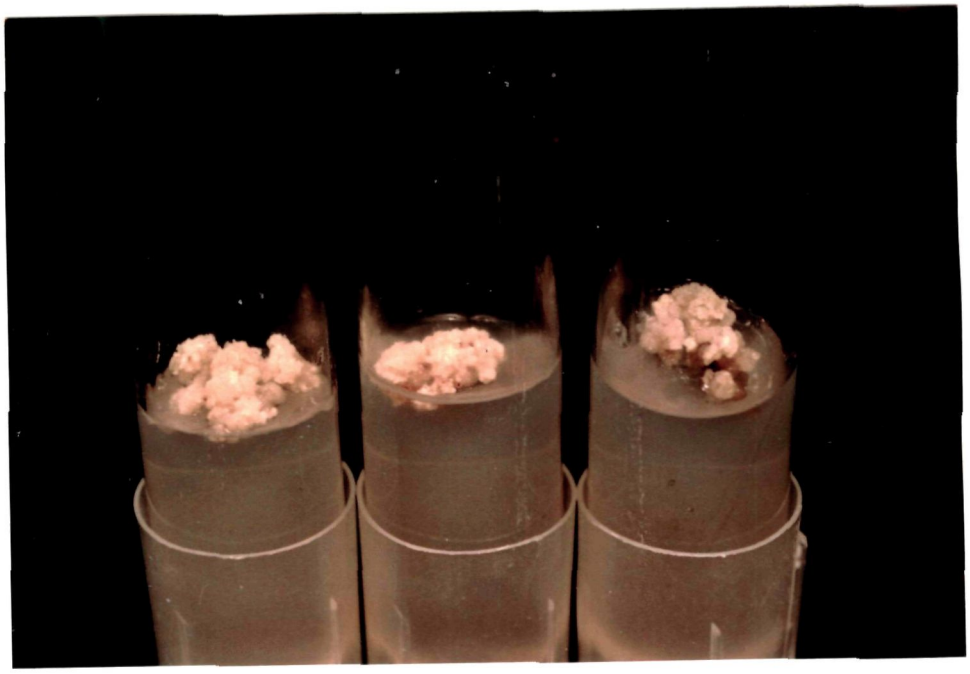


Fig.3b

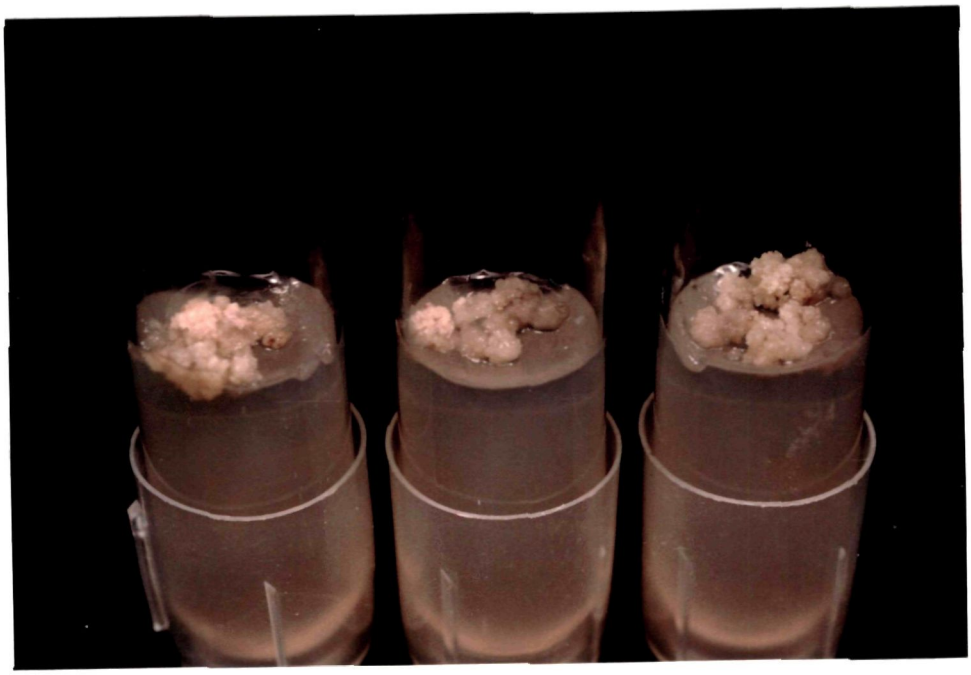


Fig.3c

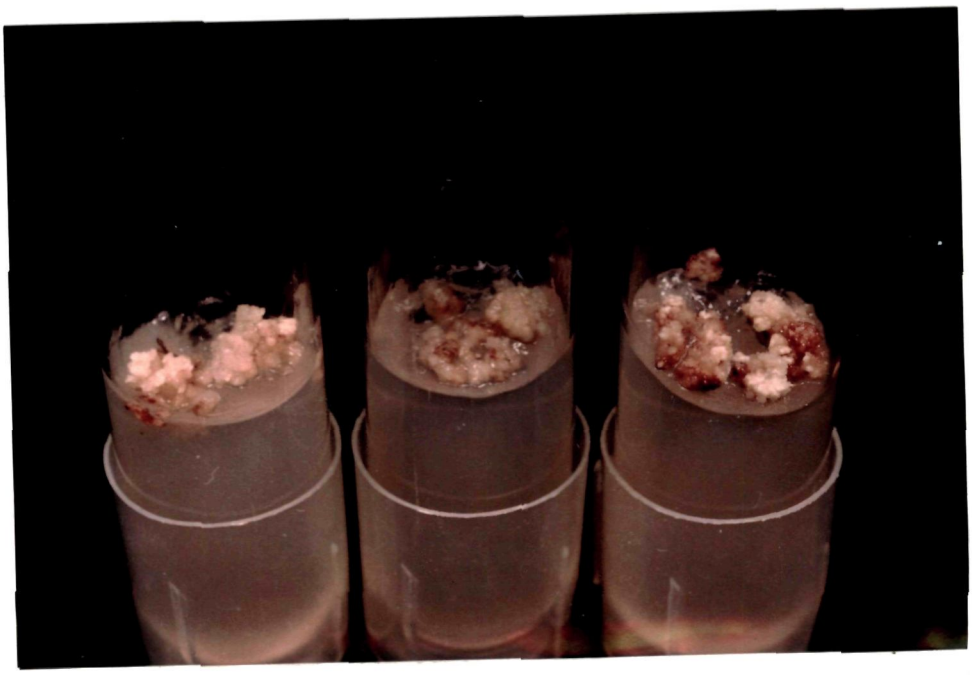


Plate II

Sugarcane cultures grown on initiation medium. After 12 days transferred to initiation medium supplemented with different concentrations of 5-AzaC, subsequently transferred to differentiation medium supplemented with respective concentrations of 5-AzaC, as described in Materials and Methods.

Fig. 3d : From left to right, sugarcane cultures in differentiation medium supplemented with 0, 2.5 and 5.0 μM 5-AzaC.

Fig. 3e : From left to right, sugarcane cultures in differentiation medium supplemented with 10, 15 and 20 μM 5-AzaC.

Fig. 3f : From left to right, sugarcane cultures in differentiation medium supplemented with 30, 40 and 50 μM 5-AzaC.

Fig.3d

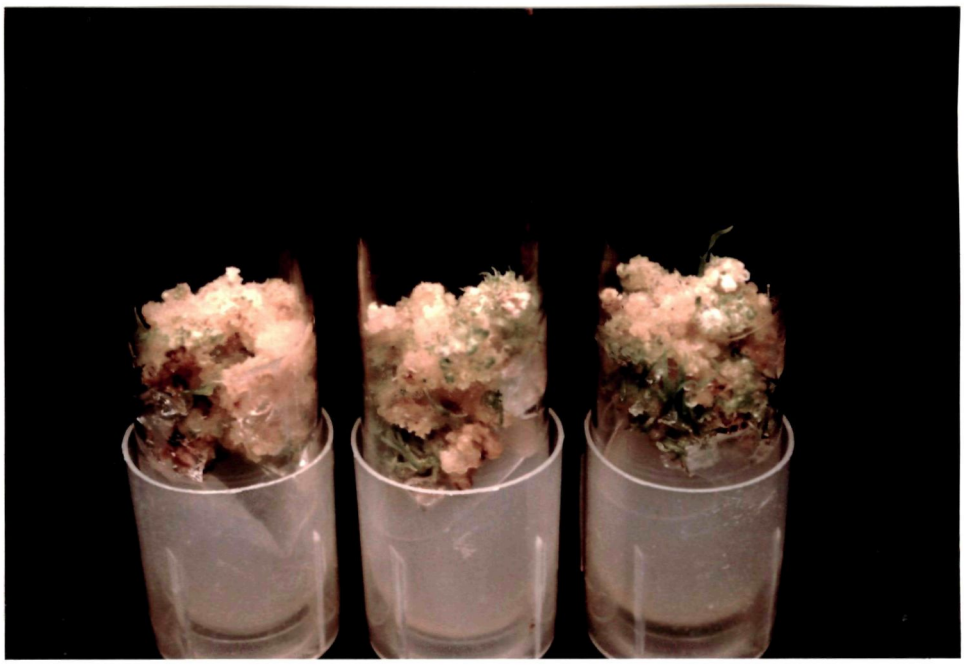


Fig.3e

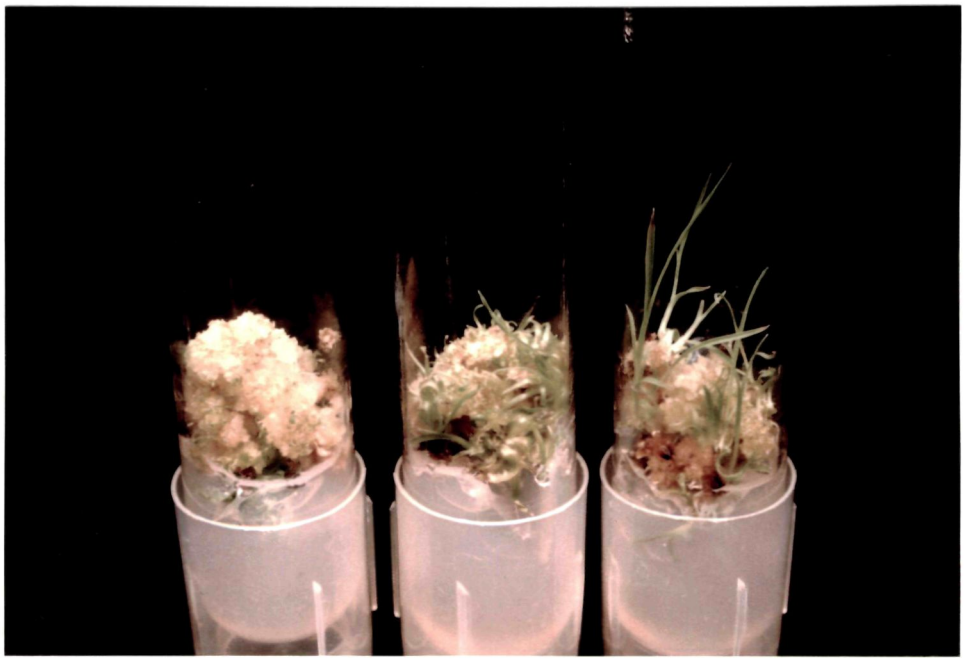


Fig.3f

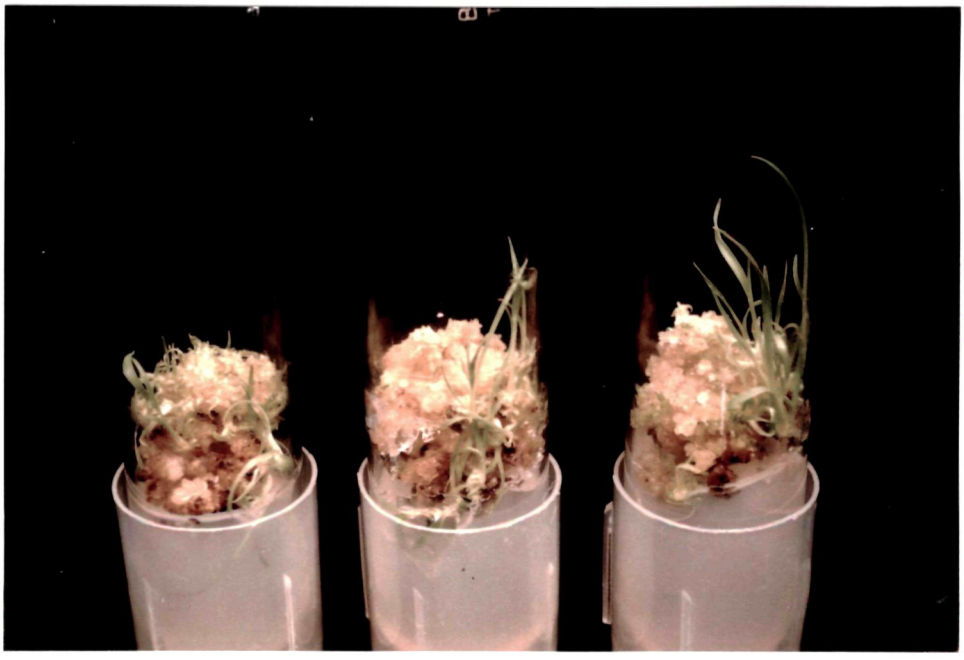


Plate III

Sugarcane callus cultures grown on initiation medium supplemented with different concentrations of 5-AzaC. After 12 days, transferred to fresh initiation medium supplemented with respective 5-AzaC concentrations, as described in Materials and Methods.

Fig. 3g : From left to right, sugarcane callus in initiation medium supplemented with 0, 2.5 and 5.0 μM 5-AzaC.

Fig. 3h : From left to right, sugarcane callus in initiation medium supplemented with 10, 15 and 20 μM 5-AzaC.

Fig. 3i : From left to right, sugarcane callus in initiation medium supplemented with 30, 40 and 50 μM 5-AzaC.

Fig.3g

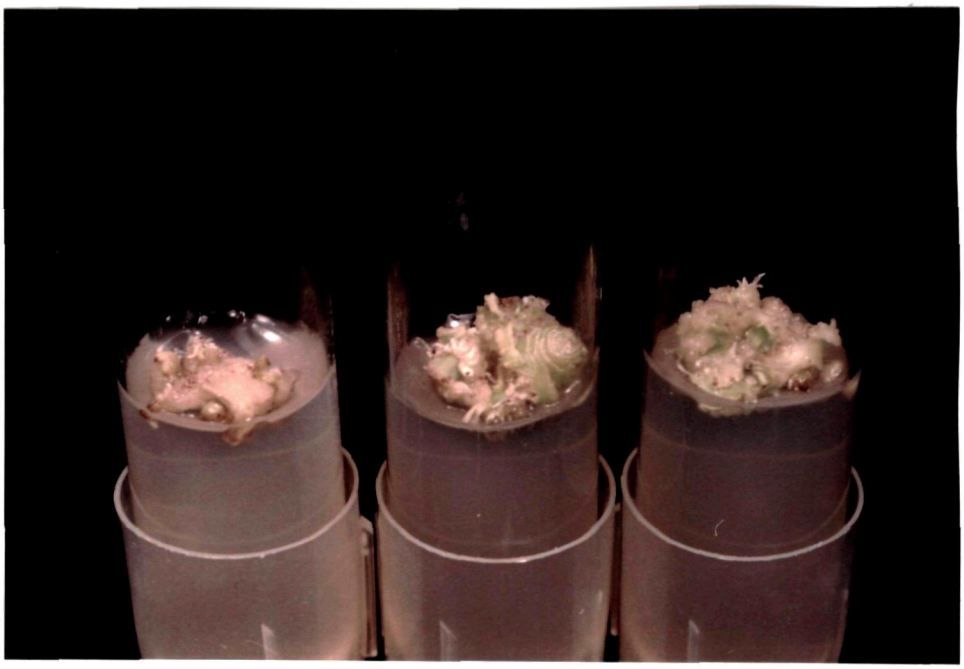


Fig.3h

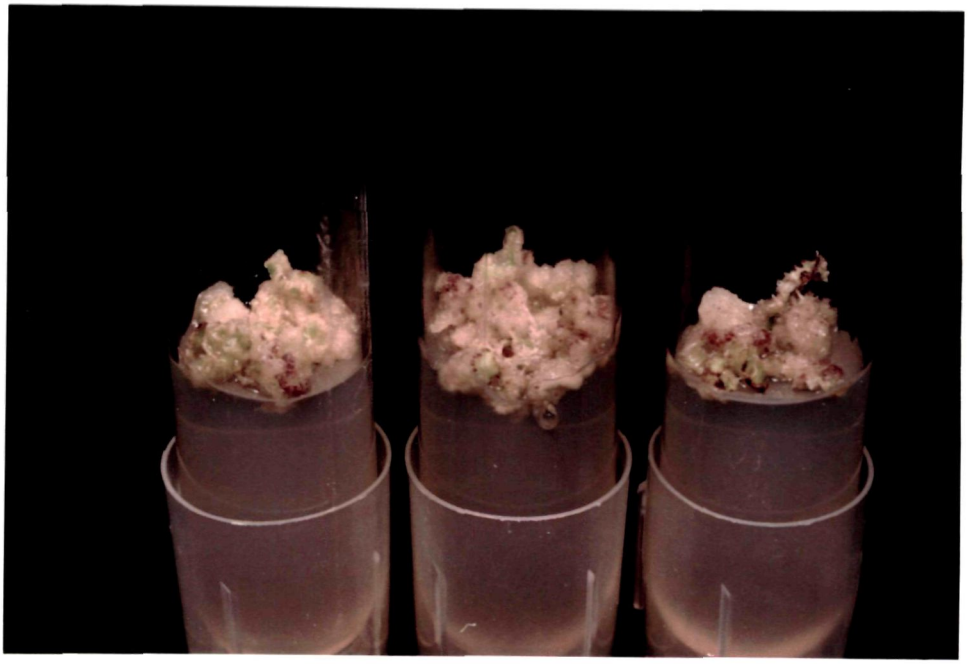


Fig.3i

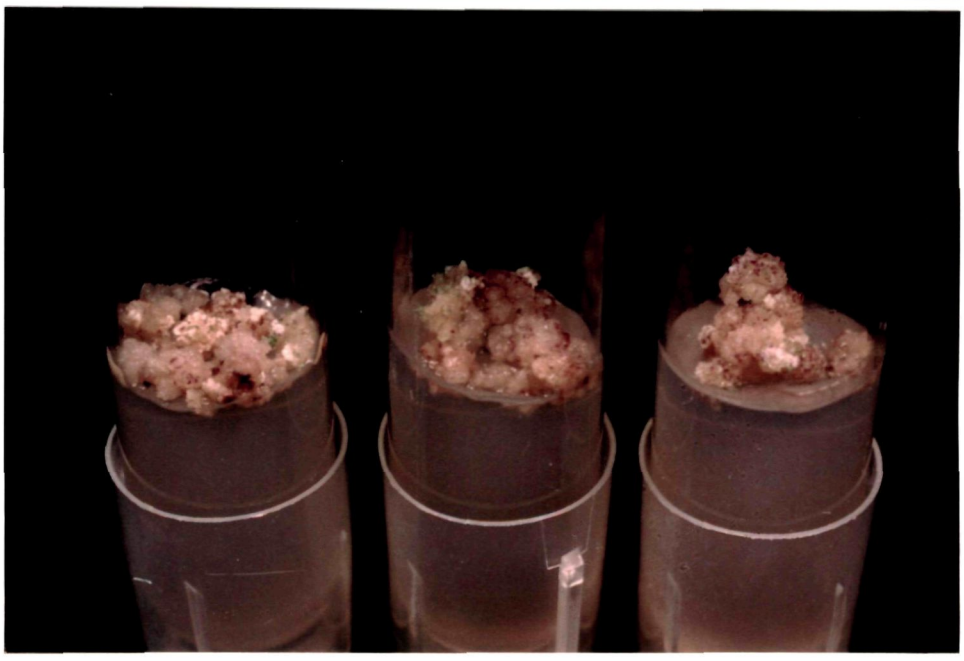


Plate IV

Sugarcane cultures grown on initiation medium supplemented with different concentrations of 5-AzaC. After 12 days, transferred to fresh initiation medium supplemented with respective 5-AzaC concentrations, subsequently transferred to differentiation medium supplemented with respective 5-AzaC concentrations, as described in Materials and Methods.

Fig. 3j : From left to right, sugarcane cultures in differentiation medium supplemented with 0, 2.5 and 5.0 μM 5-AzaC.

Fig. 3k : From left to right, sugarcane cultures in differentiation medium supplemented with 10, 15 and 20 μM 5-AzaC.

Fig. 3l : From left to right, sugarcane cultures in differentiation medium supplemented with 30, 40 and 50 μM 5-AzaC.

