NUCLEAR-PLASTIDIC INTERACTIONS IN *Pennisetum glaucum* (L.) R. Br: REGULATION OF CHLOROPHYLL BIOSYNTHESIS, GENE EXPRESSION AND ORGANISATION

A Thesis Submitted

for the degree of
Doctor of Philosophy

K. KARUNASRI

School of Life Sciences
University of Hyderabad
Hyderabad- 500 046 India

FEBRUARY 1996

Enrolment Number: OL-5095

S T A T E M E N T

I hereby state that the work presented in this thesis entitled "Nuclear-plastidic interactions in *Pennisetum glaucum* (L.) R. Br: Regulation of chlorophyll biosynthesis, gene expression and organization" has been carried out by me under the supervision of Prof. N.C. Subrahmanyam, School of Life Sciences, University of Hyderabad, Hyderabad - 500 046, India, and that this work has not been submitted for any degree or diploma of any other university earlier.

K. Karunasri

(K. Karunasri)

Subrahmanyam 9.2.96

Prof. N.C. Subrahmanyam, FNA, FNAAS.FASC.
School of Life Sciences
University of Hyderabad.

C E R T I F I C A T E

This is to certify that Ms. K. Karunasri, has carried out the research work embodied in the present thesis under my supervision and guidance for the full period prescribed under the Ph.D. ordinance of this university. I recommend her thesis entitled **"Nuclear-plastidic** interactions in *Pennisetum glaucum* (L.) R. Br: Regulation of chlorophyll biosynthesis, gene expression and organization", for submission for the degree of Doctor of Philosophy of this University.

Subrahmanyam 9.2.96

Prof. N.C. Subrahmanyam
FNA, FAAS, FASc
School of Life Sciences
University of Hyderabad

Dean
School of Life Sciences
University of Hyderabad

A C K N O W L E D G E M E N T S

I have no words to express my profound gratitude to Prof. N.C. Subrahmanyam, *FNA, FAAS, FASc.* guide and supervisor of this work. I am indebted to him for his valuable and timely suggestions, discussion and help in successful completion of this work.

I am grateful to Prof. K.K. Tewari, Director, ICGEB, New Delhi for encouraging me and extending the facilities to carryout part of this work at ICGEB.

I am indebted to Dr. M.K. Reddy, a scientist at ICGEB for his continued interest, timely help and moral support in conducting the experiments at ICGEB inspite of his busy schedule.

I thank Prof. A.R. Reddy, Dean School of Life Sciences, University of Hyderabad for the facilities provided to me during the course of my Ph.D.

I thank Prof. V.S. Rama Das *FNA,FASc,FAAS* for allowing me to utilise the low temperature fluorescence spectroscopy facility.

My colleagues Dr. Rajeswari Ramanan, Dr. Nagesh, Mr. Kranthi Kumar, Mr. George Thomas, Ms. Sudha, Ms. Leela and Dr. M.V. Sailaja take their due credit for the help rendered to me.

I also thank Mr. Laxman and Mr. Anthaiah for their devoted assistance and skillful maintenance of the valuable genetic stocks used in the study.

Support of technical staff of School of Life Sciences, University of Hyderabad is greatly acknowledged.

My thanks to all the faculty members in the School of Life Sciences who have helped me.

Special thanks are to Drs. Navin Khanna, **Sunil** Mukherjee and Suresh Nair for their cooperation and kind help extended to me during my stay at **ICGEB.**

I appreciate and **acknowledge,** Mr. Kedar Singh and Mr. Chandan Singh of PHB, ICGEB for their assistance in conducting experiments.

Unaccountable moral support and help rendered by my **friends,** Dr.M.V. Sailaja, P. **Padma,** Dr.A.S. Sailaja, Dr.S. Rama Devi, Ms.R. Suguna and **Mr.C.S.S.** Kasyapa are highly acknowledged.

I thank Mr K.Srinivas and Mr David **Raju** for typing this manuscript.

I am grateful to all the members of my family for their encouragement, continued support and understanding without which this would have remained a dream.

I appreciate the concern, countenance and endurance of my husband.

K. KARUNASRI

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

Δ-ALA	Delta aminolevulinic acid
BME	β-mercapto ethanol
Cab	Chlorophyll a/b binding
CHISAM	Chloroform isoamyl alcohol
Chlide	Chlorophyllide
CS	Chlorophyll synthetase
DAPI	4,6-Diamidino-2-phenylindole
dATP	deoxy Adenosine triphosphate
dCTP	deoxy Cytosine triphosphate
DEPC	Diethylpyrocarbonate
dGTP	deoxy Guanosine triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dTTP	deoxy Thymidine triphosphate
EDTA	Ethylene diamine tetra acetic acid
FNR	Ferredoxin oxidoreductase
HEPES	N-2-hydroxyethyl, piperazine-N-2-ethane sulfonic acid
kb	Kilobase
kD	Kilo daltons
LSU	Large subunit of Rubisco
MES	2-Morpholino ethanesulfonic acid
MOPS	Morphilino propanesulfonic acid
Pchlide	Protochlorophyllide
PCR	Polymerase chain reaction
PCOR	Protochlorophyllide oxidoreductase
PMSF	Phenylmethylsulfonylfluoride
rbcL	gene coding for large subunit of Rubisco
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rpo A	gene coding for α-subunit of RNA polymerase
rpo B	gene coding for β-subunit of RNA polymerase
Rubisco	Ribulose bisphosphate carboxylase oxygenase

SDS	Sodium dodecylsulfate
SSC	Sodium saline citrate
SSU	Small subunit of Rubisco
STE	Sodium Tris-EDTA
TAE	Tris acetate EDTA
TBE	Tris borate EDTA
TCA	Trichloro acetic acid
TE	Tris EDTA buffer
Tris	Tris (hydroxy methyl) amino methane
UV	Ultraviolet

ABSTRACT

Coordinated expression of plastid and nuclear genomes determines the developmental status of the plastid (Taylor 1989). A mutation either in plastid genome or in nuclear genome leads to significant changes in the plastid phenotype (Kirk and Tilney-Bassett 1978). Following mutation, normal and mutant plastids sort out from one another during successive cell divisions of heteroplastidic cells in a regular manner to produce variegated plants (Kirk and Tilney Bassett 1978). Several nuclear gene induced plastid mutations are known in pearl millet Subrahmanyam et *al* (1986) demonstrated a pattern dependent maternal plastid inheritance.

Several nuclear induced plastid mutants of higher plants and algae are known to block different steps in the chlorophyll biosynthesis and affect the development of photosynthetic apparatus (Kirk and Tilney-Bassett 1978). Direct evidence for the role of nuclear genes in the regulation of plastid development comes from the study of mutants with defects in chloroplast biogenesis such as structural organization of thylakoid membranes (Martienssen et *al* 1987), assembly of multiprotein complexes (Rochaix and Erickson 1988), plastid transcription (Jenssen *et al* 1986), plastid RNA processing and translation (Taylor 1989). An understanding of the control of plastid gene expression will be useful to decipher the mechanisms coordinating the expression of the plastid and nuclear genes to developmental signals.

Chlorophyll deficient mutants controlled by independent loci are well characterised in pearl millet (Karunasri and Subrahmanyam, 1994). Normal and chlorophyll-deficient phenotypes with the same genotype under identical growth conditions and chlorophyll-deficient (yellow/white) phenotypes differing in their genotypes with respect to one or more independent loci can be readily produced from the appropriate variegated (stripe) plants. The present study was undertaken to: 1) identify the nature of block (s) in chlorophyll biosynthesis among chlorophyll-deficient phenotypes 2) study the regulation of gene expression in normal and chlorophyll-deficient siblings among different genotypes and 3) compare the plastid genome organization among chlorophyll deficient mutants.

Low temperature (77 K) fluorescence spectroscopy was employed to identify the blocks in the chlorophyll biosynthetic pathway. The green seedlings of all the accessions exhibited fluorescence emission maxima at 657, 685 and 733 nm at an excitation wavelength of 438 nm. The 657 emission peak corresponds to a spectral form of protochlorophyllide (Pchlide) (Lebdev et *al* 1985; Boddi et *al*1992), while the 678 emission maximum is of chlorophyllide (Chlide). The F_{685} emission peak corresponds to chlorophyll fluorescence from photosystem II (PSII) and F from LHCPI of photosystem I (PSI). The yellow seedlings showed emission maxima at 657 and 678 nm and the white seedlings displayed single peak with emission maximum at 657. The proposed block in the chlorophyll biosynthetic pathway of white seedlings is in the conversion of Pchlide to Chlide, whereas in yellow seedlings the

conversion of Chlide to chlorophyll is impaired. It is proposed that *vi* and *vi* control the reduction of Pchlide to Chlide while the conversion of Chlide to chlorophyll is controlled by vi_1 and *vi*. loci.