Fig.3j

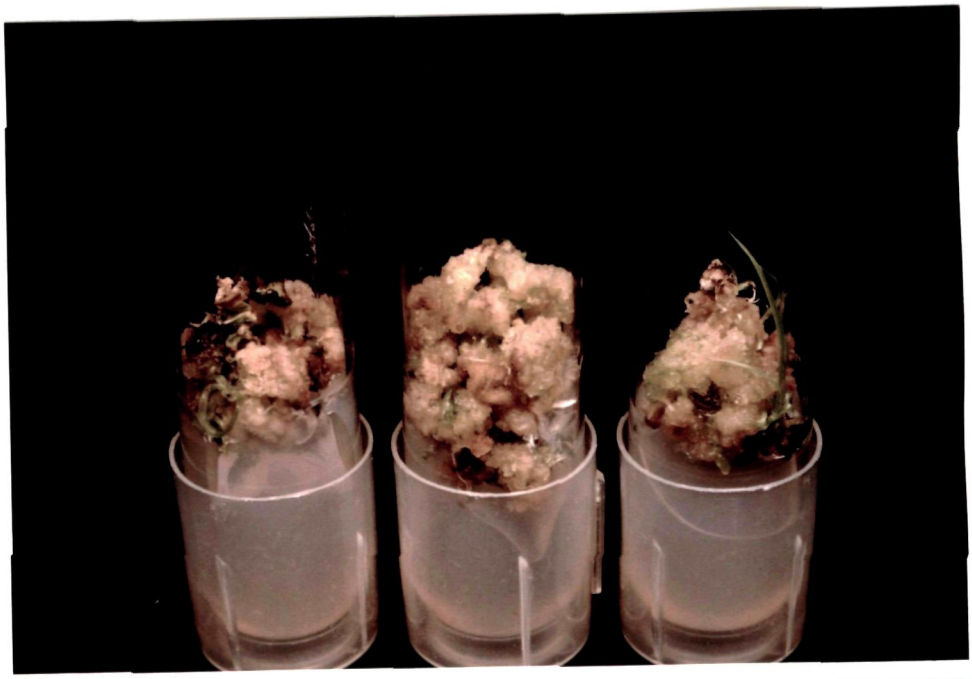


Fig.3k

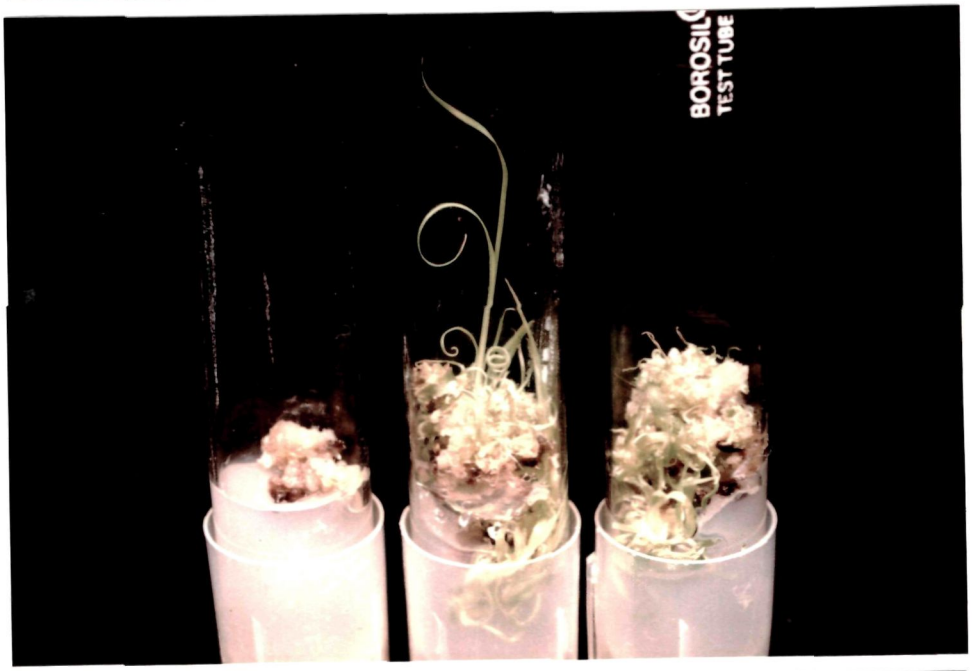
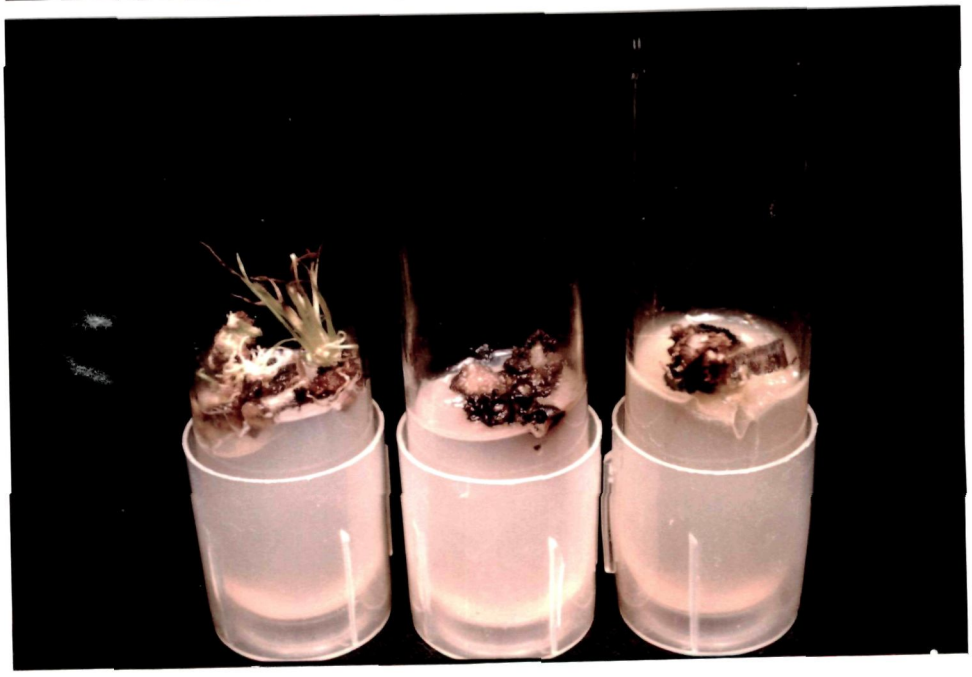


Fig.3l



Biochemical Study of the Effect of 5-AzaC on Phospho*enol*pyruvate Carboxylase on Day 15 of Subculture

Introduction :

Sugarcane, a NADP-malic enzyme type C_4 photosynthetic plant (Edwards and Huber, 1981), exhibits the characteristic Kranz leaf anatomy. Characteristically it fixes atmospheric CO_2 into C_4 dicarboxylic acids in the mesophyll cells. This primary CO_2 fixation is carried out by phospho*enol*pyruvate carboxylase (PEPC; EC 4.1.1.31) in the mesophyll cytoplasm. Subsequently, CO_2 is delivered in the bundle sheath cells as malate and released by NADP-malic enzyme (NADP-ME : EC 1.1.1.40), to be utilized by ribulose bisphosphate carboxylase/oxygenase (RuBISCO : EC 4.1.1.39) via C_3 photosynthetic carbon reduction cycle (Edwards and Huber, 1981; Hatch, 1987).

Transcription of C_4 photosynthetic genes are reported to be induced by light in a cell-specific manner (Bedbrook *et al*, 1978; Link *et al*, 1978; Broglie *et al*, 1984; Nelson *et al*, 1984; Sheen and Bogorad, 1985, 1986, 1987a,b). Methylated cytosine, which accounts for approximately 30% of the total genomic cytosine in the plants (Gruenbaum *et al*, 1981) and is methylated predominantly at the CpG and CpNpG islands (Adams and Burden, 1983), is shown to be related to the levels of gene expression (Doerfler, 1983). C-residue methylation in maize has been implicated in the light mediated differential expression of the photosynthetic genes (Ngernprasirtsiri *et al*, 1989). It has also been implicated in the inactivation of inserted genes in transformed plant cells (Amasino

et al, 1984; Gelvin *et al*, 1984; Peerbolte *et al*, 1986; Hepburn *et al*, 1988). Transcriptional regulation involves interaction(s) of a large number of *trans*-acting factors with specific *cis*-acting DNA sequences (Yanagisawa and Izui, 1990; Kano-Murakami *et al*, 1991; Matsuoka and Numazawa, 1991) wherein cytosine methylation provides one such control mechanism.

As it is generally accepted that a correlation exists between gene methylation and gene expression, a possibility then exists of general activation of genes by a hypomethylating agent like 5-azacytidine (5-AzaC). 5-AzaC, an anticancer agent (Van Groeningen *et al*, 1986; Glover *et al*, 1987), has been used as a tool to study the role of DNA methylation on gene activation and expression (Van Slogteren *et al*, 1984; Jones 1985a,b; Brown *et al*, 1989; Klaas *et al*, 1989; Zhen *et al*, 1991; Lim *et al*, 1992). A majority of the studies on gene activation by 5-AzaC in plants are limited to activation of otherwise silent T-DNA genes (Amasino *et al*, 1984; Van Slogteren *et al*, 1984; Peerbolte *et al*, 1986; Hepburn *et al*, 1988).

The present chapter deals with the effects of 5-AzaC on total soluble protein synthesis, PEPC activity and the synthesis of the PEPC protein in the mesophyll cells and bundle sheath strands from the leaves of 15 days old sugarcane cultures maintained *in vitro*.

Results :

Ngernprasirtsiri *et al* (1989), presented evidence and suggested cytosine methylation to be an important factor in light regulation and differential expression of PEPC gene(s) in maize. By manipulating the extent of C-residue methylation in plants with the

use of the hypomethylating drug 5-AzaC, it may become possible to alter the expression of the PEPC gene(s) in both the MC and BS cells independent of the light conditions. To test this hypothesis we assayed PEPC enzyme activity, PEPC protein content and total soluble protein content in the cell free lysates from the mesophyll cells and the bundle sheath strands from the leaves of sugarcane plants grown under the experimental conditions described in the chapter on Materials and Methods.

Total soluble proteins :

An overall proliferation of total soluble proteins in the mesophyll cells and the bundle sheath strands from both short-term and long-term 5-AzaC treated sugarcane plants was observed. In the mesophyll cells from light grown plants the increase in protein content was 18% and 13% respectively in response to short-term and long-term 5-AzaC treatments as compared with control (Fig. 4a, CLA:CL and AL:CL). Corresponding increase in the protein content in the mesophyll cells from dark grown plants was 131% and 164% respectively (Fig. 4a, CDA:CD and AD:CD).

In the bundle sheath strands of light grown plants the total soluble proteins increased by 181% (Fig. 4b, CLA:CL) in response to short-term 5-AzaC treatment. On the contrary the total protein content declined by about 55% in response to long-term 5-AzaC treatment (Fig. 4b, AL:CL). The protein content in the bundle sheath strands from dark grown plants exhibited a phenomenal increase of 1517% (Fig. 4b, CDA:CD) and 1042% (Fig. 4b, AD:CD) respectively in short-term and long-term 5-AzaC treated plants.

Enzyme Activity :

The PEPC activity as compared with the control plants was 74% higher in the mesophyll cells isolated from light grown sugarcane plants subjected to short-term 5-AzaC treatment (Fig. 4c, CLA:CL). It was lower by 17% in light grown plants exposed to long-term 5-AzaC treatment (Fig. 4c, AL:CL). There was, however, no change in PEPC activity in mesophyll cells from dark grown plants subjected to short-term 5-AzaC treatment (Fig. 4c, CDA:CD). Whereas the PEPC activity in the mesophyll cells from light grown and long-term 5-AzaC treated plants was lower by 17%, it was almost two fold higher in long-term 5-AzaC treated plants grown in constant darkness as compared with their respective controls (Fig 4c, AL:CL and AD:CD). No PEPC activity was detected in the BS strands.

Specific Activity :

The specific activity of PEPC in mesophyll cells of long-term 5-AzaC treated plants was lower than their controls. While in light grown plants it was lower by 25%, in the dark grown plants it was lower by about 30% (Fig. 4d, AL:CL and AD:CD). On short-term 5-AzaC exposure while the light grown plants registered a 50% increase in the PEPC specific activity, in the dark grown plants there was a 60% decline in PEPC specific activity as compared to the corresponding controls (Fig. 4d, CLA:CL and CDA:CD).

Immunoblot Analysis :

An increase in PEPC protein content in mesophyll cells was observed in both light and dark grown sugarcane plants subjected to long-term 5-AzaC treatment (Fig. 4e). No change was recorded in the

PEPC protein content upon short-term 5-AzaC treatment of plants grown in the presence of light (Fig 4e, CLA:CL). However, in short-term 5-AzaC treated plants grown in constant darkness, the PEPC protein content was about 54% lower than the control (Fig. 4e, CDA:CD).

Discussion :

Hodges *et al* (1986), rationalized that if tissue culture response in maize is as a consequence of the presence of nuclear genes, then the fact that there is differential effect of 5-AzaC, dependent on whether the tissue is green or not, may be relevant. Brown *et al* (1989), suggested that the differential response of maize tissue cultures to 5-AzaC treatment, could be due to the role played by certain chloroplast-encoded factors also, along with nuclear gene expression. In the present study also, *in vitro* grown sugarcane cultures respond differentially to 5-AzaC treatment, dependent on whether these plants are grown in light or in constant darkness.

The observed overall proliferation of proteins in the mesophyll cells and the bundle sheath strands from sugarcane leaves in response to 5-AzaC treatment and its independence of light is in general agreement with earlier reports (Doerfler, 1983). This phenomenon can essentially be attributed to de-repression of otherwise silent genes. The observation that the hypomethylating drug 5-AzaC induces general proliferation of proteins has lead to experiments designed to study the cause-effect relationship of 5-AzaC addition to plants *in tissue cultures* (Amasino *et al*, 1984; Peerbolte *et al*, 1986; Hepburn *et al*, 1988; Brown *et al*, 1989),

foreign gene expression in transgenic plants (Van Slogteren *et al*, 1984; Jones 1985b; Klaas *et al*, 1989; Zhen *et al*, 1991), expression of plant transposable elements (Kunze *et al*, 1988), etc.

PEPC is a multigene family of 3-4 members (O'Leary, 1982; McNaughton *et al*, 1989). The active PEPC is a homotetramer with a subunit molecular mass of ~100 kDa. PEPC is regulated post-translationally, in the presence of light by subunit aggregation (Uedan and Sugiyama, 1976; Mares *et al*, 1979; Walker *et al*, 1986a,b), and phosphorylation (Bakrim *et al*, 1992; Jiao and Chollet, 1992). In the present study, the observation that PEPC protein abundance is similar in CLA and CL plants, concomitant with 74% higher enzyme activity in the former, could be the result of proper post-translational modification(s) of the PEPC protein to an active form (Fig. 4e and 4c, CLA:CL). Similar observation could be made with regards to CDA and CD PEPC protein abundance and its activity (Fig. 4e and 4c, CDA:CD).

An entirely different picture is presented when PEPC protein abundance and its activity in both light and dark grown plants subjected to long-term 5-AzaC treatment is considered. The high abundance of PEPC protein as well as the enzyme activity in both cases is comparable (Fig. 4e and 4c, Al:CL and AD:CD). This suggests the possible lapse in post-translational modification of PEPC to a fully active form.

From the above observations it appears that there is up-regulation of PEPC activity and/or its post-translational modifications in both light and dark grown plants only if subjected to a short-term exposure to 5-AzaC. However, on long-term 5-AzaC

treatment, while PEPC gene expression is enhanced (evidenced by high PEPC protein abundance), lack of effective post-translational modifications for e.g., inability of PEPC protein to attain proper quaternary structure which would lead to aggregation of the subunits, and/or lack of phosphorylation of the Serine residue, could be responsible for lack of concomitant enhancement of PEPC activity.

Plate V

Total protein profile on day 15 of subculture.

Fig. 4a : Total protein profile in mesophyll cells on day 15 of subculture.

Fig. 4b : Total protein profile in bundle sheath strands on day15 of subculture.

Fig.4a

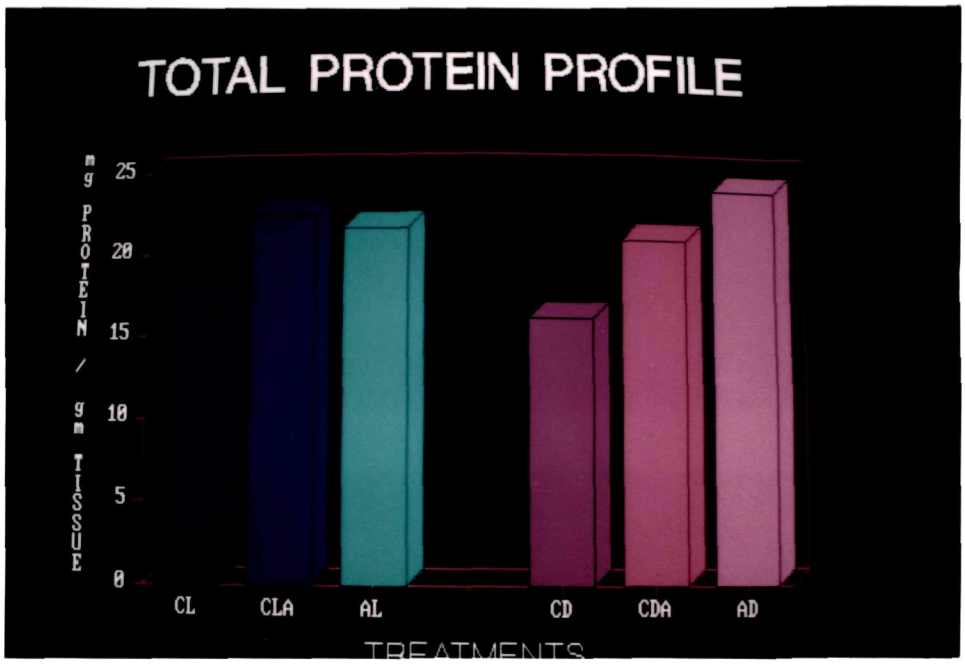

TOTAL PROTEIN PROFILE
mg PROTEIN / gm TISSUE
25
20
15
10
5
0
CL
CLA
AL
CD
CDA
AD
TREATMENTS

Fig.4b

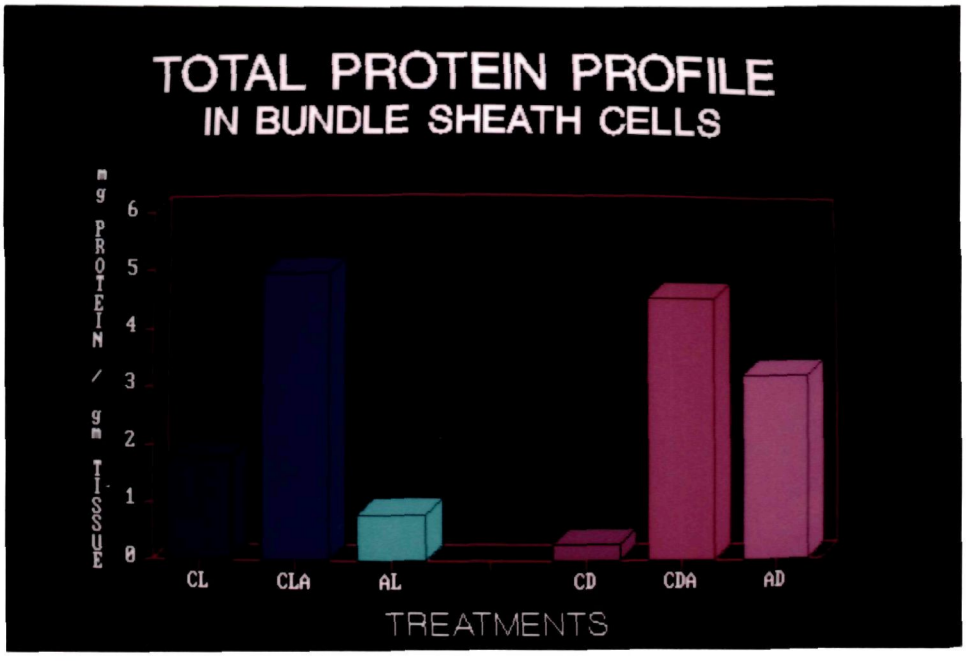

TOTAL PROTEIN PROFILE
IN BUNDLE SHEATH CELLS
mg PROTEIN / gm TISSUE
6
5
4
3
2
1
0
CL
CLA
AL
CD
CDA
AD
TREATMENTS

Plate VI

Phospho*enol*pyruvate on day 15 of subculture.

Fig. 4c : PEPC enzyme activity on day 15 of subculture.

Fig. 4d : PEPC specific activity on day 15 of subculture.

Fig. 4e : PEPC immunoblot analysis on day 15 of subculture.

Fig.4c

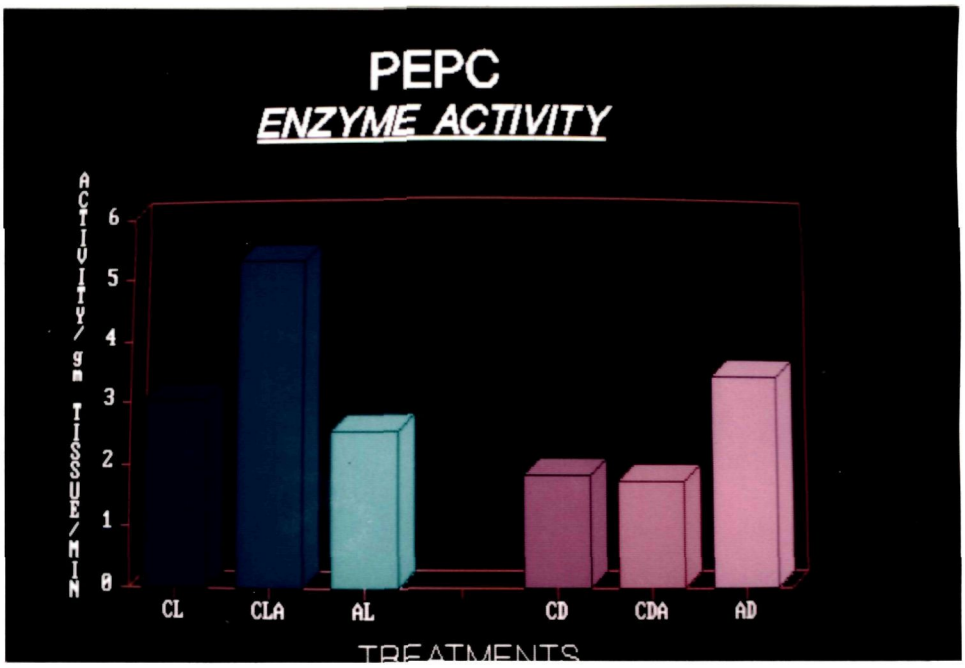


Fig.4d

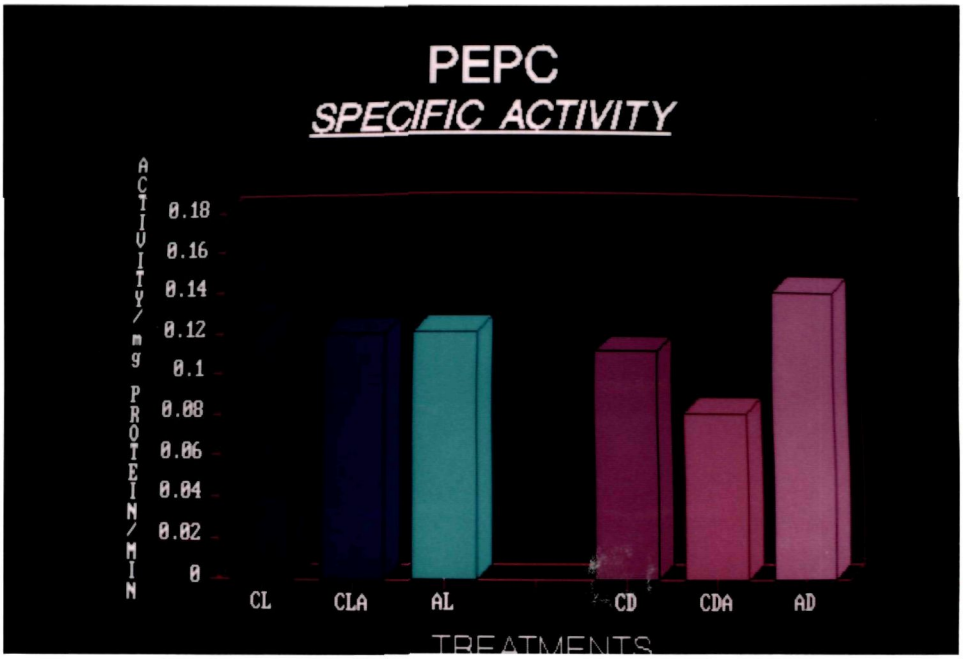
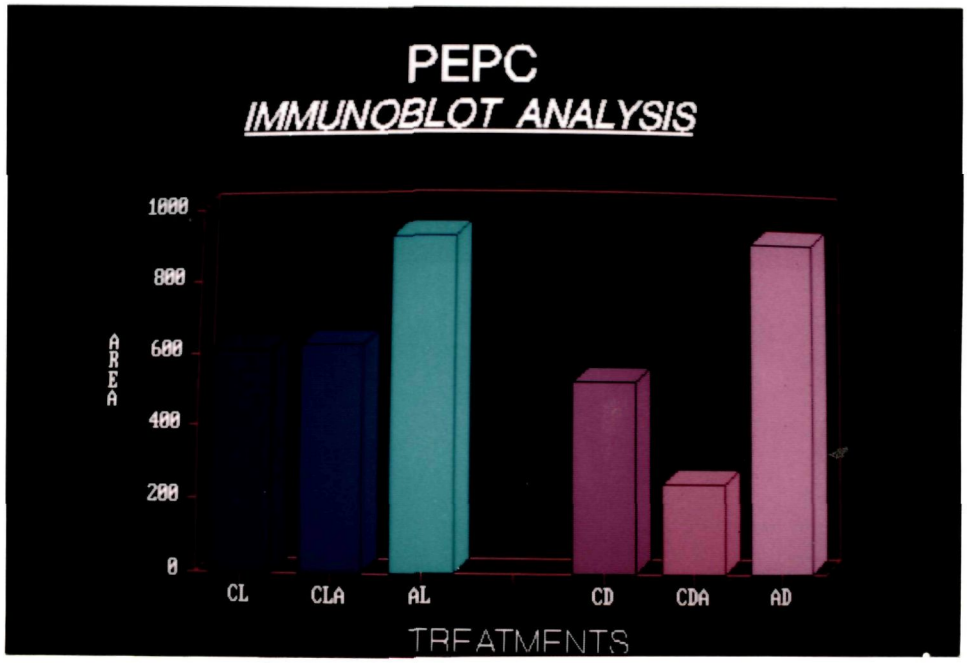


Fig.4e



Time Course Analysis of the Influence of 5-AzaC on the Enzyme Activities of PEPC (Mesophyll Cytoplasmic Enzyme), PPDK (Mesophyll Chloroplastic Enzyme) and NADP-ME (Bundle Sheath Chloroplastic Enzyme)

Introduction :

The interesting features of C_4 photosynthetic plants include two distinct cell-types, often associated with dimorphic chloroplasts. Thus C_4 photosynthetic plants have high photosynthetic rates and growth rates, low photorespiration rates and markedly reduced rates of water loss. These features are interrelated and result from modified metabolic processes for photosynthesis (Hatch, 1976; Hatch, 1978; Edward and Huber, 1981).

The improved photosynthetic efficiency of C_4 photosynthetic plants, such as sugarcane, is possible due to kranz anatomy, wherein atmospheric CO_2 is trapped in the outer mesophyll cells, in the form of a C_4 acid, and subsequently released in the inner bundle sheath cells, where it is utilized by RuBISCO. The sequential CO_2 trapping involving the two cell-types is as follows :

In Mesophyll cells :

$$\text{Pyruvate} + \text{ATP} + \text{Pi} \xrightarrow{\text{PPDK}} \text{PEP} + \text{AMP} + \text{PPi}$$

$$\text{PEP} + CO_2 \xrightarrow{\text{PEPC}} \text{Oxalacetate}$$

$$\text{Oxalacetate} + \text{NADPH} \xrightarrow{\text{NADP-MDH}} \text{Malate} + \text{NADP+}$$

The malate formed in mesophyll chloroplasts is transferred to bundle sheath cells.

In Bundle Sheath cells :

$$\text{Malate} + \text{NADP+} \xrightarrow{\text{NADP-ME}} CO_2 + \text{NADPH}$$

$$CO_2 + \text{RuBP} \xrightarrow{\text{RuBISCO}} 2\ (\text{3-phosphoglycerate})$$

All the enzymes are regulated by light. Light induces PEPC, PPDK and NADP-MDH expression in mesophyll cells and NADP-ME and RuBISCO expression in bundle sheath cells. PEPC, PPDK and NADP-ME gene expression has been studied previously at the molecular and biochemical level (Andrews and Hatch, 1969; Kobayashi, *et al* , 1980; O'Leary, 1982; Edwards and Nakamoto, 1985; Jenkins, *et al* , 1987; Sheen and Bogorad, 1987a,b; Cameron and Bassett, 1988; Jiao and Chollet, 1988; Stiborova, 1988; McNaughton *et al* , 1989; Ngernprasirtsiri, *et al* , 1989; Grammatikopoulos and Manetas, 1990; Jawali, 1990; Matsuoka, 1990; Kano-Murakami, *et al* , 1991; Matsuoka and Numazawa, 1991; Rajeevan, *et al* , 1991; Sheen, 1991; Bakrim, *et al* , 1992; Drincovich, *et al* , 1992; Iglesias, *et al* , 1993; Rajagopalan, *et al* , 1994). Subsequently, it was suggested that the differential expression of these enzymes in the two cell-types was due to differential methylation of their genes (Sheen and Bogorad, 1987a; Ngernprasirtsiri *et al* , 1989).

To address the questions, a) 'Whether C-residue methylation is responsible for differential expression of the PEPC, NADP-ME and PPDK genes in the two cell-types?' and, b) 'Whether C-residue methylation interact with light in modulating the expression of these genes?', sugarcane cultures were raised in the presence and

absence of 5-AzaC, and a time course (day 12-18) analyses of the three enzymes was carried out in mesophyll cells and bundle sheath strands of sugarcane plants grown *in vitro*.

In the present study no odd place presence of enzyme activities was observed as PEPC and PPDK enzyme activities were restricted to the mesophyll cells and NADP-ME activity was restricted to the bundle sheath cells, irrespective of the plants grown in absence or presence of 5-AzaC.

Results :

Total Soluble Protein Content in Mesophyll Cells :

In light, total soluble protein content in the mesophyll cells of both CL and CLA plants was on increase from day 12 onwards, reaching peak values on day 16, thereafter till day 18 the protein content drops slightly. On day 16 the protein content in CLA-MC (29.2 mg/gm fr. wt.) was 20% higher than in CL-MC (24.2 mg/gm fr. wt.). By day 18, there was two times more total soluble protein in CLA-MC (44 mg/gm fr. wt.) than in CL-MC (21.4 mg/gm fr. wt.)(Fig. 5a, CLA:CL).

However, in long-term 5-AzaC treated (AL) plants, two fold higher total soluble proteins on day 12 (25.2 mg/gm fr. wt.), drops down to ~81% of the CL-MC total soluble protein content by day 18 (Fig. 5a, AL:CL).

In plants grown under conditions of constant darkness, total soluble proteins content of long-term 5-AzaC (AD) plants was found to be higher than control (CD) plants throughout the duration of time course period (Fig. 5b, AD:CD). Total soluble proteins content

in short-term 5-AzaC (CDA) plants was higher by ~30% on day 15 and by day 18, it was ~60% higher when compared to control plants (Fig. 5b, CDA:CD).

Total Soluble Protein Content in Bundle Sheath Cells :

CL-BS total soluble protein content varied from as low as 1.2 mg/gm on day 12 to as high as 6.0 mg/gm on day 17. Short-term 5-AzaC treatment to light-grown (CLA) plants resulted in higher total soluble protein content from day 14 onwards. However, CLA-BS total soluble protein content was highest on day 15, when it was about 81% more than CL-BS. By day 18 CLA-BS total soluble protein content dropped down to the same level as CL-BS (Fig. 5c, CLA:CL). Total soluble protein content of AL-BS was less than CL-BS through out the duration of time course period (Fig. 5c, AL:CL).

On the other hand, in plants grown under the conditions of constant darkness, CDA-BS total soluble proteins content was higher than CD-BS total soluble protein content throughout the time course period, barring on day 17 (Fig. 5d, CDA:CD). The maximum difference was observed on day 15, when CDA-BS total soluble protein content was about 16 fold higher than CD-BS. By day 18, however, the CDA-BS total soluble protein level dropped down to CD-BS total soluble protein level (Fig. 5d, CDA:CD). As against this, AD-BS total soluble protein content was highest on day 14, where it was about 6 fold higher than CD-BS (Fig. 5d, AD:CD). By day 18, AD-BS total soluble protein content too dropped down to the same level as CD-BS.

PEPC Enzyme Activity :

PEPC activity in control plants grown in light (CL) showed a continuously declining trend from day 12 onwards. CLA plants also exhibited a similar developmental pattern. However, the PEPC activity in CLA plants was higher on all days by 24% to 74% than CL plants (Fig. 5e, CLA:CL). On the other hand, the PEPC activity in AL-plants demonstrated a steady increase on all days, though the activity remained by and large lower than in CLA plants throughout (Fig. 5e, AL:CL).

In plants grown under the conditions of constant darkness, there was no substantial difference in the developmental pattern of PEPC activity in CDA plants as compared to activity in CD plants throughout the duration of time course. The enzyme activity in the two plant types was also comparable (Fig. 5f, CDA:CD). On the other hand, in long-term 5-AzaC treated (AD) plants, PEPC activity was higher than either in CD or CDA plants throughout the duration of the time course period and maximum activity was achieved on day 15. (Fig. 5f, AD:CD).

PEPC Specific Activity :

In light, the PEPC specific activity essentially showed a downward trend during the time course period for CLA as well as CL plants (Fig. 5g, CLA:CL). This followed the same trend as for the enzyme activities (Fig. 5e, CLA:CL). Since the PEPC activity in CLA plants was by and large higher than in CL plants the specific activity was also correspondingly higher in CLA plants than CL plants. However, the low PEPC specific activity in AL plants was as

a consequence of low PEPC activity in these plants as well as high protein content in AL plants during the initial period of the time course. On the other hand, high PEPC specific activity in AL plants during the later period was due to higher PEPC activity and also due to lower AL-MC protein content (Figs. 5a, 5e and 5g, AL:CL).

In plants grown under the conditions of constant darkness, CDA-PEPC specific activity showed a very similar developmental trend as CD-PEPC specific activity (Fig. 5h, CDA:CD). AD-PEPC specific activity was, in general, lower than CD-PEPC specific activity since AD-MC plants had much higher total soluble protein content than CD-MC plants (Figs. 5b, 5f and 5h AD:CD).

PPDK Enzyme Activity :

In light, no appreciable change in the PPDK activity in short-term 5-AzaC treated (CLA) plants as compared to control (CL) plants was recorded. Rather the trend was for the CLA-PPDK activity to be slightly lower than CL-PPDK activity (Fig. 5i, CLA:CL). The AL-PPDK activity was higher than CL-PPDK activity throughout the duration of the time course period (Fig. 5i, AL:CL). However, the overall developmental trend of PPDK activity in CLA and AL were very similar to that of CL plants.

A similar situation was encountered in plants grown under conditions of constant darkness, with the difference that here CDA-PPDK activity was slightly higher than CD-PPDK activity in the initial period of the time course (Fig. 5j, CDA:CD). In the case of long-term 5-AzaC treated (AD) plants, PPDK activity was found to be higher than CD-PPDK activity throughout the duration of time course period (Fig. 5j, AD:CD).

PPDK Specific Activity :

In general, both in the presence and absence of light, short-term 5-AzaC treated (CLA:CDA) plants showed declining trend in PPDK specific activity as compared to the control plants (Figs. 5k and 5l; CLA:CL and CDA:CD). On the other hand, long-term 5-AzaC treated (AL, AD) plants showed high and increasing specific activity than the control plants (Figs. 5k and 5l; AL:CL and AD:CD). The PPDK specific activity on day 12 and 13 in long-term 5-AzaC treated plants was low because the plants had lower PPDK activities. Indeed PPDK activity in these plants picked up and reached a peak on day 15 in both the presence and the absence of light.

NADP-ME Enzyme Activity :

In light, both short-term (CLA) and long-term 5-AzaC treatment (AL), did not appreciably influence NADP-ME activity in sugarcane plants. While CLA-ME activity was higher than CL-ME only on day 13 and 15, for rest of the duration of time course period, both CLA-ME and AL-ME activities were lower than CL-ME (Fig. 5m, CLA:CL and AL:CL).

In plants grown under conditions of constant darkness, however, short-term 5-AzaC application led to generally higher NADP-ME activity compared to control plants (Fig. 5n, CDA:CD). On the other hand, AD-ME activity was lower than CD-ME throughout except on day 15 and 16 (Fig. 5n, AD:CD).

NADP-ME Specific Activity :

In light, both long-term and short-term 5-AzaC treated plants had by and large lower NADP-ME specific activity than control

plants, except on day 13 (Fig. 5o, CLA:CL and AL:CL). This was due to the fact that day 13 CL BS total soluble protein content was very high.

In plants grown under conditions of constant darkness, the specific activities of all the control and 5-AzaC treated plants were very low (Fig. 5p), except for CD-ME on day 13. This was again due to the fact that day 13 CD-BS total soluble protein content was very low.

Discussion :

Ngernprasirtsiri *et al* (1989), using isoschizomeric pairs of restriction endonucleases, showed that certain C_4-photosynthetic genes are differentially methylated in the mesophyll and bundle sheath cell-types of maize. They showed that whereas PEPC and PPDK were methylated in the bundle sheath cells, RuBISCO small and large subunit genes were methylated in the mesophyll cells. Since, PEPC and PPDK are expressed only in mesophyll cells and RuBISCO is expressed exclusively in bundle sheath cells of green NADP-ME type C_4-photosynthetic plants, it was suggested that C-residue methylation was responsible for the differential expression of these genes. We raised sugarcane plants both in the absence and presence of 5-AzaC, which gets incorporated in the replicating DNA and thus inhibits C-residue methylation.

The two cell-types, viz mesophyll and bundle sheath cells were separated and PEPC, PPDK and NADP-ME activities assayed. Purity of our cell extract preparation was checked by microscopic examinations, and chlorophyll a/b ratio of the BS and MC cell extracts. Since, no PEPC and PPDK activities were detected in the

bundle sheath preparations, and no NADP-ME activity was detected in the mesophyll cell extracts, irrespective of whether the plants were grown in the presence or absence of 5-AzaC, confirmed the purity of the extracts.

Total Soluble Protein Content :

DNA methylation is known to regulate gene expression in eucaryotes (Holliday and Pugh, 1975; Riggs, 1975; Sager and Kitchin, 1975; Bird, 1978; McGhee and Ginder, 1979). In plants, 5-mC is found to be as high as 50 mol% (Thomas and Sherratt, 1956; Dunn and Smith, 1958; Shapiro and Chargaff, 1960; Doskocil and Sorm, 1962; Vanyushin *et al* , 1968; Vanyushin *et al* , 1970; Drozhdenyuk *et al* , 1977; Adams *et al* , 1979; Deumling, 1981). Further, in plants, there is different methylation gradient in different cell-types of same age (Ngernprasirtsiri *et al* , 1989), or along different developmental age (Watson *et al* , 1987).

Addition of 5-AzaC, which cannot be methylated , leads to its incorporation into the DNA. In this state, it inhibits DNA methyltransferases (Christman *et al* , 1980; Jones and Taylor, 1981; Creusot *et al* , 1982; Jones *et al* , 1983; Gabbara and Bhagwat, 1995).

5-AzaC addition to the medium results in a general protein proliferation. However, absence of DNA methylation appears to be a necessary prerequisite but not a sufficient precondition for gene activation (Van der Ploeg and Flavell, 1980; Kuhlman and Doerfler, 1982). Hence, it is not expected that all dormant cellular genes can be turned on by 5-AzaC. Further, 5-AzaC appeared to be refractory to the DNA methyltransferase inhibition at CAG/CTG sequences, as

opposed to CG doublets and CCG/CGG triplets in tobacco (Kovaric *et al* , 1994). Thus 5-AzaC cannot be considered as a general methyltransferase inhibitor. The reason why total soluble protein content in AL-plants is lower than CL-plants is not clear. It could be due to 5-AzaC depletion from the medium or due to habituation of sugarcane cultures grown and maintained in 5-AzaC and its metabolism. Hodges *et al* (1986), working with maize tissue culture found that response to 5-AzaC was different in light and dark-grown cultures. They suggested that, if tissue culture response is related to presence of nuclear genes, then the fact that there is a differential response to 5-AzaC dependent on whether the tissue is etiolated or green, could be due to the absence or presence of certain factors. These factors may or may not be chloroplast encoded (Hodges *et al* , 1986). Hence, it could be that under long-term 5-AzaC treatment in the presence of light, certain factors are expressed, which may or may not be chloroplast-encoded, but which are in fact inhibitors of either gene expression or protein synthesis . The fact that total soluble protein content in AD-plants is higher than CL-plants by day 18 may be due to the absence of these factors.

We have observed that total soluble protein content, especially in bundle sheath cells, varied widely from day to day. Indeed the total soluble protein content from CL-BS varied from as low as 1.2 mg/gm on day 12 to as high as 6.0 mg/gm on day 17. Compared to mesophyll cells, the bundle sheath cells had less total soluble protein content. This was because there were less bundle sheath cells per unit square leaf area compared to mesophyll cells. However, the fact that there is a transient increase in total

soluble protein content in bundle sheath cells, both in light and dark on 5-AzaC treatment suggests that though bundle sheath cells are more internal than mesophyll cells and are characterized by heavy cell wall, this has not caused any hindrance on 5-AzaC entry into them and general gene expression.

PEPC Activity :

Overall, 5-AzaC application seems to enhance PEPC enzyme activity in mesophyll cells of sugarcane plants, both over a short-term as well as a long-term period. However, in light, the increase in PEPC activity was apparent more in short-term 5-AzaC treated plants.

The native C_4 PEPC molecule is a homotetramer with a monomer molecular mass of 100, 000 (Uedan and Sugiyama, 1976; Mares *et al* , 1979; Stiborova and Leblova, 1983a, 1986; Huber *et al* , 1986; Stiborova *et al* , 1986; Walker *et al* , 1986a,b). PEPC can exist in different oligomeric forms depending on factors like pH (Walker *et al* , 1986; Stiborova and Leblova, 1986; Stiborova *et al* , 1986), ionic strength (Walker *et al* , 1986; Stiborova and Leblova, 1986; Wagner *et al* , 1987), and temperature (Chou *et al* , 1986; Wu and Wedding, 1987). CAM-PEPC from *Crassula argentea* is tetrameric at night and dimeric during the day. Both forms exhibit the enzymic activity, but differ in pH optima, kinetics and the sensitivity to effectors (Wu and Wedding, 1985a,b).

Jawali (1990), found that active C_4 PEPC from maize to be tetrameric form. The dimeric form, found in leaves incubated in dark before enzyme extraction, could be converted to tetrameric form in the presence of light. The enzyme from a few other C_4 plants shows

decrease in activity with time during the assay (Manetas and Gavalas, 1982; Manetas *et al* , 1988; Gavalas and Manetas, 1988). This is attributed to the dissociation of the enzymes during assay to less active forms.

McNaughton *et al* (1989), on the other hand reported that in maize, both C_4-PEPC and C_3-PEPC can exist in tetrameric and dimeric forms, and the sensitivity kinetics to malate of C_4-PEPC tetrameric and dimeric forms was less than C_3-PEPC tetrameric or dimeric forms. They concluded that changes in oligomerization state of PEPC are not directly involved in its light regulation in maize.

Light also causes PEPC phosphorylation (Jiao and Chollet, 1988, 1992; Jiao *et al* , 1991; Bakrim *et al* , 1992; Wang and Chollet, 1993), and induces subtle conformational changes in the enzyme (Huber *et al* , 1986). The enzyme responsible for PEPC phosphorylation is phospho*enol*pyruvate carboxylase protein serine kinase (PEPC-PK) (Jiao *et al* , 1991; Bakrim *et al* , 1992; Jiao and Chollet, 1992; Wang and Chollet, 1993). PEPC-PK is light inducible. Jiao *et al* (1991), reported that PEPC-PK regulated *in vivo* involves net *de novo* cytoplasmic protein synthesis in light and subsequent degradation in darkness. The single *in vitro* and *in vivo* phosphorylation site has been identified as serine 15 in maize (Jiao and Chollet, 1990; Jiao *et al* , 1991), serine 8 in sorghum (Jiao *et al* , 1991; Wang *et al* , 1992) and serine 11 in tobacco (Wang and Chollet, 1993). In the case of CAM-PEPC from *Mesembryanthemum crystallinum*, serine 11 is phosphorylated in the active (night) form while the same is dephosphorylated in the day (Jiao and Chollet, 1991; Baur *et al* , 1992; Nimmo *et al* , 1993). Phosphorylation of C_4-

PEPC makes it less sensitive to malate inhibition. In dark, C_4-PEPC is dephosphorylated by a type 2A protein phosphatase, in which state PEPC becomes more susceptible to inhibition by malate.

Jiao and Chollet (1992), proposed that the calvin cycle supplies mesophyll cells with (a) a putative signal (phosphorylated metabolite, amino acid) that interacts with the cytoplasmic protein synthesis event to effect the light activation of PEPC-PK and concomitant phosphorylation of PEPC, and (b) high levels of known positive effectors (e.g. glucose-6-phosphate, triose-phosphate) that interact directly with PEPC. The combined result of this complex regulatory cascade is to effectively desensitize PEPC to feedback inhibition by the millimolar levels of L-malate required for rapid diffusive transport to the bundle sheath during high rates of C_4 photosynthesis.

On the other hand, Iglesias *et al* (1993), while trying to explain light/dark regulation of C_4-PEPC suggested the role of inorganic phosphate. Regulation of PEPC by reversible phosphorylation is already documented. Further, Podesta *et al* , (1990), reported that Pi could activate C_4-PEPC by mediating pH-dependent increases in the enzyme's affinity for PEP. Since PEPC is dephosphorylated by an okadaic acid-sensitive protein phosphatase 2A, to a less active, more malate sensitive form, and since sucrose-6-phosphate synthase is also dephosphorylated but activated by a protein phosphatase 2A that is inhibited by Pi (Huber *et al* , 1989). Iglesias *et al* , (1993), suggested that the former phosphatase is also regulated by Pi, and that the same phosphatase dephosphorylates both target proteins. If so, an effective mechanism based on Pi regulation of protein phosphatase(s) would be operative in the

cytosol of C_4 mesophyll cells, coordinating the rates at which CO_2 is initially fixed and the newly fixed carbon is converted into sucrose.

Langdale *et al* (1991) and Yanagisawa *et al* (1991), found a number of GC motifs in, or near maize *ppc* genes. Indeed long GC repeats were reported as far as 3.5 kb upstream of maize *ppc* genes (Langdale *et al* , 1991). Although as yet no specific function has been ascribed to them, but since plants have about 30% C in GpC and GpNpC sequences, these motifs gain importance as far as PEPC regulation and differential expression is concerned. Indeed, Kano-Murakami *et al* (1991), did find a plant nuclear protein *PEPI*, which is exclusively present in green leaves of maize, and which interacts with a GC-rich repeat in the C_4-PEPC promoter region. The tissue specificity of *PEPI* suggests that *PEPI* may function to coordinate PEPC gene expression with respect to light and tissue specificity. Similarly, Yanagisawa and Izui (1990, 1993), reported leaf-specific protein factors that interacted with maize *ppc* promoters.

Hence, C_4-PEPC expression is influenced by a complex regulatory cascade which involves *cis*-acting elements interacting with specific *trans*-acting factors at the transcription level, and subunit aggregation / disaggregation and phosphorylation/dephosphorylation at the post-translational level. Gene methylation seems to be only a small part in this regulatory cascade, and so far as the differential regulation of PEPC is concerned, mere C-residue methylation does not seem to be responsible for it.

However, in our studies, we did notice that PEPC activity in mesophyll cell extracts increases upon 5-AzaC application. As shown

in the previous chapter (Fig. 4e), although there is a much higher PEPC protein content in 15 day old AL plants as compared with CL plants, there is correspondingly much lower PEPC activity. This could be due to non-assembly of PEPC protein to tetrameric form and/or non-phosphorylation. Similarly, there is hardly 2% increase in PEPC protein content in 15 day old CLA-plants, yet they registered a 74% higher PEPC activity. This disproportionately high increase in PEPC activity vis-à-vis the abundance of PEPC protein might be due to high aggregation to the active tetrameric state and/or high phosphorylation status. However, the increase in PEPC activity in light, especially under short-term 5-AzaC treatment suggests that 5-AzaC leads to expression of, besides others, proteins responsible for post-translational modification of C_4-PEPC. On the other hand, the increase in PEPC protein content in 15 day old AD-plants is concomitant with the increase in PEPC activity in these plants (Fig. 4e and 4c AD:CD). However, since no C_4-PEPC is expressed in dark, this would suggest that C_3-PEPC expression increases in response to 5-AzaC treatment in dark grown sugarcane plants.