The overall transcription of plastid genome was low in the chlorophyll-deficient siblings. Northern analysis revealed aberrant transcripts for plastid encoded RNA polymerase (RNAP-P) genes (*rpoA*, *rpoB)* in yellow and white seedlings differing in their abundance compared to the normal green seedlings. The transcript abundance and size varied among different yellow and white seedlings. The 16s rRNA, transcripts of *rbcL* and *psbA* were absent in yellow and white seedlings unlike the *rpoA* and *rpoB* transcripts suggesting a differential transcription of plastid genes in chlorophyll-deficient seedlings. Abundance of the aberrant transcripts could be a consequence of altered transcriptional levels and/or impaired post-transcriptional and processing in the absence of plastid translational machinery. The abundant levels of aberrant transcripts of plastid *rpo* genes indicate that RNA polymerase activity is of nuclear origin. The plastid encoded large subunit (LSU) of Rubisco, nuclear encoded LHCP-II of PS II and ferredoxin oxidoreductase (FNR) were detected following immunoblotting with polyclonal antibodies raised against the respective proteins. The LSU was present in all green seedlings irrespective of their genotype while it was absent in yellow and white seedlings. Four subunits of LHCP-II with molecular sizes 3 0 kD; 28 kD; 27 kD and 24 kD were found in green seedlings. Yellow seedlings showed 27 kD and 24 kD polypeptides

while the white seedlings displayed 28 kD polypeptide only. FNR was detectable in all green and yellow seedlings but was absent in all white seedlings. FNR catalyzes the reduction of plastid specific NADP to NADPH, an essential compound of the ternary complex (PCOR-NADPH-Pchlide) for the reduction of Pchlide to Chlide. It is thus proposed that *vi* or vi. control FNR synthesis.

Southern blot of DNA from green and yellow/white seedlings from stripe mutants were hybridized with plastid encoded 16 rDNA, *rbcL*, *rpoA* and *rpoB* probes from rice. With 16s rDNA probe Bgl I digests accessions showed a 2.9 kb prominent band in all the genotypes irrespective of phenotypes while GWS 14 white showed 2-additional bands of 1.5 kb and 1.2 kb. Bam HI digests revealed 2 more (3.2 kb and 0.4 kb) bands of equal intensity. No differences were observed between normal and yellow siblings within yellow stripe mutants whereas considerable differences were found between white stripe mutants VCM 36 and GWS 14.

Hybridization of Bgl I digests with *rbcL* probe in all the accessions revealed 12.3 kb fragment. IP 5009 and GWS 14 showed an additional band corresponding to 10.1 kb. Bam HI and Bgl I + Bam HI double digests showed two bands corresponding to 6.2 kb and 3.3 kb differing in their signal intensity in IP 9712 and VCM 36. The 3.3 kb fragment was absent in the double digest of VCM 36 white, while 6.2 kb fragment was absent in Bam HI digest.

Bgl I digests probed with *rpoA* showed a single fragment (18.2 kb) suggesting no Bgl I site within the gene whereas Bam HI digests showed 1.5 kb and 1.3 kb fragments with the intensity of 1.3 kb fragment being 3-fold higher. Hybridization of Bgl I

digests with *rpoB* probe showed no differences between normal and mutant seedlings within each genotype, but differences between yellow stripe mutants IP 5009 and IP 9712 and between white stripe mutants VCM 36 and GWS 14 were found. Signal intensity among the bands varied between genotypes. Bam HI digests showed no differences between green and mutant seedlings within each genotype with the exception of VCM 36 white in which 0.5 kb fragment was absent.

These results indicate RFLPs in the *rpoB* and *16s rDNA* regions of the plastome among the different pearl millet genotypes.

INTRODUCTION

Coordinated expression of plastid and nuclear genomes determines the developmental status of the plastid. The fact that many nuclear mutants block chloroplast development has been interpreted as an argument for the regulation of chloroplast development by nuclear genes. Genetic information in the nucleus therefore ultimately regulates the expression of the plastid genome (Taylor 1989). A mutation either in plastid genome (Borner et al 1972, 1973; Rao 1974; Kirk and Tilney-Bassett 1978) or in nuclear genome (Ratnaswamy 1960; Miller 1968; Tilney-Bassett 1963, 1965, 1970a, b; Krishna Rao and Koduru 1978; Koduru and Rao 1980, 1983; Henny 1982; Appa Rao and Mengesha 1984; Subrahmanyam et al 1986; Reddy and Subrahmanyam 1988 a, b; Karunasri and Subrahmanyam 1994) leads to significant changes in the plastid phenotype.