PPDK Activity :

PPDK is a homotetramer with a subunit molecular mass of 94,000 (Sugiyama, 1973). PPDK in the absence of $Mg2^+$ (Sugiyama, 1973), dissociates to the inactive dimeric form. The enzyme is also inactive at low temperatures, but the activity is fully restored upon rewarming (Shirahashi *et al* , 1978; Hatch, 1979). PPDK is also inactivated in the presence of thiol compounds (Hatch and Slack, 1968). PPDK from sugarcane is sensitive to sulfhydryl blocking

reagents, particularly the reagents that bind to vicinal thiol groups (Hatch and Slack, 1969).

PPDK activation/inactivation involves a unique mechanism of covalent modification that is catalyzed by a single bifunctional PPDK-regulatory protein (Burnell and Hatch, 1983, 1985; Budde *et al* , 1986; Hatch, 1987; Chollet *et al* , 1990). The regulatory protein dark-inactivates PPDK through ADP-dependent phosphorylation of a single threonine residue. Light-dependent dephosphorylation (activation) is not the standard hydrolysis catalyzed by a protein phosphorylase, rather the regulatory protein utilizes Pi as a substrate to form PPi and active PPDK (Burnell and Hatch, 1983, 1985; Hatch, 1987; Chollet *et al* , 1990).

Sheen (1990a), reported that maize chloroplastic C_4-PPDK gene (C4ppdkZm1) appears to be created by the addition of an exon encoding the chloroplast transit peptide at a site upstream of a cytosolic PPDK gene (cyppdkZm1).

Sheen (1990b), using freshly isolated maize mesophyll protoplasts and a transient expression method, showed that the transcription activity of photosynthetic gene promoters are specifically and coordinately repressed by the photosynthetic endproducts sucrose and glucose and by the exogenous carbon source acetate. Hence, as in unicellular bacteria and yeast, genes involved in metabolic pathways are also subjected to regulation by the fluctuation of internal and external metabolite in multicellular higher plants. The metabolic repression of photosynthetic genes overrides other forms of regulation, e.g., light, tissue type and developmental age .(Simpson *et al* , 1986; Kuhlemeier *et al* , 1987;

Schell, 1987; Ha and An, 1988; Benfey and Chua, 1989; Ueda *et al* , 1989) because it is executed in young leaf cells (Sheen, 1990b). Further, when the concentration of sucrose was decreased from 300 mM to the lower levels found in normal photosynthetically active cells (30 mM) (Gerhard *et al* , 1987), sugar repression was no longer significant. Therefore, it is possible that sugar inhibition is a type of feedback regulation and only occurs when the accumulation of sugars in cells is above certain physiological levels (Sheen, 1990b).

Matsuoka (1990), reported several putative regulatory sequences at the 5' upstream region of PPDK, including two GC boxes and three long directly repeated sequences. Matsuoka and Numazawa (1991), identified a *cis*-acting site which gave a strong binding to the protein *PPD1*, which is present only in green leaves. The fact that this protein was absent in all other tissues as also in etiolated leaves of maize, suggests that this protein might be one of the factors responsible for light-induced increase in PPDK mRNA in maize.

The PPDK activity seems to be modulated at the transcriptional and post-translational level. Control at transcriptional level would be indicated in the amount of enzyme formed, and at the post-translational level, by the amount of active enzyme found. In the present study short-term 5-AzaC treatment did not affect the enzyme activity, neither when plants were grown in continous light, nor when these were grown in constant darkness. However, in both the light and dark grown plants , a substantial increase in the PPDK activity on long-term 5-AzaC treated plants was recorded. This

suggests that PPDK is up-regulated by 5-AzaC, albeit only on long-term application.

CL-PPDK activity on an average was about 60-70% higher than CD-PPDK activity. Yet, AL-PPDK and AD-PPDK activities were comparable. So much so that AD-PPDK activity throughout the duration of the time course period was higher than CL-PPDK activity. This would suggest increase in C_4-PPDK expression in sugarcane upon long-term 5-AzaC treatment. PPDK activity in leaves declines in the dark and is rapidly recovered following illumination. Because of the fact that activation occurs without any lag period, it was suggested that the increase represented reactivation of an inactive enzyme rather than enzyme synthesis (Slack, 1968). Sheen and Bogorad (1987), reported the existance of a tight temporal correlation between protein and mRNA accumulation by light for PPDK, except that mRNA accumulation is transient for PPDK whereas the amount of protein remains constant after accumulation. The protein appears to be relatively stable while the size of mRNA pool change. The reason why PPDK activity of short-term 5-AzaC treated plants show no change compared to control plants, remains speculative. However, in long-term 5-AzaC treated plants, the quantum of 5-mC is definitely going to be lower than in short-term 5-AzaC treated plants. It is possible that there is an increased expression of *trans*-acting factors like *PPD1* in these plants, which lead to higher expression of PPDK.

NADP-ME Activity :

NADP-dependent malic enzyme acts in a wide range of metabolic pathways in plants (Drincovich *et al* , 1992). The enzyme catalyzes reversible oxidative decarboxylation of L-malate in the presence of

NADP and a bivalent metal ion to produce pyruvate, NADPH and CO_2. In animals, cytosolic NADP-ME generates reducing power for the biosynthesis of fatty acids (Frenkel, 1975; Wise and Rubin, 1984).

In plants, two forms of this enzyme are known to occur and have important metabolic roles (Pupillo and Bussi, 1979; Scagliarini *et al* , 1988). The cytosolic form is thought to participate in the regulation of intracellular pH (Davies and Patil, 1974; Pupillo and Bussi, 1979) and/or in the provision of reducing power that can be used in processes requiring NADPH (Edwards and Huber, 1981). The chloroplast stromal form is found specifically in the bundle sheath chloroplasts of NADP-ME type C_4 photosynthetic plants. The enzyme plays a key role in C_4 photosynthetic metabolism because it generates reducing power and CO_2 in the bundle sheath chloroplasts where RuBISCO and the calvin cycle operates (Edwards and Huber, 1981; Edwards and Andreo, 1992). The enzyme has a high specificity and low K_m for $NADP^+$ (Edwards and Andreo, 1992). NADP-ME exists as a tetramer with subunit molecular mass ranging from 62-68 kD, which may undergo changes in oligomerization and exhibit hysteresis (Edwards and Andreo, 1992). The enzyme requires Mg^{2+} or Mn^{2+} as a cofactor.

Studies of chemical modification of the enzyme suggested that histidine (Jawali and Bhagwat, 1987) and argenine (Rao *et al*, 1991) residues are required for activity. Evidence for the existence of sulfhydryl groups at or near the NADP-binding site of maize NADP-ME was demonstrated using the relatively selective reagent bromopyruvate (Drincovich and Andreo, 1992). Drincovich *et al* (1992), subsequently provided evidence for the existence of two

essential and proximal cysteinyl residues in NADP-ME from maize leaves.

The maize chloroplastic NADP-ME is encoded in the nucleus and contains a transit peptide for directing organellar transport (Rothermel and Nelson, 1989). Rajeevan *et al* (1991) cloned NADP-ME from leaves of *Flaveria trinervia* (C_4), *Flaveria linearis* (C_3-C_4), and *Flaveria pringlei* (C_3). C_4 enzyme activities in intermediate species are generally 2- to 5-fold higher than C_3 types, but 10- to 20-fold lower than C_4 types (Ku *et al* , 1983; Bauwe, 1984; Cameron and Bassett, 1988). Although these differences in enzyme activity could reflect structural changes and/or environmental influences, studies indicate that the level of subunits for several C_4 enzymes differs in parallel with activity measurements between species (Cameron and Bassett, 1988; Cameron *et al* , 1989). Subsequently, Rajeevan *et al* (1991), reported that expression of NADP-ME transcripts in *Flaveria* sp. representing C_3, C_3-C_4 and C_4 types of photosynthesis parallels data from enzyme activity and subunit levels, suggesting that control of NADP-ME expression is at the level of transcription or post-transcription.

We have found that in light, short-term 5-AzaC treated plants gave a rather inconsistant to NADP-ME activity response. The activity fluctuated widely day to day. CLA-ME activity was more than CL-ME on day 13 (143%) and on day 15 (130%). AL-ME activity was significantly lower than CL-ME activity throughout the duration of time course period. On the other hand, in plants grown under the conditions of constant darkness, CDA-ME consistently registered higher activity than CD-ME. Significantly, AD-ME activity, barring on days 15 and 16, was lower than CD-ME activity throughout. We

found that in dark, both in mesophyll and bundle sheath cells, there was a considerable increase in total soluble protein content in AD-plants. Yet this increase does not reflect in increase in NADP-ME activity barring on days 15 and 16 (where there is 2- and 4-fold increase in activity). This would suggest that C_4 NADP-ME gene is not affected by 5-AzaC treatment. Though there is an increase in NADP-ME activity in CDA-plants, unlike PPDK, the net activity at any given timepoint was much lower than the CL-ME activity. Hence, this increase in NADP-ME activity in dark could be due to the increase in cytosolic NADP-ME expression, rather than the C_4 specific NADP-ME. Sheen and Bogorad (1987), suggested that NADP-ME was under the control of light, and there is both increase in mRNA as well as protein when etiolated maize leaves were exposed to light. We have found that the NADP-ME activity in CL-plants is about 10-fold higher than CD-plants. As far as the role of cytosine methylation and light is concerned, at least in the case of NADP-ME, C-residue methylation inhibition proved to be detrimental to NADP-ME expression in light.

Plate VII

Total soluble protein profile in mesophyll cells from day 12 to day 18.

Fig. 5a : Total soluble protein profile in mesophyll cells of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5b : Total soluble protein profile in mesophyll cells of dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5a

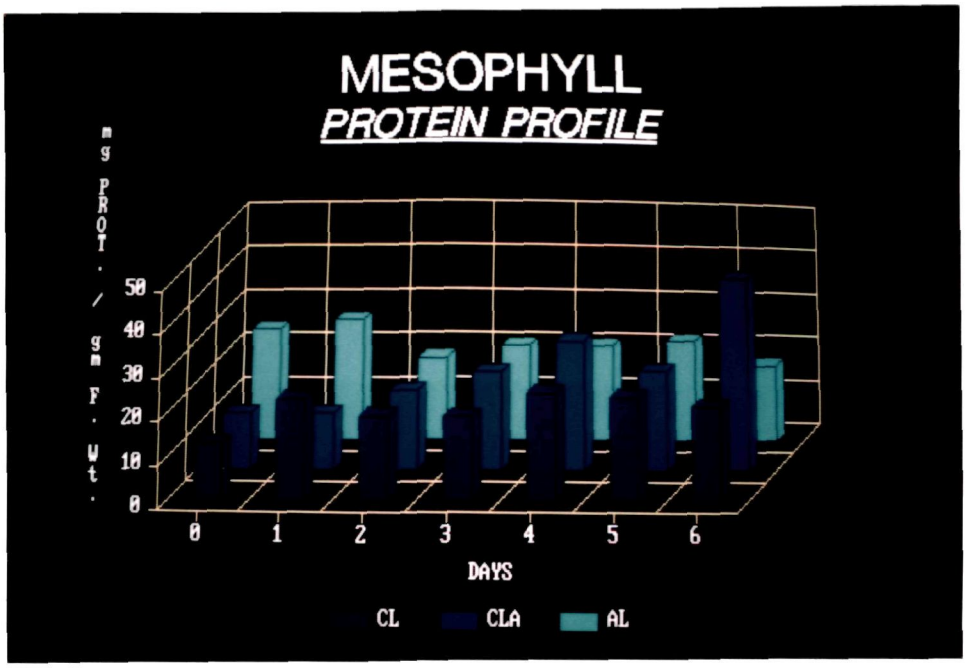

MESOPHYLL
PROTEIN PROFILE
mg PROT. / gm F. Wt.
50
40
30
20
10
0
0
1
2
3
4
5
6
DAYS
CL
CLA
AL

Fig5b

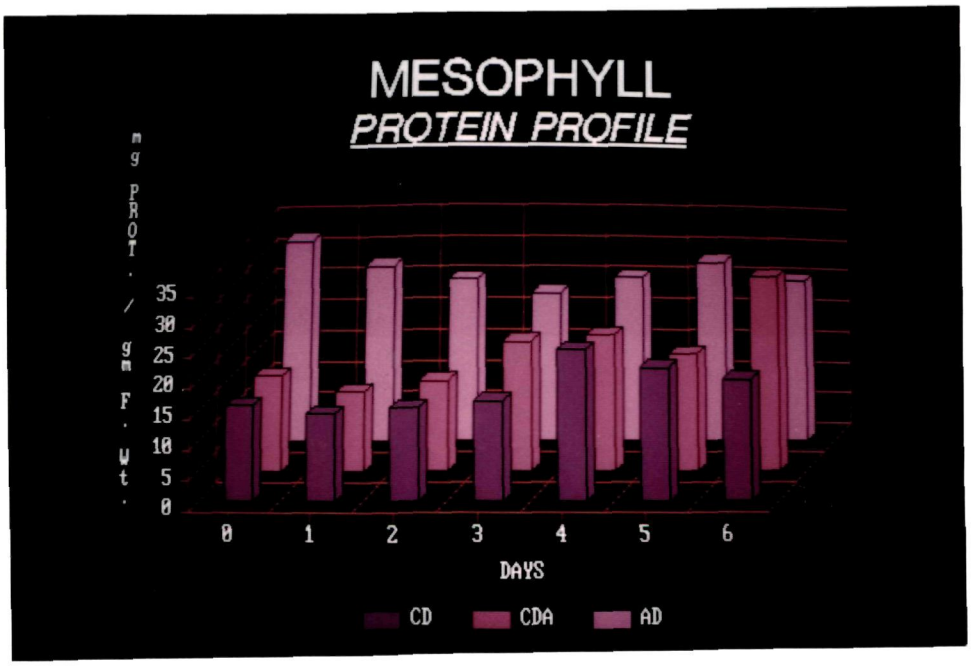

MESOPHYLL
PROTEIN PROFILE
mg PROT. / gm F. Wt.
35
30
25
20
15
10
5
0
0
1
2
3
4
5
6
DAYS
CD
CDA
AD

Plate VIII

Total soluble protein profile in bundle sheath strands from day 12 to day 18.

Fig. 5c : Total soluble protein profile in bundle sheath strands of light-grown plants from 0-6 days (day12 to day 18).

Fig. 5d : Total soluble protein profile in bundle sheath strands of dark-grown plants from 0-6 days (day12 to day 18).

Fig.5c

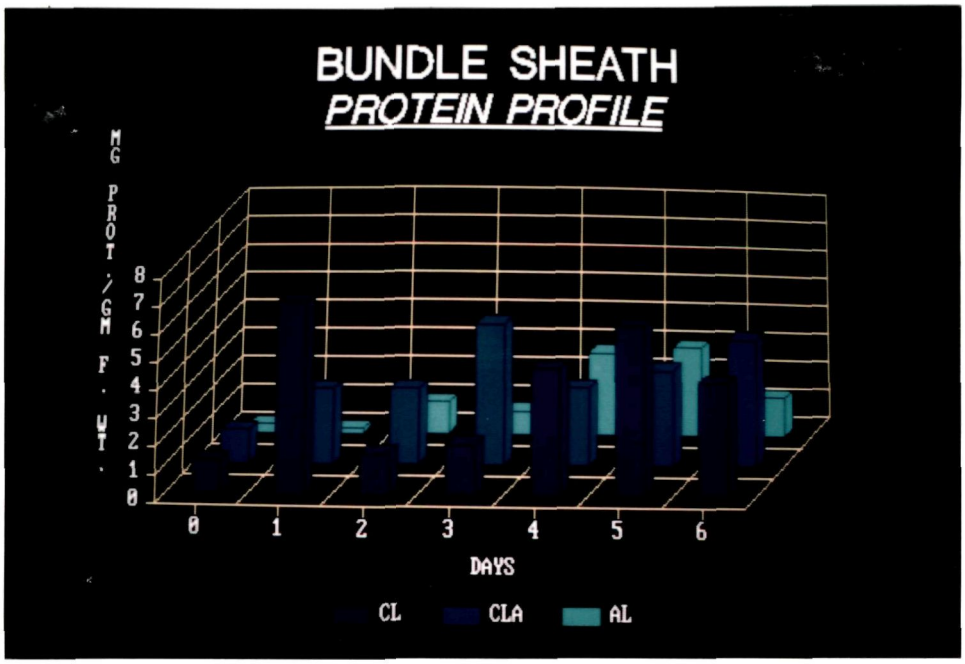

BUNDLE SHEATH
PROTEIN PROFILE
MG PROT./GM F. WT.
0 1 2 3 4 5 6 7 8
0 1 2 3 4 5 6
DAYS
CL CLA AL

Fig.5d

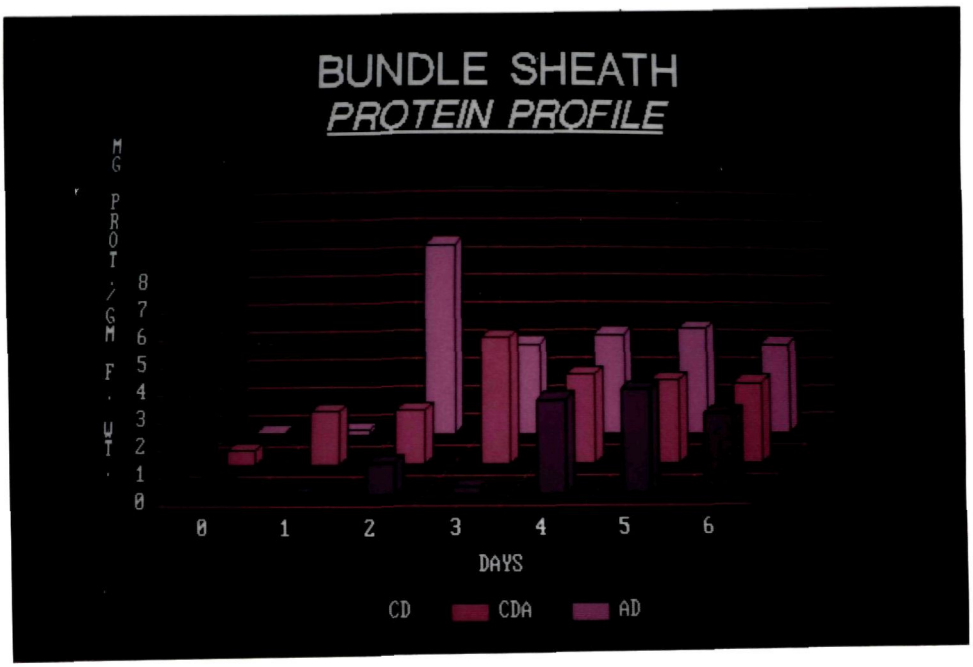

BUNDLE SHEATH
PROTEIN PROFILE
MG PROT./GM F. WT.
0 1 2 3 4 5 6 7 8
0 1 2 3 4 5 6
DAYS
CD CDA AD

Plate IX

Phospho*enol*pyruvate enzyme activity from day12 to day 18.

Fig. 5e : PEPC enzyme activity in mesophyll cells of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5f : PEPC enzyme activity in mesophyll cells of dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5e

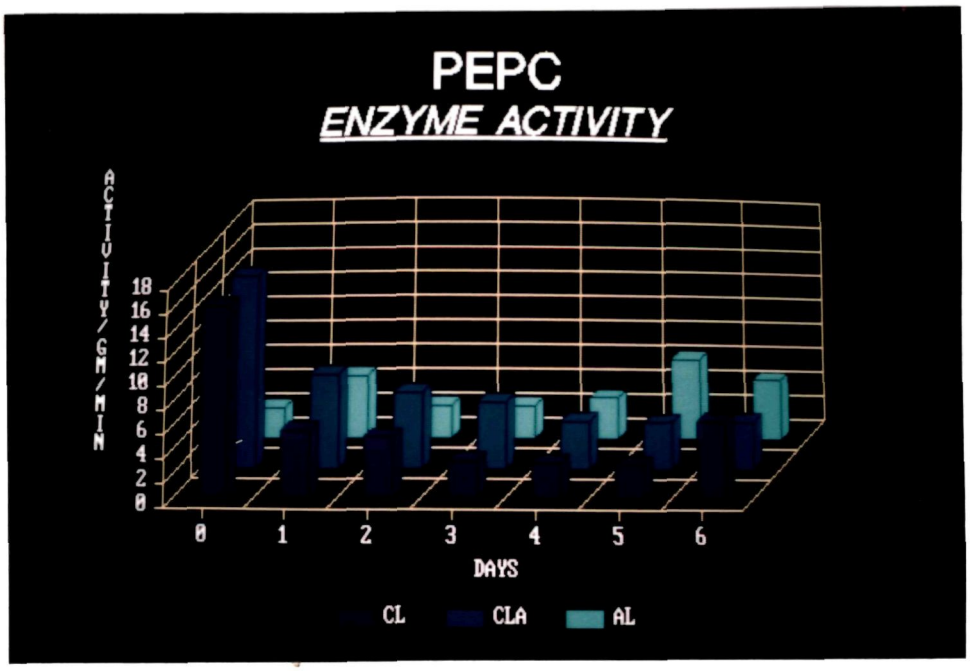

PEPC
ENZYME ACTIVITY
ACTIVITY/GM/MIN
18
16
14
12
10
8
6
4
2
0
0
1
2
3
4
5
6
DAYS
CL
CLA
AL

Fig.5f

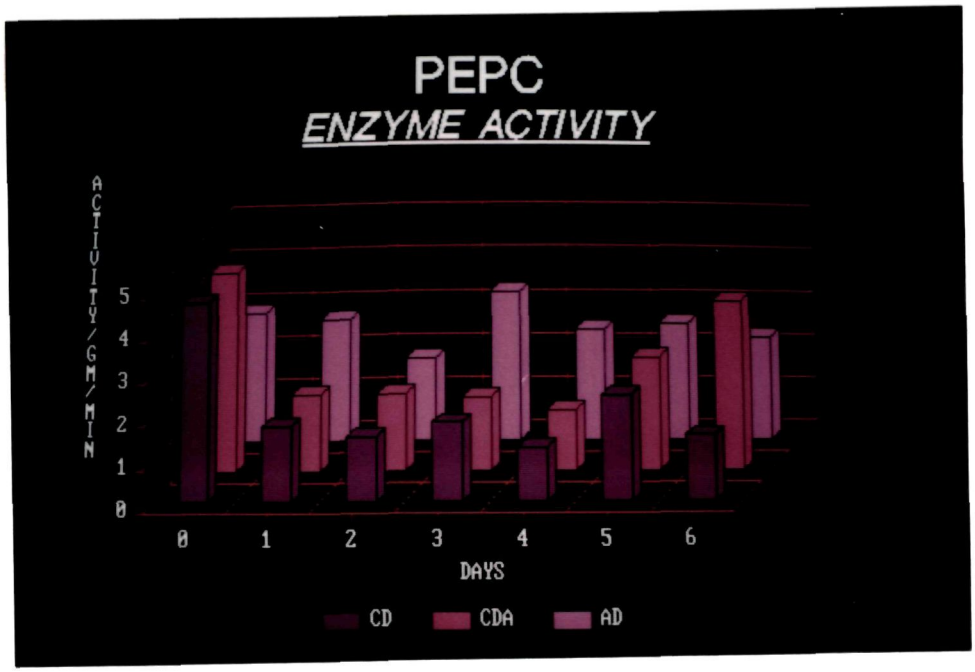

PEPC
ENZYME ACTIVITY
ACTIVITY/GM/MIN
5
4
3
2
1
0
0
1
2
3
4
5
6
DAYS
CD
CDA
AD

Plate X

Phospho*enol*pyruvate specific activity from day 12 to day 18.

Fig. 5g : PEPC specific activity in mesophyll cells of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5h : PEPC specific activity in mesophyll cells of dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5g

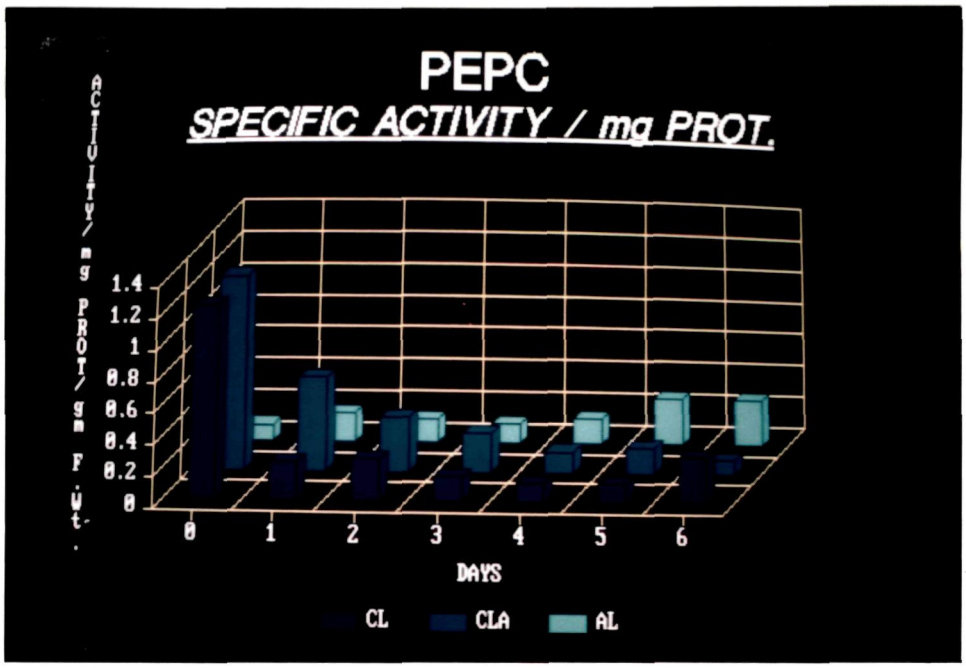

PEPC
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F.Wt.
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
DAYS
CL CLA AL

Fig.5h

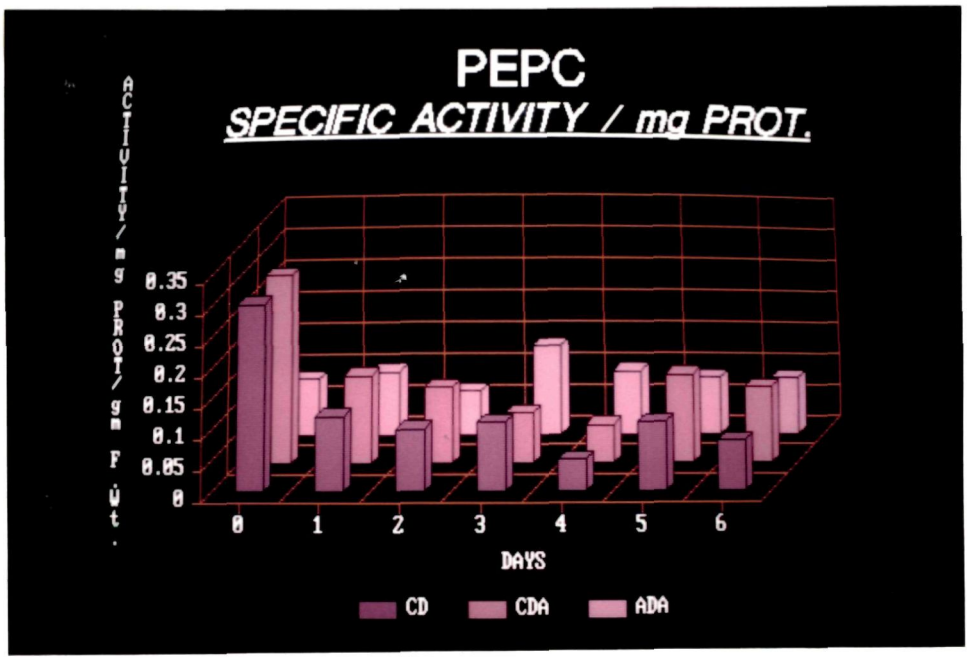

PEPC
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F.Wt.
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0 1 2 3 4 5 6
DAYS
CD CDA ADA

Plate XI

Pyruvate *ortho*phosphate dikinase Enzyme activity from day 12 to day 18.

Fig. 5i : PPDK enzyme activity in mesophyll cells of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5j : PPDK enzyme activity in mesophyll cells of dark-grown plants from 0-6 days (day12 to day 18).

Fig.5i

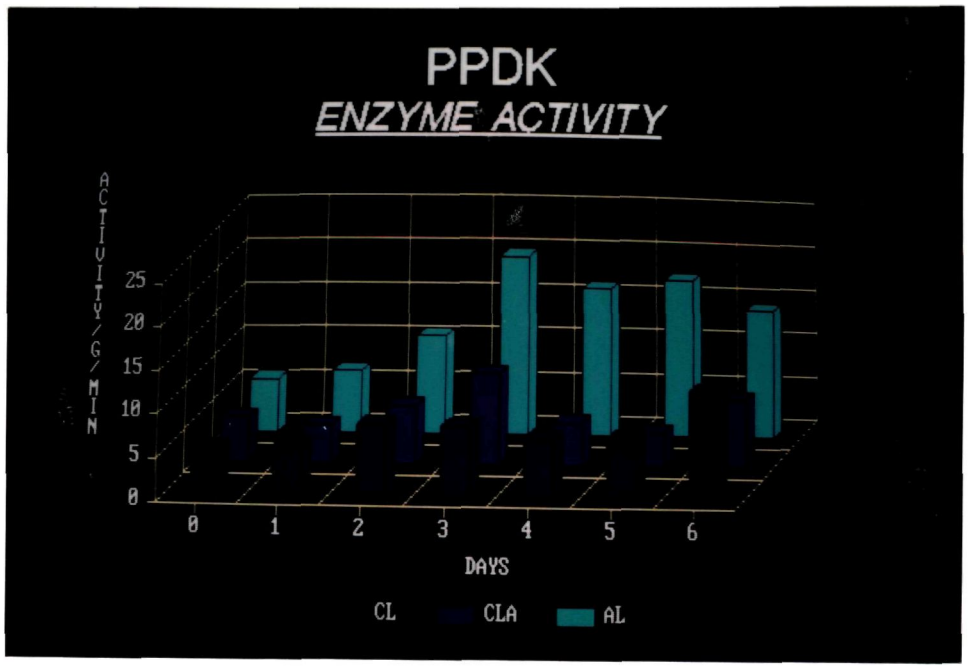

PPDK
ENZYME ACTIVITY
ACTIVITY/G/MIN
25
20
15
10
5
0
0
1
2
3
4
5
6
DAYS
CL
CLA
AL

Fig.5j

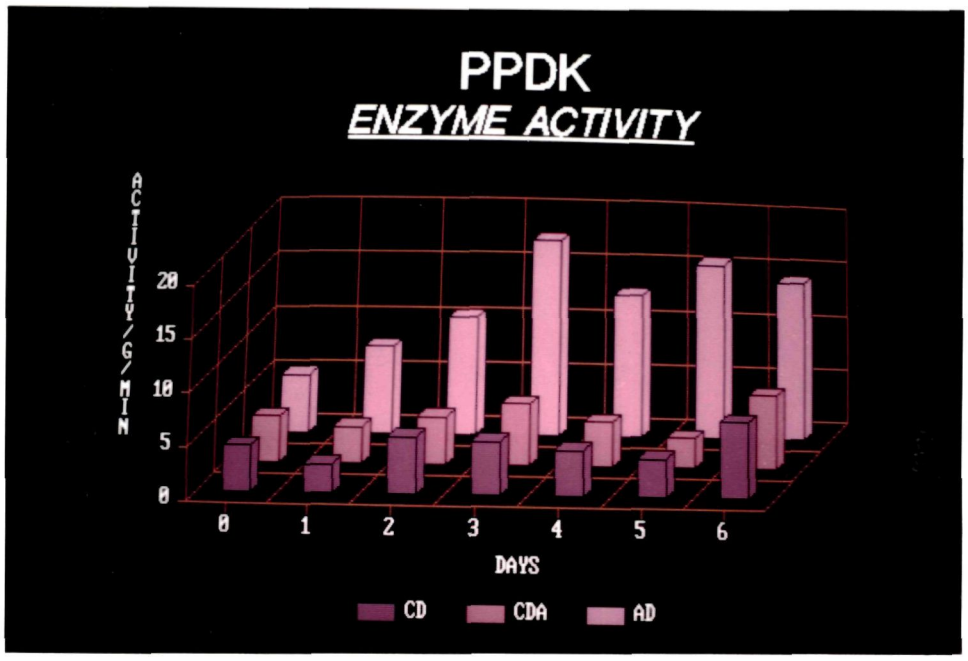

PPDK
ENZYME ACTIVITY
ACTIVITY/G/MIN
20
15
10
5
0
0
1
2
3
4
5
6
DAYS
CD
CDA
AD

Plate XII

Pyruvate *ortho*phosphate dikinase specific activity from day 12 to day 18.

Fig. 5k : PPDK specific activity in mesophyll cells from light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5l : PPDK specific activity in mesophyll cells from dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5k

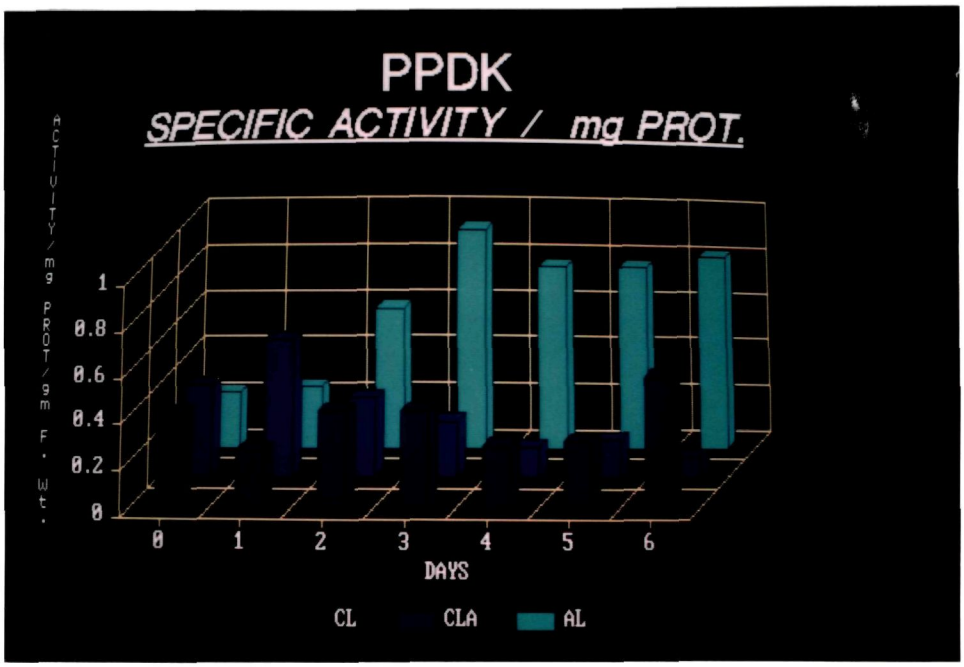

PPDK
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F. Wt.
1
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
DAYS
CL CLA AL

Fig.5l

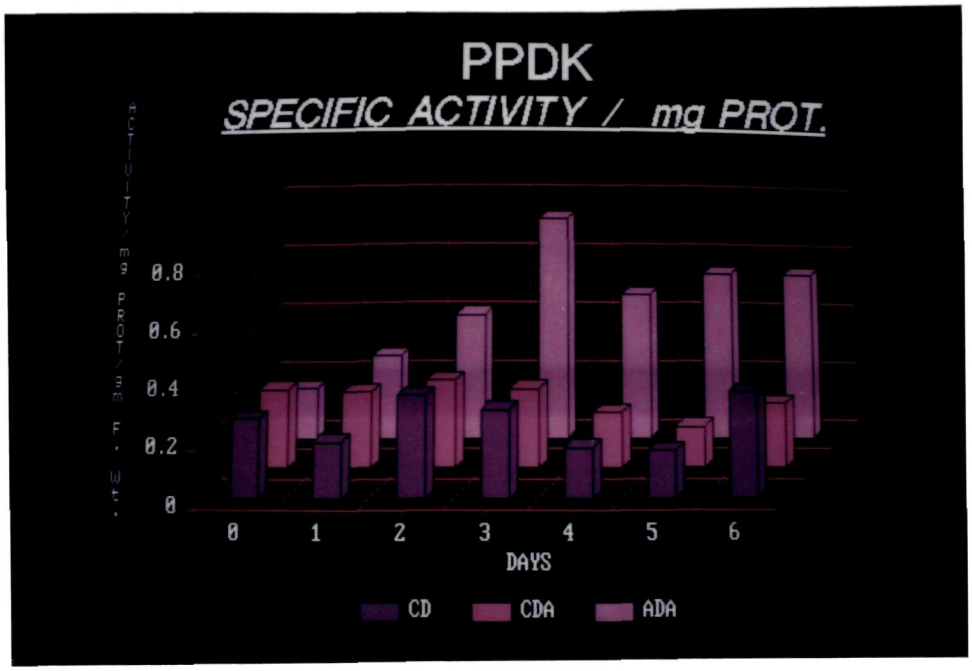

PPDK
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F. Wt.
0.8
0.6
0.4
0.2
0
0 1 2 3 4 5 6
DAYS
CD CDA ADA

Plate XIII

NADP dependent malic enzyme enzyme activity from day 12 to day 18.

Fig. 5m : NADP-ME enzyme activity in bundle sheath strands of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5n : NADP-ME enzyme activity in bundle sheath strands of dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5m

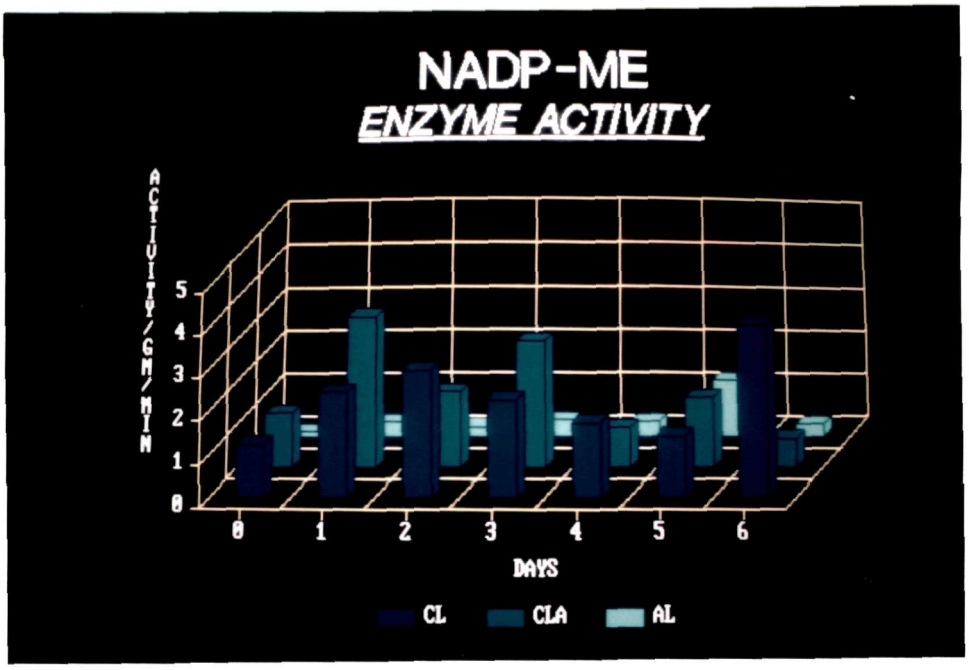

NADP-ME
ENZYME ACTIVITY
ACTIVITY/GM/MIN
0
1
2
3
4
5
0
1
2
3
4
5
6
DAYS
CL
CLA
AL

Fig.5n

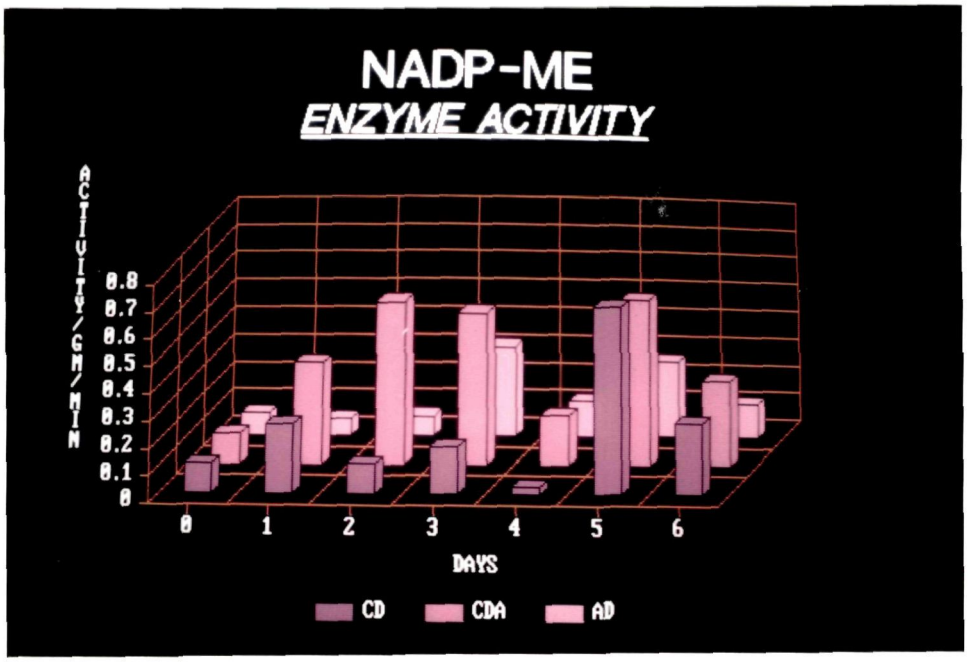

NADP-ME
ENZYME ACTIVITY
ACTIVITY/GM/MIN
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0
1
2
3
4
5
6
DAYS
CD
CDA
AD

Plate XIV

NADP dependent malic enzyme specific activity from day 12 to day 18.

Fig. 5o : NADP-ME specific activity in bundle sheath strands of light-grown plants from 0-6 days (day 12 to day 18).

Fig. 5p : NADP-ME specific activity in bundle sheath strands of dark-grown plants from 0-6 days (day 12 to day 18).

Fig.5o

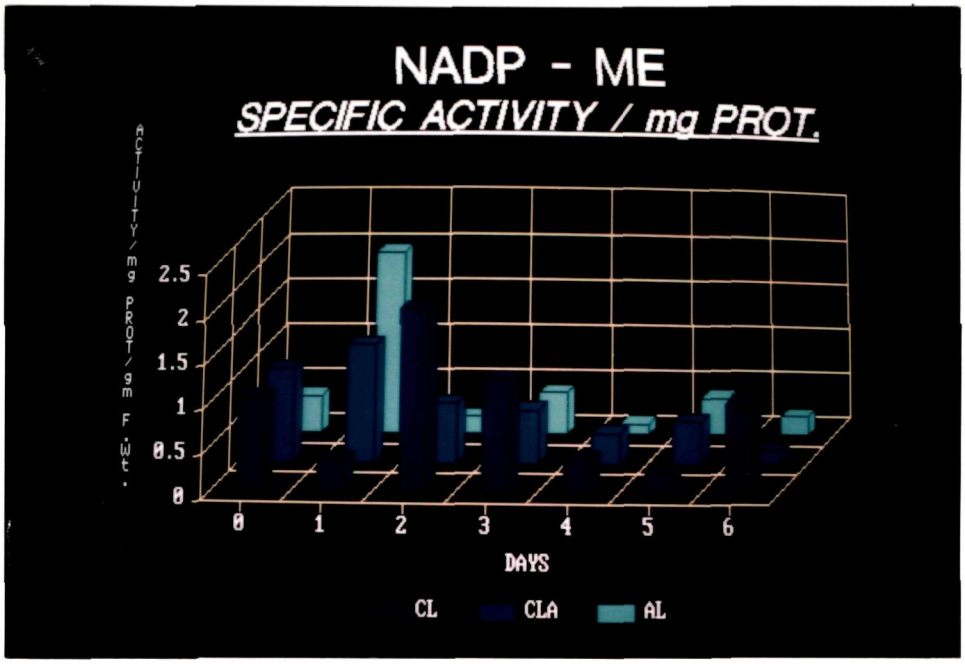
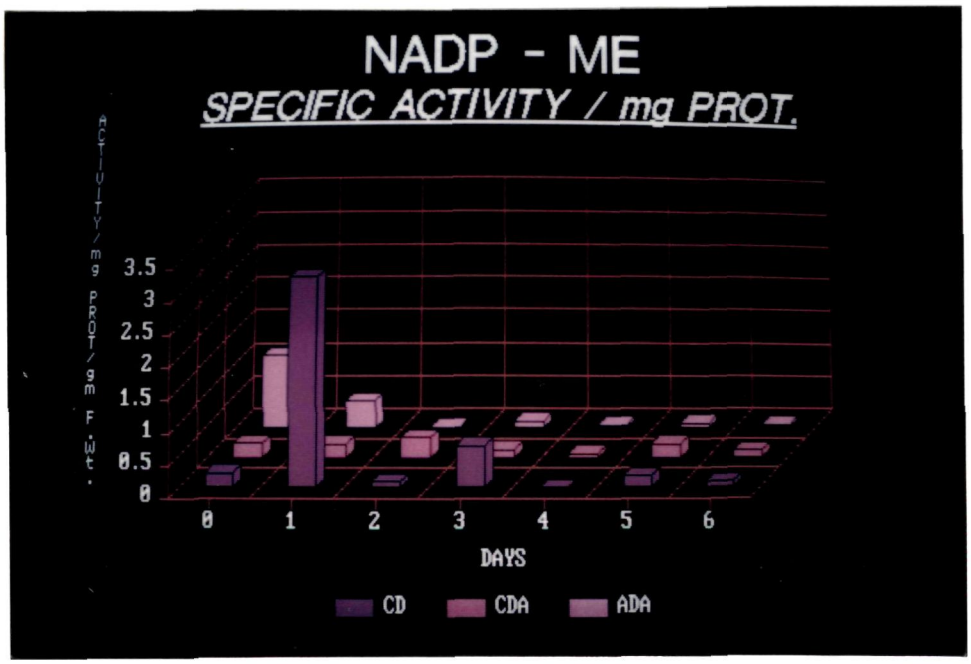

NADP - ME
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F.Wt.
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
DAYS
CL
CLA
AL

Fig.5p


NADP - ME
SPECIFIC ACTIVITY / mg PROT.
ACTIVITY/mg PROT/gm F.Wt.
3.5
3
2.5
2
1.5
1
0.5
0
0
1
2
3
4
5
6
DAYS
CD
CDA
ADA

Sequence Analysis of PEPC cDNA from Sugarcane

Introduction :

Three to four members of a nuclear gene family encode different isoforms of phosphoenolpyruvate carboxylase : C_4-specific, C_3 or etiolated, CAM and root isoforms. C_4 leaf PEPC is encoded by a single gene, *ppc*, in C_4 monocots maize and sorghum (Hudspeth *et al*, 1986; Hudspeth and Grula, 1989; Matsuoka and Minami, 1989; Yanagisawa and Izui, 1989; Cretin *et al*, 1990, 1991; Lipiniec *et al*, 1992, 1993), and by multiple genes in the C_4-dicot *Flaveria trinervia* (Poetsh *et al*, 1991). The only PEPC gene reported from sugarcane is the housekeeping C_3-PEPC gene (Henrik *et al*, 1992).

The selective expression of *ppc* in C_4-mesophyll cells is proposed to be due to nuclear factors, DNA methylation and a distinct gene promoter (Sheen and Bogorad, 1987a,b; Ngernprasirtsiri *et al*, 1989; Yanagisawa and Izui, 1990, 1993; Kano-Murakami *et al*, 1991; Kano-Murakami and Matsuoka, 1992; Nelson and Langdale, 1992; Schafner and Sheen, 1992;). In maize, the genes for different isozymes of PEPC are dispersed on separate chromosomes, for example, C_4-form on chromosome nine; C_3-form on chromosome seven and root-form on the fifth or fourth chromosome (Izui *et al*, 1992). The multiplicity of leaf-specific C_4 *ppc* genes in *F. trinervia* (C_4 dicot), unlike the single one in maize or sorghum (C_4 monocots), raises the question of whether this is a unique property of dicots or an exception (Hermans and Westhoff, 1990). Nelson and Langdale (1992), reported that maize *ppc* gene does not contain regulatory L-, I-, or G-box motifs, which are found in genes encoding

photosynthetic enzymes such as RuBISCO. On the other hand, it contains RS1-3 motifs in the 5'-flanking regions, which interacts with nuclear factors (Yanagisawa and Izui 1990, 1992). However, maize *ppc* gene does contain a number of short repetitive sequences in the 5'-flanking region, which is a characteristic of light-regulated genes (Hudspeth *et al*, 1986; Hudspeth annd Grula, 1989). The monocot *ppc* gene of maize and sorghum lack typical TATA or CAAT box sequences, instead, a slightly modified TATTT and a CCAAT sequence is present in the 5'-upstream region of the gene (Hudspeth and Grula, 1989; Matsuoka and Minami, 1989; Cretin *et al*, 1991). It is not known whether these modified elements are functional. Matsuoka and Minami (1989), reported that a consensus sequence CCTTATCCT at the position -659 to -651 may be important for the control of gene expression by light. Kano-Murakami *et al* (1991), on performing methylation interference analysis and DNA binding assays using synthetic oligonucleotides, identified that CCCTCTCCACATCC sequence of the maize 5'-upstream region of *ppc* gene specifically binds to a green leaf specific protein *PEP-I*.

cDNAs for *ppc* were prepared from leaves of maize (Hudspeth *et al*, 1986; Izui *et al*, 1986; Yanagisawa *et al*, 1988), *F. trinervia* (Hermans and Westhoff, 1990, Poetsch *et al*, 1991) and sorghum (Cretin *et al*, 1990, 1991). The coding region of sorghum *ppc* shows 88% sequence identity with that of maize but only 40-50% with that of *E. coli* and *Anacystis nidulans* (Ishijima *et al*, 1985; Izui *et al*, 1986; Cretin *et al*, 1990). The cDNA analysis indicates that most of the conserved sequences are in the C-terminal region of the maize enzyme, suggesting that this domain may comprise part of the catalytic site, while the domains formed by the N-terminal region

might be involved in allosteric regulation of the gene (Izui *et al*, 1986).

At the protein level, the C_4 PEPC from maize is composed of 970 amino acids in maize (Izui *et al*, 1986; Hudspeth and Grula, 1989; Matsuoka and Minami, 1989), 952 amino acids in sorghum (Cretin *et al*, 1990; Lipiniec *et al*, 1992), 966 amino acids in *F. trinervia* (Poetsch *et al*, 1991) and 883 aminoacids in *E. coli* (Fujita *et al*, 1984; Ishijima *et al*, 1985). While there exists a 60-90% sequence identity between the C_4-PEPC from maize, sorghum and *F. trinervia* and CAM PEPC of ice plant (*Mesembryanthemum crystallinum*) (Cushman *et al*, 1989; Hudspeth and Grula, 1989; Hermans and Westhoff, 1990, Cretin *et al*, 1991), there is only 40-50% sequence identity between maize PEPC and that of procaryotic origin from *E. coli* and *Anacistis nidulans* (Ishijima *et al*, 1985).

Results and Discussion :

We made a cDNA library from sugarcane leaves in λGEM4 vector from Promega™. Putative PEPC cDNAs were subsequently screened and isolated.

Genomic Southern :

Sugarcane leaf genomic DNA was digested with EcoR I and Hind III and double digested with EcoR I/Hind III. The fragments were separated on a 0.8% TBE gel (Fig. 6a) and lifted onto Hybond™ membrane. The membrane was probed using the 1.2 kb fragment from maize *ppc* gene (a kind gift from Dr. L. Bogorad). We got the Hind III lane giving a prominent band at ~3.8 kb and two other bands at ~2.7 kb and ~750 bp regions. The EcoR I lane showed two bands, one

~5.5 kb and the other ~4.4 kb region. The double digested lane showed three bands, the topmost at ~4.0 kb region, followed by a band at ~3.2 kb and one other band at ~700 bp region (Figs. 6a and 6b).

cDNA Library Preparation :

Protocols of Hoge *et al* (1982), De Vries *et al* (1983) and Govers *et al* (1985), was modified slightly to extract total RNAs from green sugarcane leaves. We found that the addition of 1mM ATA to the RNA extraction buffer significantly reduced RNA degradation during isolation, and subsequent storage at -20°C or -70°C. ATA binds ireversibly to RNases and inactivates it. For the isolation of PolyA$^+$ mRNA, an oligo-dT cellulose column was packed in a blue tip. We found that once PolyA$^+$ RNA binds to the column, the flow rate decreased considerably and the column appeared to get choked. Hence, the column was kept in an eppendorf tube and spun in SIGMA refrigerated centrifuge at 1500 x g for 2 min. This modification not only ensured that binding/washing/elution was carried out perfectly, but this treatment was also mild enough to ensure that the oligo-dT cellulose did not collapse during the whole operation.

After purification, the integrity of PolyA$^+$ mRNA was checked on denaturing formaldehyde gel. PolyA$^+$ mRNA purification was confirmed by the disappearance of rRNA bands. Subsequently, fractions of PolyA$^+$ mRNA ranging in size from ~2-4 kb were isolated, pooled and used further for cDNA synthesis.

cDNA synthesis was carried out essentially according to Promega's RiboClone® cDNA synthesis system. cDNA synthesis was followed by running a tracer reaction simultaneous to both first and

second strand synthesis reactions using α ^{32}P-dCTP. We got 26.4% mRNA converted to first strand cDNA and 98% of above was converted to double stranded form, as evidenced by TCA precipitation of the two tracer reactions. Subsequently, EcoR I adapters, which were synthesized using two oligonucleotide sequences as described earlier were ligated to the flushed ends of the double stranded cDNAs. The cDNAs were then digested with Xba I to generate staggered ends and facilitate directional cloning into λGEM-4 EcoR I/Xba I arms.

Recombinant λGEM-4 DNA was packaged in E.coli LE392 using Promega's Packagene® system (Rosenberg *et al*, 1985; Rosenberg, 1987). The system employs an *E.coli* C bacterial host, LE392, which lacks all known restriction systems, hence the libraries prepared in these are not biased by restriction of genetic material. The λGEM-4 was packaged at efficiency of 3 x 106 pfu/ml.

Screening :

Recombinant λGEM-4 were plated to a density of about 500-600 pfu/plate and were used for plaque lifts on S&S nitrocellulose paper. PEPC cDNA clones were screened out by probing with a C_4 PEPC cDNA fragment from maize (Fig. 6c). The positive clones were picked and plated at a density of 50-100 pfu/plate to facilitate secondary screening (Fig. 6d). Further work was done only on those plaques which gave intense dark spots on hybridization.

Around 40 plaques were isolated after final screening. The DNA from each was isolated, and pooled together to give a mixture of 3-5 plaque DNAs. The mixing of DNA was done to facilitate faster screening. The DNA was then subjected to SpeI digestion. Since λGEM-

4 has a full copy of plasmid pGEM-I, and since our inserts are cloned within the EcoRI and XbaI sites of the multiple cloning site of the plasmid, digestion with SpeI released full copy of plasmid pGEM I along with the inserts (Fig. 6e). Presence of PEPC cDNA was confirmed by southern analysis of the gel (Fig. 6f). PCR of the recombinant plaque DNA using Sp6 and T7 promoter primers was done (Fig. 6g). As further confirmation that the isolated clones are PEPC cDNAs, the PCR fragments generated above were subsequently probed with the maize *ppc* probe (Fig. 6h). The recombinant plasmids were isolated, self-ligated and transformed in *E.coli* JM109. Further work was performed using these plasmids.

Subsequent work was concentrated on two of these clones labelled pJM27 and pJM32, as these clones had the longest insert sizes of ~1.2 kb each. Each plasmid was digested with Hind III, EcoR I and double digested with Hind III and EcoR I (fig. 6i). An internal Hind III site ~550 bp from the 3′ end was observed (Fig. 6i). The fragments were subsequently lifted onto Hybond™ membranes and probed with the maize *ppc* cDNA probe (Fig. 6j). Since only the inserts gave intense dark bands on the autoradiogram, the presence of PEPC cDNA clones in these plasmids was confirmed (Fig. 6j).

Restriction mapping of both pJM27 and pJM32 confirmed that these are the same clones. They have internal BamH I, Hind III, Not I, Pst I and Sma I sites, while no sites were found for Apa I, Bgl II, Cla I, EcoR V, Hinc II,Kpn I, Nco I, Nsi I, Sac I, Sal I and Xho I (Figs. 6k, 6l and 6m).

Sequencing :

As both pJM27 and pJM32 were of the same size and generated similar restriction map, sequencing was performed only with pJM27. Sequencing was performed both from the 5' end and the 3' end using SEQUENASE Ver 2® kit procurred from Amersham. The sequences were separated on a 55 cm, 6% denaturing PAGE gel.

We were able to read a total of around 450 bp from either ends using two gel loads. The long poly T tract did not interfere with the sequencing, nor did it result in any compression on the gel (Fig. 6n).

Analysis :

The 5' end of pJM27 is from within the ORF of all the reported PEPC sequences. It shows maximum homology with a sorghum *ppc* gene (BLAST and DNASIS output). The 3' sequence on the other hand constitutes about 60 codons and around 300 bases at the 3' noncoding sequence. The 3' sequences of pJM27 also give maximum identity with the sorghum *ppc* gene (BLAST and DNASIS output).

Both the 5' and the 3' sequences were sent to the National Center for Biotechnology Information at the National Library of Medicine, Bethesda, USA for doing BLAST search. BLAST (Basic Local Alignment Search Tool) algorithm is a heuristic for finding ungapped, locally optimal sequence alignments (Altschul *et al*, 1990). The BLAST search confirmed that the cDNA pJM27 is a C_4 *ppc* mRNA as it shows highest identities with C_4 *ppc* sequences from sorghum and maize, both at the 5' and the 3' ends. While the 5'

sequence gave 96% identity with sorghum *ppc* gene, the 3' sequence gave 85% sequence identity.

Subsequently, both 5' and 3' sequences were aligned against all the known PEPC sequences right from procaryotic origin to C_4 PEPC gene using Hitachi's DNASIS package. The K-tuple value used was 1, which gives the most sensitive results. The portions of the sequences which gave maximum homology with pJM27 5' and 3' sequences, were excised and used as an input in the programme CLUSTALV (Higgins and Sharp, 1988, 1989). CLUSTALV programme used this input file to give a multiple alignment of all the sequences. A total of 29 sequences comprising procaryotic PEPC, gymnosperm PEPC, C_3, C_3-C_4 intermediates and C_4 PEPC of monocot and dicot origins and CAM PEPC sequences were aligned against the pJM27 5' and 3' sequence. The multiple alignment from above 30 sequences was used in the phylogenetic analyses using PHYLIP (Phylogeny Inference Package)(Felsenstein, 1989, 1993). DNA parsimony (Eck and Dayhoff, 1966; Kluge and Farris, 1969; Fitch, 1971) analysis gave a clear clustering of C_4 PEPC sequences, which align with the pJM27 5' and 3' sequences confirming that pJM27 is a C_4 *ppc* cDNA clone from sugarcane (Figs. 6o and 6p). The DNA parsimony tree from 5' sequence of pJM27 showed a clear clustering of C_4 PEPC sequences from monocots sorghum and maize; procaryotic PEPC from *E. coli, Anacystis, Anabena* and *Corrynebacterium;* C3 PEPC sequences from monocots sorghum and maize and CAM plants; gymnosperm *Picea abies,* dicot C_3, C_3-C_4 intermediate and C_4 *Flaveria* sp. and other dicot C_3 plants including *Solanum tuberosum, Nicotiana tabacum, Medicago sativa* and *Glycine max* (Fig. 6o). However, there appears to be some ambiguity as far as C_3 PEPC from sugarcane, and *Mesembryanthemum crystallinum,* and CAM PEPC from

Kalanchoe sp. are concerned. Since all these branched out along with the dicots (Fig. 6o). CAM PEPC from ice plant was also clustered with the monocot C_3 sequences from sorghum and maize, indicating that the CAM PEPC sequences branched out probably at about the same time as the C_3 sequences (Fig. 6o).

On the other hand, the phylogenetic analysis of the 3' non-coding region again showed pJM27 clustered with the C_4 PEPC genes of maize and sorghum, a separate cluster of C_3 PEPC of maize and sorghum, a separate cluster of the *Flaveria* sp., a separate branching of the gymnosperm *Picea abies* (Fig. 6p). However, procaryotic PEPC forms two clusters, one comprising *E. coli*, *Anacystis* and *Anabaena*, and the other comprising *Corynebacterium* (Fig. 6p). Here also the 3' non-coding region of C_3 PEPC from sugarcane and ice plant are found to be clustered along with dicot C_3 PEPC sequences from *Glycine max* and *Medicago sativa*. Yet the 3' non-coding sequence of CAM PEPC from ice plant and C_3 dicot *Solanum tuberosum* seem to have branched out together (Fig. 6p). The differences between the parsimonious trees of 5' and 3' pJM27 sequences may be due to the fact that the 5' sequence, which is within the ORF, would not be expected to change much, where as the same need not be the case with the 3' non-coding sequences.

The nearest neighbor (Saitou and Nei, 1987; Kimura, 1980) analysis of 5' pJM27 sequences showed that the ambiguity found in the parsimonious tree concerning the C_3 PEPC sequence from sugarcane and CAM PEPC from ice plant, disappeared as sugarcane PEPC clustered with C_3 sequences from monocots sorghum and maize, and the CAM PEPC from ice plant formed a cluster by themselves (Fig. 6q). Phylogenetic tree generated using the Neighbor-joining method for 3'

sequences also gave a very similar result as that of 5′ sequences (Fig. 6r). Significantly, *Picea* PEPC (gymnosperm), branches out separately , and it seems to be the closest to the procaryotes (Figs. 6q and 6r). This is in agreement with the paleantological data which suggests that gymnosperms ancestor arose around 370 million years ago and the angiosperm ancestors arose about 200 million years ago (Barnabas *et al*, 1995). Since the nearest neighbor analysis is a distance matrix method of producing phylogenies, it becomes clear that monocot C_4 PEPC sequences have possibly evolved most recently, and all the ancestral parents may have been the procaryotic PEPC.

The results were confirmed by constructing near parsimonious trees using the Fitch-Margoliash criterion (Fitch and Margoliash, 1967) (Fig. 6s and 6t). This estimates parsimonies based on the "additive tree model" according to which the distances are expected to equal the branch lengths between species. Significantly, phylogenetic tree constructed using the Fitch-Margoliash criterion of sequences aligned with the 5′ sequence of pJM27 are clustered more closer together (Fig. 6s). This is because the 5′ sequence of pJM27 is from with in the ORF, and hence these sequences would be under much more stringent evolutionary pressure than that of the 3′ sequences (Figs. 6s and 6t). On the other hand, the 3′ sequences are found to be much more dispersed (Fig. 6t) because changes in the 3′ region of the gene would not cause a drastic change in PEPC gene expression. However, phylogenetic analyses confirms that C_4 *ppc* from monocots are highly conserved in both the 5′ and the 3′ regions of the gene. PEPC genes from dicot *Flaveria* sps. are also conserved.

Hence, it is confirmed that C_4 PEPC is under a much stringent evolutionary pressure than the other PEPC isoforms.

Plate XV

Gel electrophoresis of sugarcane and tobacco genomic DNAs.

Fig. 6a : Lanes from left to right :

1. Hind III marker
2. Sugarcane genomic DNA digested with Hind III
3. Sugarcane genomic DNA digested with Hind III and EcoR I
4. Sugarcane DNA digested with EcoR I
5. Tobacco DNA digested with Hind III
6. Tobacco DNA digested with Hind III and EcoR I
7. Tobacco DNA digested with EcoR I
8. Hind III marker

Fig. 6b : Southern hybridization of above with maize *ppc* cDNA clone.

Screening of PEPC cDNA clones from sugarcane leaf cDNA library.

Fig. 6c : Primary screening of sugarcane leaf cDNA library with maize *ppc* cDNA clone.

Fig. 6d : Secondary screening of sugarcane leaf cDNA library with maize *ppc* cDNA clone.

Fig.6a

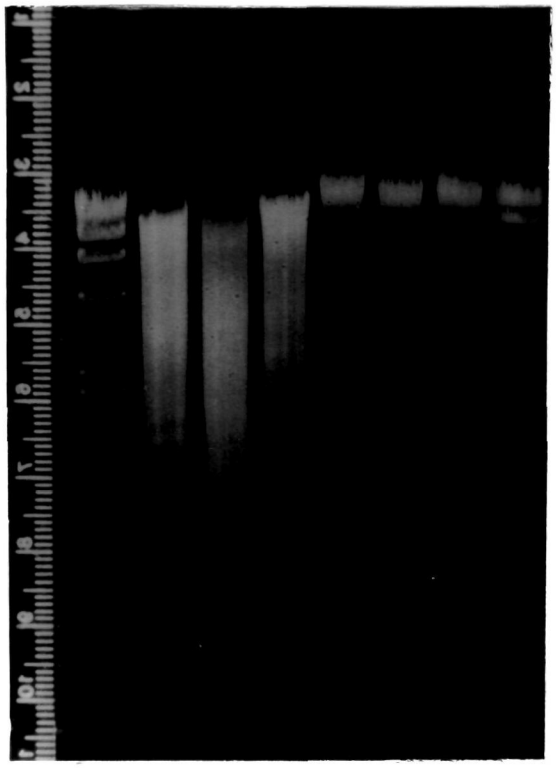


Fig.6b

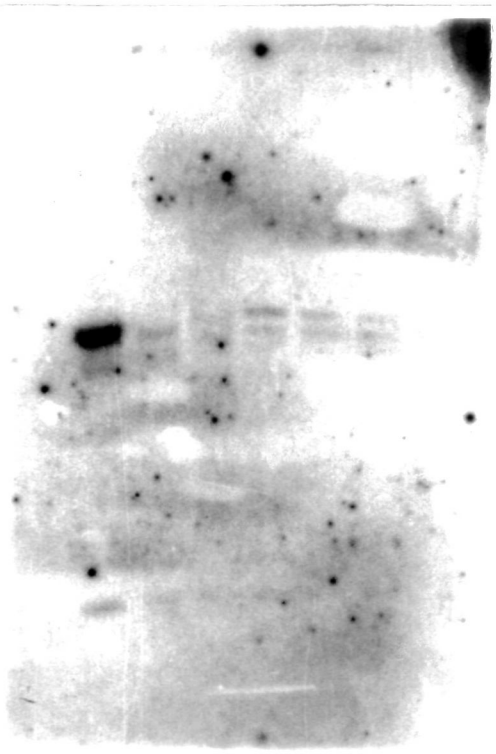


Fig.6c

Fig.6d

Plate XVI

Gel electrophoresis of recombinant λGEM 4 DNAs pooled in bunches of 2-5 and digested with Spe I, as described in the text.

Fig. 6e : Lanes 1, 6 and 11 are λ Hind III markers.

Fig. 6f : Southern hybridization of above with maize *ppc* cDNA clone.

Gel electrophoresis pattern of PCR of recombinant λGEM 4 DNAs pooled in bunches of 2-5 with Sp6 and T7 promoter primers, as described in the text.

Fig. 6g : Lanes 1 and 10 is λ Hind III marker and lanes 2 and 9 is 1 kb ladder.

Fig. 6h : Southern hybridization of above with maize *ppc* cDNA clone.

Fig.6e

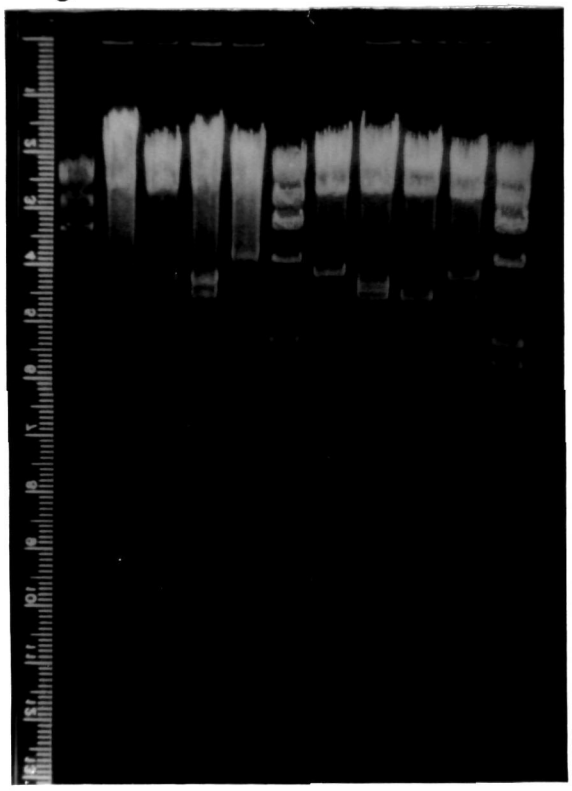


Fig.6f

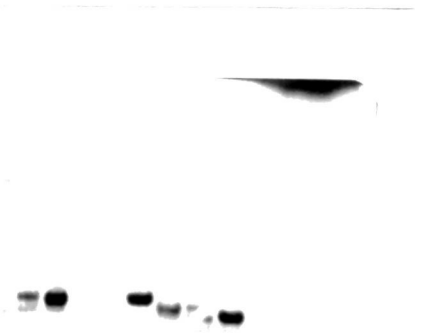


Fig.6g

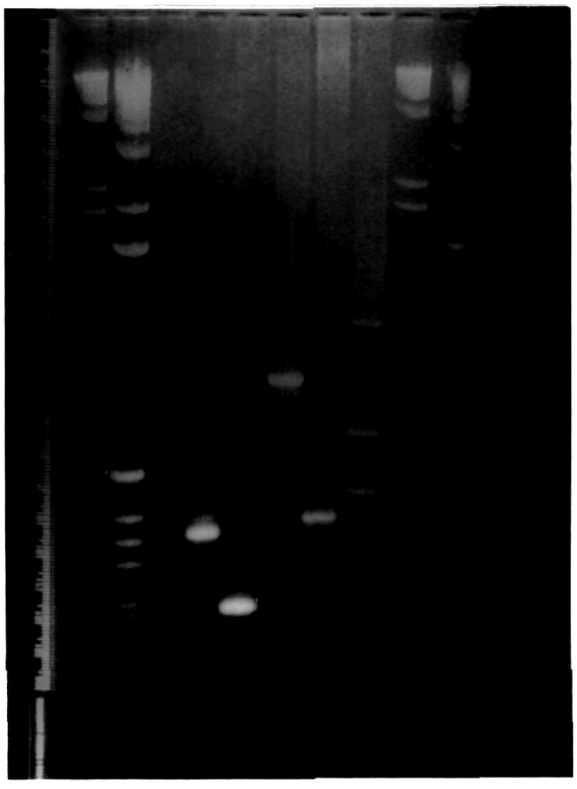


Fig.6h

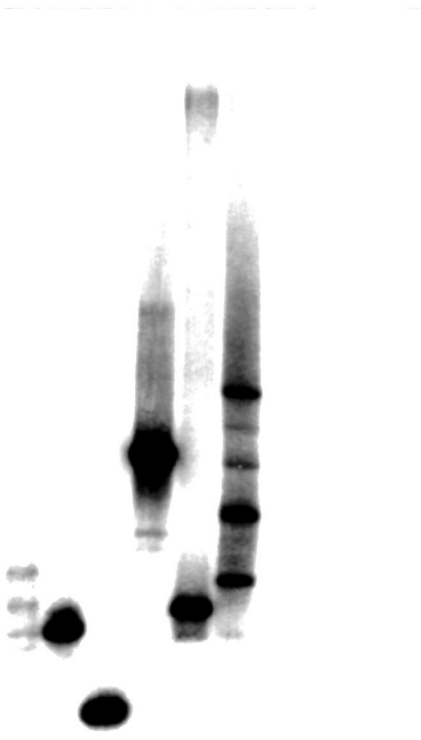


Plate XVII

Restriction mapping of pJM27 and pJM32 cDNA clones.

Fig. 6i : Lanes from left to right :

1. λ Hind III marker
2. pJM27 digested with Hind III
3. pJM27 digested with Hind III and EcoR I
4. pJM27 digested with EcoR I
5. Uncut pGEM I plasmid
6. pJM32 digested with Hind III
7. pJM32 digested with Hind III and EcoR I
8. pJM32 digested with EcoR I

Fig. 6j : Southern hybridization of above with maize *ppc* cDNA clone.

Fig.6i

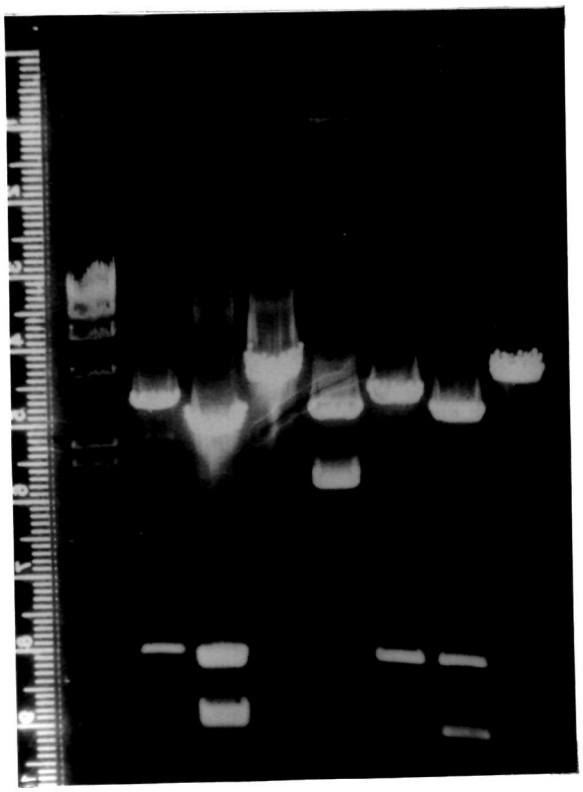


Fig.6j

Plate XVIII

Restriction mapping of pJM32.

Fig. 6k : Lanes left to right :

1. pJM32 digested with Sac I
2. pJM32 digested with Hinc II
3. pJM32 digested with BamH I
4. pJM32 digested with Sal I
5. pJM32 digested with Sma I
6. pJM32 digested with Pst I

Fig. 6l : Lanes from left to right :

1. pJM32 digested with Xba I
2. pJM32 digested with EcoR I and Sma I
3. λ Hind III marker
4. pJM32 digested with EcoR I and Not I
5. pJM32 digested with EcoR I and Apa I

Fig. 6m : Lanes from left to right :

1. pJM32 digested with Apa I
2. pJM32 digested with Bgl II
3. pJM32 digested with Cla I
4. pJM32 digested with EcoR V
5. pJM32 digested with Kpn I
6. pJM32 digested with Nco I
7. pJM32 digested with Not I
8. pJM32 digested with Nsi I
9. pJM32 digested with Xho I
10. λ Hind III marker
11. pJM32 digested with BamH I and EcoR I
12. pJM32 digested with BamHI and Hind III
13. pJM32 digested with BamH I and Pst I
14. pJM32 digested with Pst I and EcoR I
15. pJM32 digested with Pst I and Hind III

Fig.6k

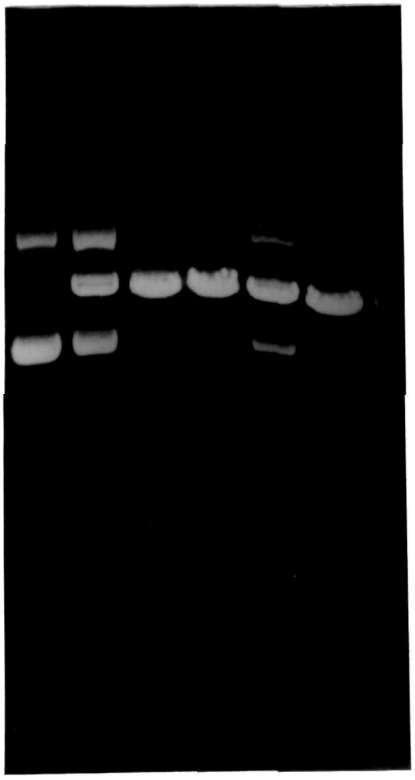


Fig.6l

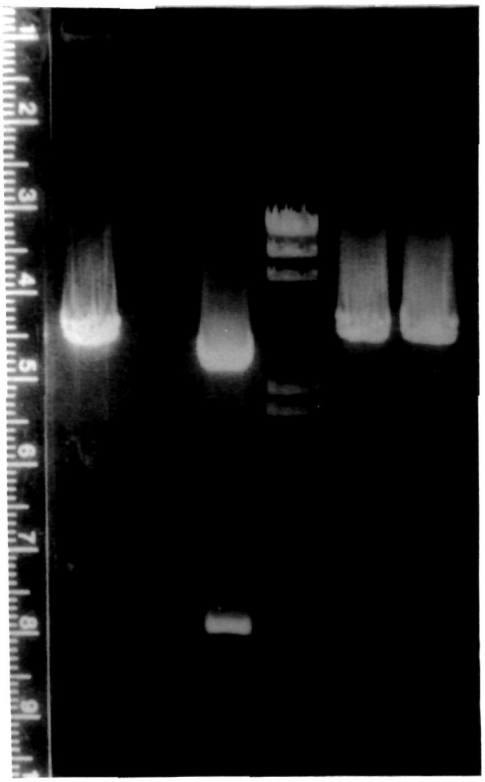


Fig.6m

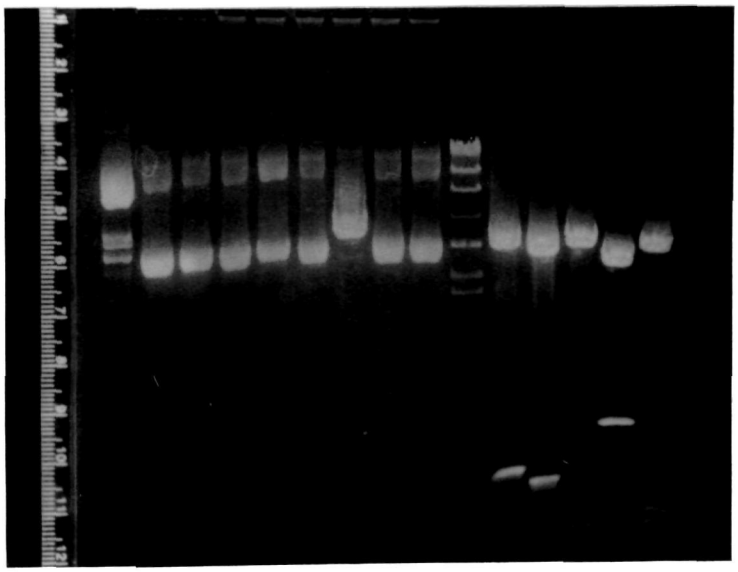


Plate XIX

Sequencing gel electrophoresis of pJM27.

Fig. 6n : The order of the lanes is G, A, T and C. First four lanes are long run of sequencing reaction using Sp6 sequencing primer. The next four lanes are short run of sequencing reaction using Sp6 sequencing primer. The next four lanes are long run of sequencing reaction using T7 sequencing primer. The last four lanes are short run of sequencing reaction using T7 sequencing primer.

Fig.6n

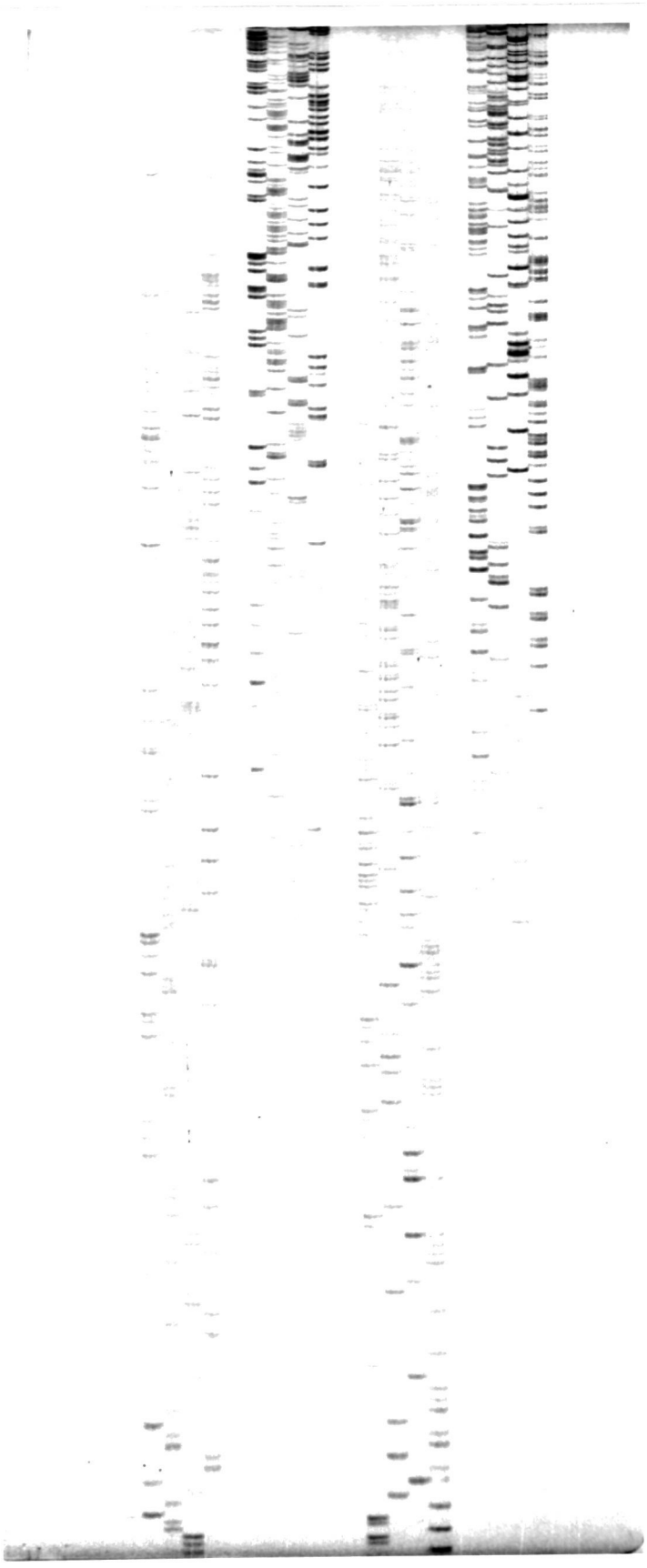


Plate XX

Fig. 6o : Phylogenetic tree generated by DNA parsimony analysis of the 5′ sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
KBPPC2	*Kalanchoe blossfeldiana*	CAM	Gehrig *et al*, 1995
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC2	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989c
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989

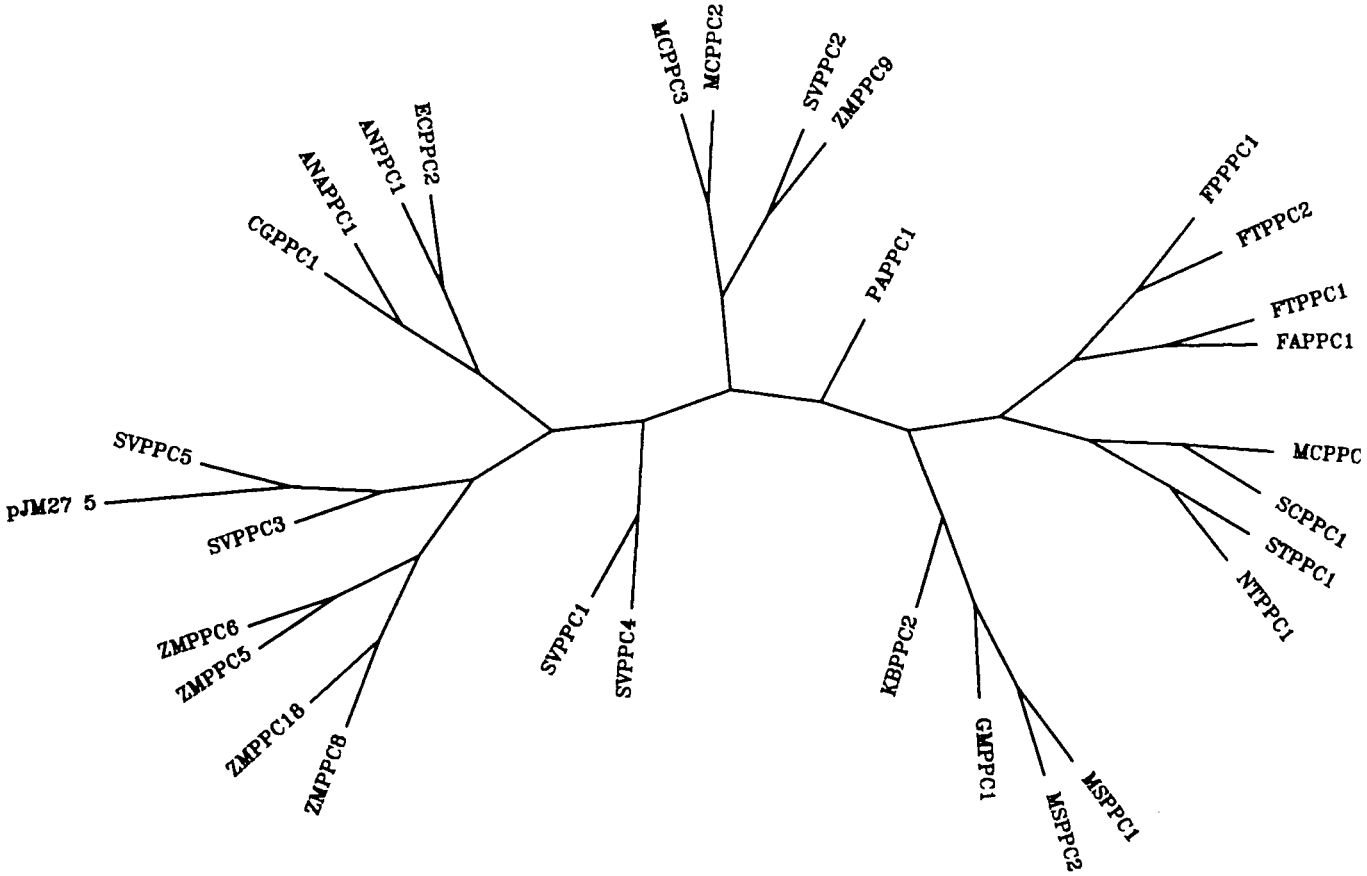
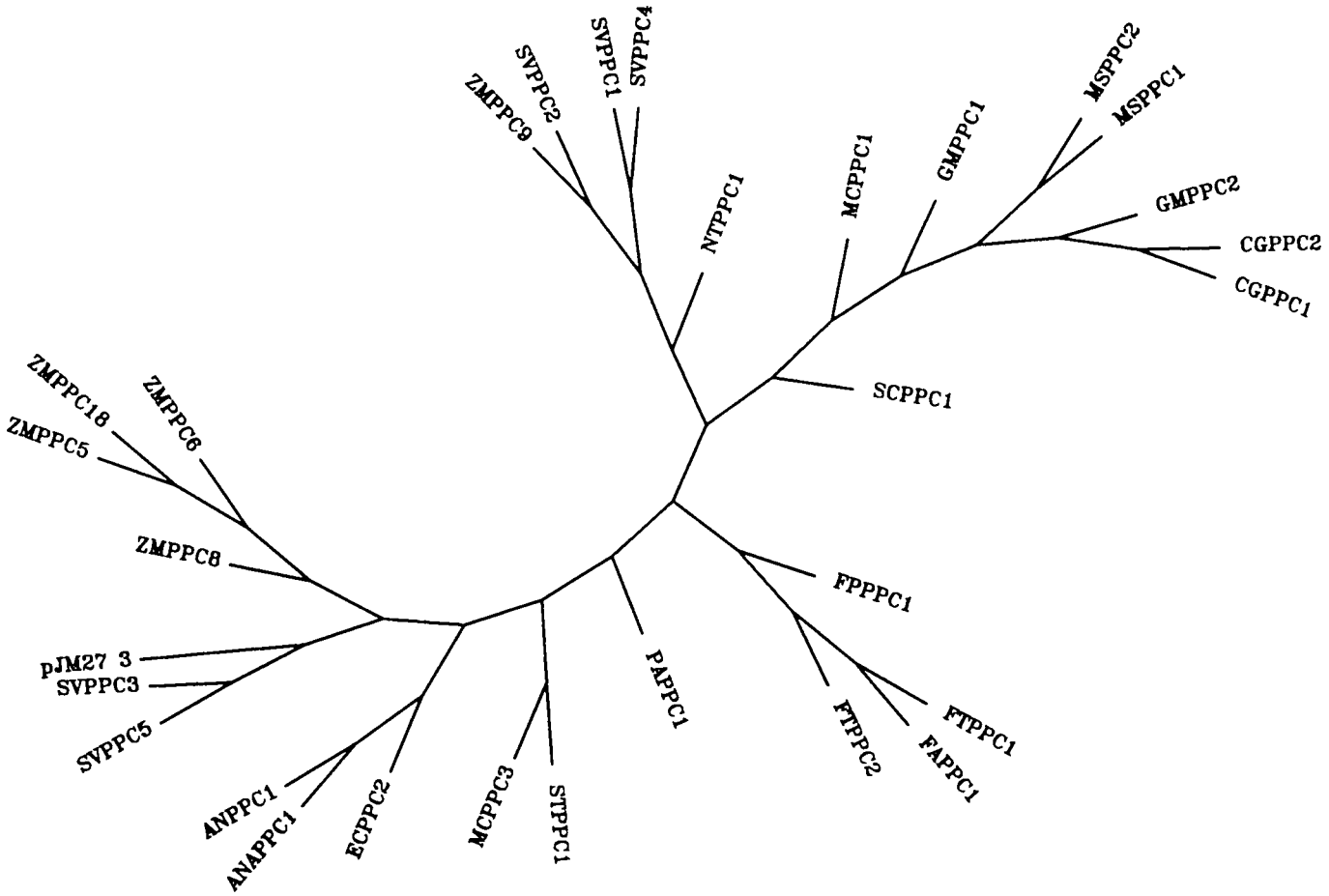

MCPPC3
MCPPC2
SVPPC2
ZMPPC9
ECPPC2
ANPPC1
ANAPPC1
CGPPC1
PAPPC1
FPPPC1
FTPPC2
FTPPC1
FAPPC1
SVPPC5
pJM27 5
SVPPC3
MCPPC
SCPPC1
STPPC1
NTPPC1
ZMPPC6
ZMPPC5
ZMPPC18
ZMPPC8
SVPPC1
SVPPC4
KBPPC2
GMPPC1
MSPPC2
MSPPC1

Plate XXI

Fig. 6p : Phylogenetic tree generated by DNA parsimony analysis of the 3′ sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
CGPPC2	*Corynebacterium glutamicum*	procaryotic	O'Regan *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
GMPPC2	*Glycine max*	C_3-dicot	Tello *et al*, 1993
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989


ZMPPC9
SVPPC2
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SVPPC4
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GMPPC1
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MSPPC1
GMPPC2
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CGPPC1
SCPPC1
ZMPPC18
ZMPPC6
ZMPPC5
ZMPPC8
FPPPC1
pJM27 3
SVPPC3
SVPPC5
PAPPC1
FTPPC1
FTPPC2
FAPPC1
ANPPC1
ANAPPC1
ECPPC2
MCPPC3
STPPC1

Plate XXII

Fig. 6q : Phylogenetic tree generated by Neibour-joining analysis of 5′ sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
KBPPC2	*Kalanchoe blossfeldiana*	CAM	Gehrig *et al*, 1995
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC2	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989c
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989

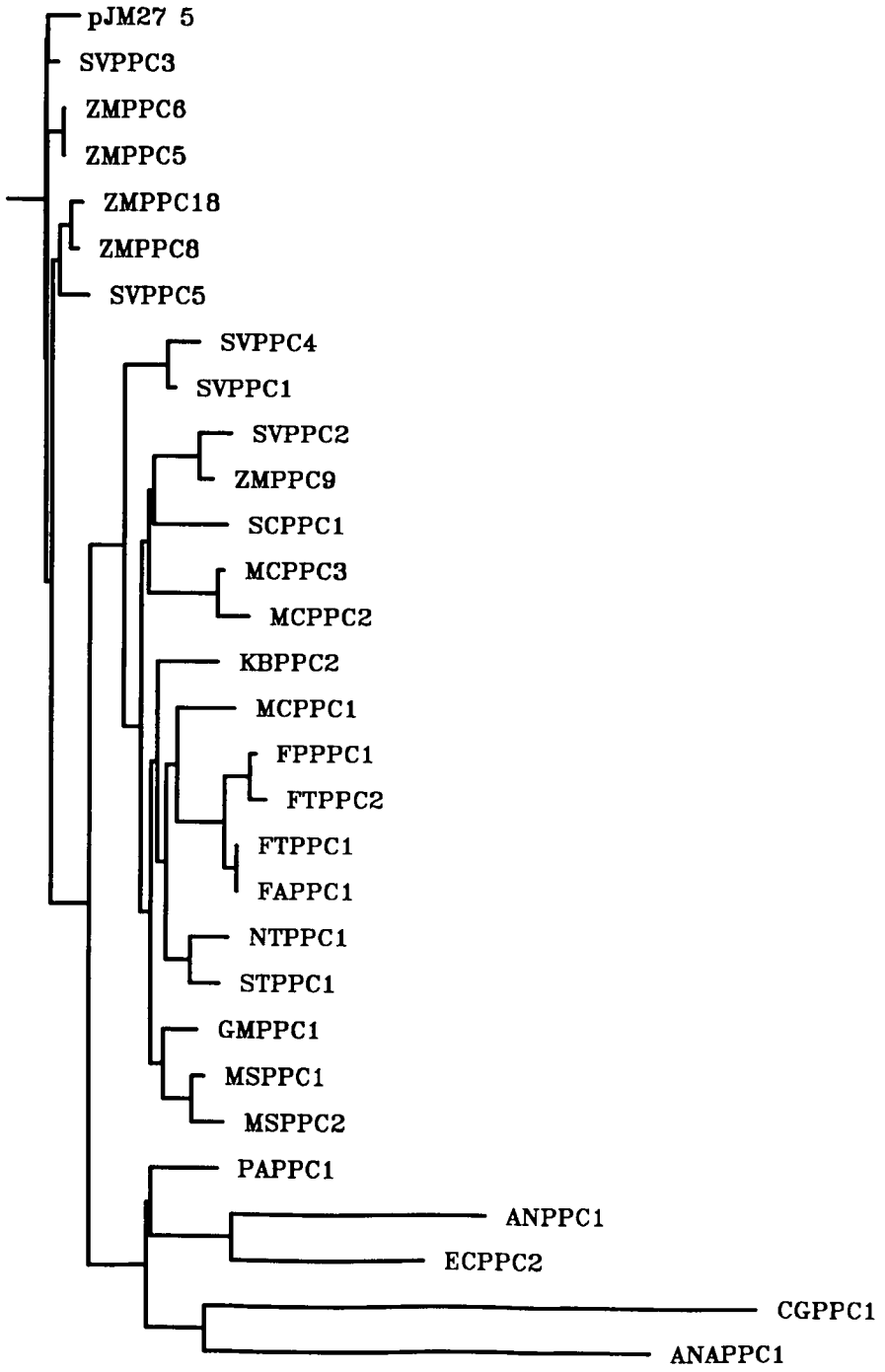

pJM27 5
SVPPC3
ZMPPC6
ZMPPC5
ZMPPC18
ZMPPC8
SVPPC5
SVPPC4
SVPPC1
SVPPC2
ZMPPC9
SCPPC1
MCPPC3
MCPPC2
KBPPC2
MCPPC1
FPPPC1
FTPPC2
FTPPC1
FAPPC1
NTPPC1
STPPC1
GMPPC1
MSPPC1
MSPPC2
PAPPC1
ANPPC1
ECPPC2
CGPPC1
ANAPPC1

Plate XXIII

Fig. 6r : Phylogenetic tree generated by Neibour-joining analysis of 3' sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
CGPPC2	*Corynebacterium glutamicum*	procaryotic	O'Regan *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
GMPPC2	*Glycine max*	C_3-dicot	Tello *et al*, 1993
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989

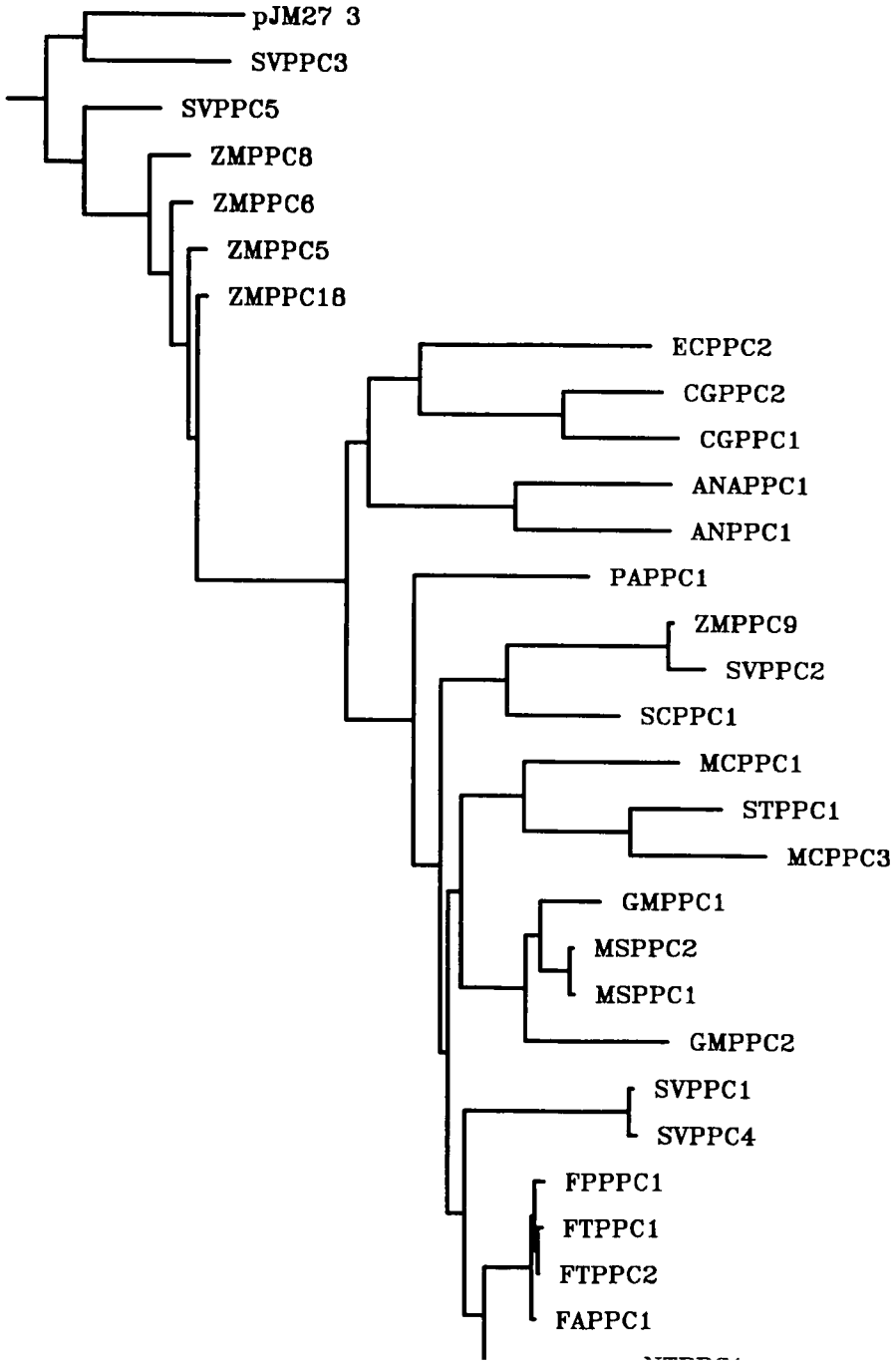

pJM27 3
SVPPC3
SVPPC5
ZMPPC8
ZMPPC6
ZMPPC5
ZMPPC18
ECPPC2
CGPPC2
CGPPC1
ANAPPC1
ANPPC1
PAPPC1
ZMPPC9
SVPPC2
SCPPC1
MCPPC1
STPPC1
MCPPC3
GMPPC1
MSPPC2
MSPPC1
GMPPC2
SVPPC1
SVPPC4
FPPPC1
FTPPC1
FTPPC2
FAPPC1

Plate XXIV

Fig. 6s : phylogenetic tree generated by Fitch-Margoliash analysis of 5′ sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
KBPPC2	*Kalanchoe blossfeldiana*	CAM	Gehrig *et al*, 1995
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC2	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989c
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989

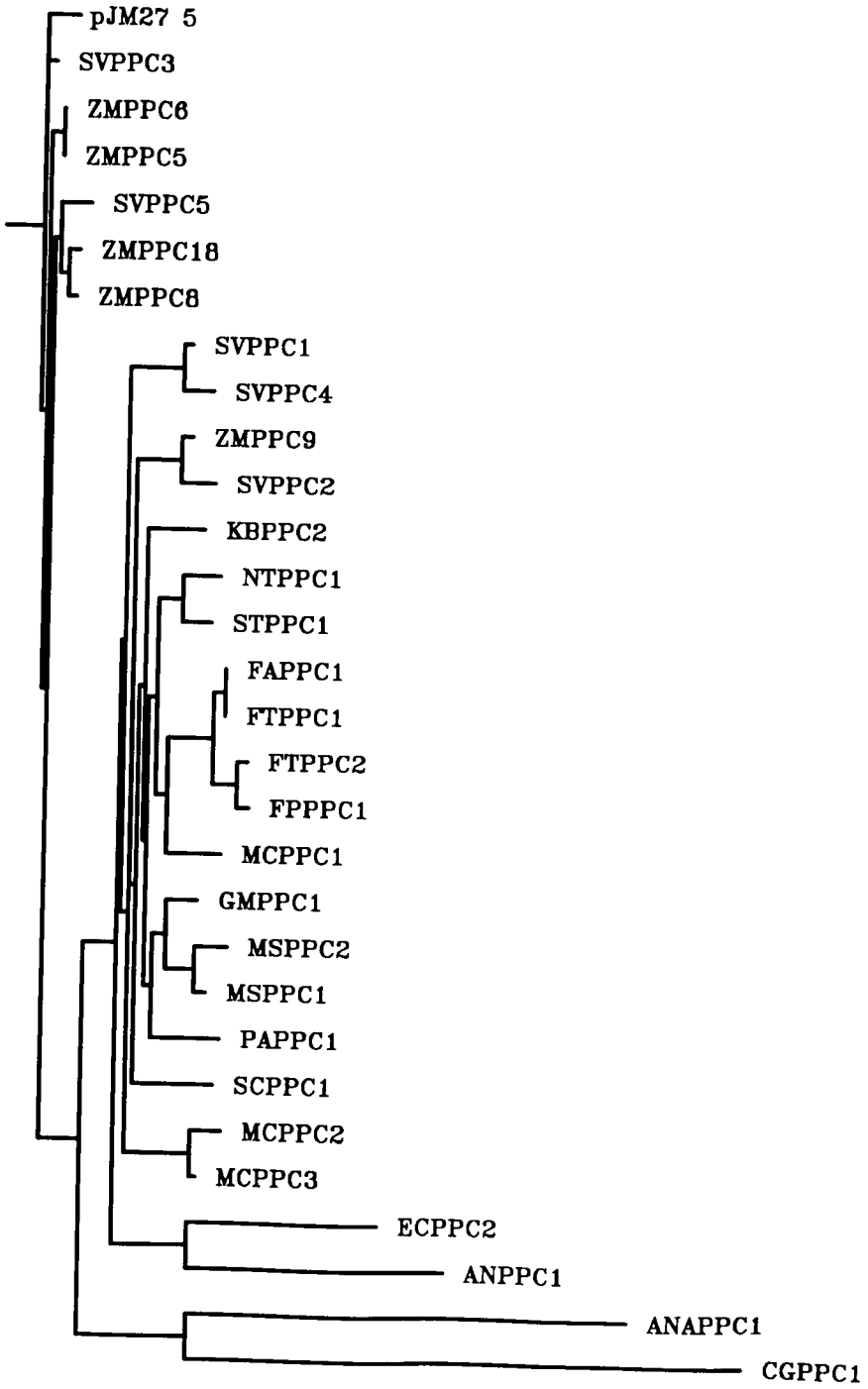
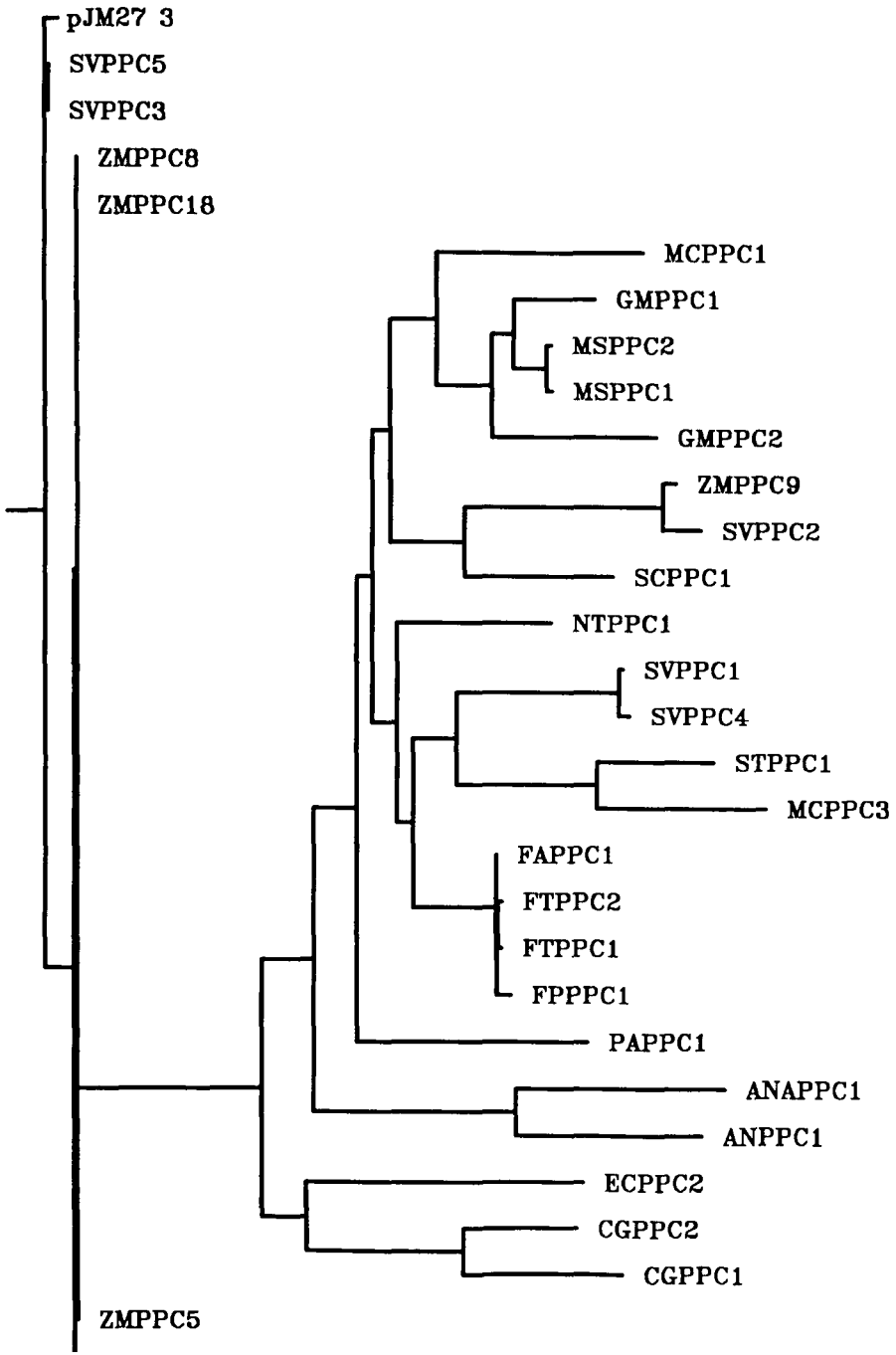

pJM27 5
SVPPC3
ZMPPC6
ZMPPC5
SVPPC5
ZMPPC18
ZMPPC8
SVPPC1
SVPPC4
ZMPPC9
SVPPC2
KBPPC2
NTPPC1
STPPC1
FAPPC1
FTPPC1
FTPPC2
FPPPC1
MCPPC1
GMPPC1
MSPPC2
MSPPC1
PAPPC1
SCPPC1
MCPPC2
MCPPC3
ECPPC2
ANPPC1
ANAPPC1
CGPPC1

Plate XXV

Fig. 6t : Phylogenetic tree generated by Fitch-Margoliash analysis of 3′ sequences of pJM27.

Name	**Organism**	**Type**	**Authors**
ANAPPC1	*Anabaena variabilis*	procaryotic	Luinenberg and Coleman, 1992
ANPPC1	*Anacystis nidulans*	procaryotic	Katagiri *et al*, 1985
CGPPC1	*Corynebacterium glutamicum*	procaryotic	Eikmanns *et al*, 1989
CGPPC2	*Corynebacterium glutamicum*	procaryotic	O'Regan *et al*, 1989
ECPPC2	*E. coli*	procaryotic	Fujita *et al*, 1984
FAPPC1	*Flaveria australasica*	C_4-dicot	Bauwe, 1993
FPPPC1	*Flaveria pringlei*	C_3-C_4-dicot	Hermans and Westhoff, 1992
FTPPC1	*Flaveria trinervia*	C_4-dicot	Poetsch *et al*, 1991
FTPPC2	*Flaveria trinervia*	C_3-C_4-dicot	Hermans and Westhoff, 1992
GMPPC1	*Glycine max*	C_3-dicot	Sugimoto *et al*, 1992
GMPPC2	*Glycine max*	C_3-dicot	Tello *et al*, 1993
MCPPC1	*Messembryanthemum crystallinum*	C_3	Cushman and Bohnert, 1989a
MCPPC3	*Messembryanthemum crystallinum*	CAM	Cushman and Bohnert, 1989b
MSPPC1	*Medicago sativa*	C_3-dicot	Pathariana *et al*, 1992
MSPPC2	*Medicago sativa*	C_3-dicot	Pathariana and Gantt, 1996
NTPPC1	*Nicotiana tabacum*	C_3-dicot	Koizumi *et al*, 1991
PAPPC1	*Picea abies*	Gymnosperm	Relle and Wild, 1994
SCPPC1	*Saccharum* hybrid	C_3-monocot	Henrik *et al*, 1992
STPPC1	*Solanum tuberosum*	C_3-dicot	Merkelbach *et al*, 1993
SVPPC1	*Sorghum vulgare*	C_3-monocot	Cretin *et al*, 1991
SVPPC2	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1991
SVPPC3	*Sorghum vulgare*	C_4-monocot	Cretin *et al*, 1991
SVPPC4	*Sorghum vulgare*	C_3-monocot	Lepiniec *et al*, 1993
SVPPC5	*Sorghum vulgare*	C_4-monocot	Lepiniec *et al*, 1992
ZMPPC5	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989
ZMPPC6	*Zea mays*	C_4-monocot	Izui *et al*, 1986
ZMPPC8	*Zea mays*	C_4-monocot	Matsuoka and Minami, 1989
ZMPPC9	*Zea mays*	C_3-monocot	Kawamura *et al*, 1992
ZMPPC18	*Zea mays*	C_4-monocot	Hudspeth and Grula, 1989


pJM27 3
SVPPC5
SVPPC3
ZMPPC8
ZMPPC18
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GMPPC1
MSPPC2
MSPPC1
GMPPC2
ZMPPC9
SVPPC2
SCPPC1
NTPPC1
SVPPC1
SVPPC4
STPPC1
MCPPC3
FAPPC1
FTPPC2
FTPPC1
FPPPC1
PAPPC1
ANAPPC1
ANPPC1
ECPPC2
CGPPC2
CGPPC1
ZMPPC5

Conclusions

The scope of the thesis covers the following three aspects :

1. Tissue culture,

2. Biochemistry and

3. Molecular biology.

The significant findings of this work are as follows :

Tissue culture :

Sugarcane (*Saccharum officinarium* cv CO 740) cultures were raised in the presence and absence of 5-AzaC. We observed that 5-AzaC was highly cyto-toxic at concentrations above 40 μM. However, sugarcane gave the optimum response to callus growth and differentiation on media supplemented with 15 μM 5-AzaC. Brown *et al* (1989) reported that 5-AzaC concentration higher than 10 μM was cyto-toxic to maize tissue culture. These authors also reported that whereas the surface area of the calli formed increased under the influence of 5-AzaC, the callus does not differentiate. They concluded that 5-AzaC has no role to play in tissue culture.

On the other hand, our observations with sugarcane suggest that not only does 15 μM 5-AzaC treatment result in higher amounts of callus formed, but there is also onset of differentiation in the callus initiation medium itself. As far as the number of shoots and the length of the shoots are concerned, we found that 5-AzaC at 15

μM concentration gave a better response than the cultures grown in control medium.

Biochemistry :

5-AzaC treatment leads to an overall proliferation of total soluble content in both mesophyll and bundle sheath strands of sugarcane. The total soluble proteins increase much more in dark grown 5-AzaC plants, than the plants grown in continuous light. This is in agreement with earlier reports to the same effect.

One of the aims of the present thesis was to study the effect of 5-AzaC on the differential expression and light regulation of C_4-photosynthetic genes from sugarcane. Contrary to the suggestion of Ngernprasirtsiri *et al*, (1989), the differential expression of PEPC, PPDK and NADP-ME genes in sugarcane on 5-AzaC treatment was not abolished. However, in the case of PEPC and PPDK, there was an increase in enzyme activity at the 'right' place.

Under long-term 5-AzaC treatment, there was an enhancement of PEPC expression in mesophyll cells on day 15 of subculture reflected by increased PEPC abundance, it, however, does not result in concomitant increase in the PEPC activity. This could result due to improper folding of the PEPC protein so that it does not form proper quaternary structure. It could also be due to non aggregation of the PEPC subunits, or the non phosphorylation of serine residue in the active site of PEPC protein. It could result from any one or a combination of the above factors.

So far as the PPDK activity is concerned, the enzyme activity increases only under the influence of long-term 5-AzaC treatment,

both in light and dark. Short-term 5-AzaC treatment does not influence PPDK activity.

NADP-ME activity was not found to be influenced by 5-AzaC treatment. NADP-ME activity was found to be higher in short-term 5-AzaC treated plants grown under the conditions of constant darkness.

Hence 5-AzaC treatment, or hypomethylation of C-residues is not a panaceae for general gene activation in plants.

Molecular Biology :

We have made a cDNA library from sugarcane leaves.About 40 putative PEPC cDNA clones were screened. One of these, pJM27 has been partially sequenced from both the 5′ and the 3′ ends. pJM27 is about 1.2 kb in size. The sequence alignments and phylogenetic studies suggest that it is a C_4 PEPC clone. Ours is the first report of *ppc* cDNA clone from sugarcane. The only other PEPC sequence reported from sugarcane is the housekeeping C_3-PEPC (Henrik *et al*, 1992). Genomic Southern reveal PEPC to be a multigene family.

Using the partial sequence of pJM27 from either ends, we have generated phylogenetic trees which shows :

- The procaryotic PEPC sequences form a separate cluster.
- Angiosperms are monophyletic.
- The monocot C_4 PEPC sequences form a separate cluster.
- The dicot C_4 PEPC sequences from *Flaveria* sp. form a separate cluster indicating that the dicot C_4 PEPC sequences evolved separately.

- Significantly, the lone gymnosperm PEPC from *P. abies* branches out independently and in the distance phylogeny analysis, it lies closest to the procaryotic PEPC sequences. This is in agreement with reports that gymnosperms evolved much earlier than angiosperms (Barnabas *et al*, 1995).

- pJM27 branches out with sorghum and maize C_4 PEPC genes.

- Procaryotic PEPC is the most distant ancestor of pJM27.

- Hence it was proved that C_4 PEPC is most recently evolved.

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