Nuclear gene induced plastid mutations are elegantly summarised by Kirk and Tilney Bassett (1978). Following plastid mutation, normal and mutant plastids sort out from one another during successive cell divisions in a regular manner to produce variegated plants with defined striping pattern in leaves and shoots (Tilney-Bassett 1978) . Variegation due to plastid mutations shows non-Mendelian inheritance, thus exhibiting differences in reciprocal crosses as in *Pelargonium zonale* (Baur 1909) and *Mirabilis jalapa* (Correns 1909). Variegations due to mutable nuclear genes and nuclear gene controlled plastid mutations which follow Mendelian inheritance were reported in several plants *viz Arabidopsis* (Robbelen 1966; Redei 1973; Redei and Plurad 1973),

Capsella bursa (Sheel 1956), barley (So 1921; Imai 1928, 1935, 1936a, b; Borner et al 1976), *Oryza* (Pal and Ramanujam 1941), maize (Anderson 1923; Jenkins 1924; Demerec 1927; Rhoades 1943, 1946; Stroup 1970; Coe et al 1982), *Sorghum* (Karper and Conner 1931; Karper 1934; Miller 1968), *Epilobium* (Michaelis 1965) *Oenothera* (Epp 1973) *Petunia* (Potrykus 1970) and pearl millet (Ayyangar et *al* 1935; Kadam et *al* 1940; Gill et *al* 1969; Appa Rao and Mengesha 1984; Subrahmanyam et *al* 1986; Reddy and Subrahmanyam 1988a,b; Karunasri and Subrahmanyam 1994).

Plastid Inheritance

Plastids are known to be transmitted either maternally or biparentally (Kirk and Tilney-Bassett 1978). Biparental plastid transmission is characterized by the contribution of plastids from both parents. Biparental inheritance of plastid mutations was demonstrated in *Pelargonium zonale* (Baur 1909; Roth 1927; Imai 1936a; Tilney-Bassett 1963, 1965, 1970a,b). *Oenothera* (Scholtz 1954) and *Secale cereale* (Frost et *al* 1970).

Ayyangar et *al* (1935) reported albinism controlled by a recessive gene in pearl millet. Vinchon (1949) showed single recessive gene pair control for 8 of the 10 lethal chlorophyll deficiencies. Ratnaswamy (1960) reported a white stripe phenotype controlled by a recessive gene. Burton and Powell (1965) observed monogenic recessive inheritance in ten of the thirteen spontaneously occurring chlorophyll deficiencies and digenic recessive inheritance in the remaining.

Gill et al (1969) reported foliage striping of two mutants,

PYS7 and GYS8, in the presence of 3 complementary recessive nuclear genes while another stripe phenotype of GWS 14 is shown to be controlled by 3 different loci with duplicate, complementary and inhibitory type of gene interactions. Subsequent studies by Reddy and Subrahmanyam (1988a) on the same white stripe mutant (GWS 14) revealed the control of striping by the delayed expression of two independently assorting recessive genes. Krishna Rao and Koduru (1978) reported non-Mendelian biparental inheritance of the variegated phenotype in a white stripe mutant. Appa Rao and Mengesha (1984) isolated a yellow stripe mutant (IP 5009) controlled by a recessive gene. Subrahmanyam et al (1986) demonstrated a pattern dependent maternal plastid inheritance in the same mutant. On selfing, the variegated plants produce green, variegated and yellow or white progeny, proportions of which are dependent upon the phenotype of the parent. Heteroplastidic parental cells may give rise to homoplastidic egg cells with either normal or mutant plastids, leading to the development of green or mutant seedlings. Variegated offspring originate from heteroplastidic egg cells where sorting out is incomplete. Reddy and Subrahmanyam (1988b) further characterized and established the genetic basis of plastid alterations, the mode of transmission following intraplant, interspikelet and intergenotypic crosses of the stripe mutants. Further genetic analysis following intercrosses of different variegated lines of *Pennisetum glaucum* revealed at least 4 independent loci, any one of which in homozygous recessive condition leads to mutant phenotypes while the development of chlorophyll is accomplished by

the complementary interaction of dominant genes at these loci (Karunasri and Subrahmanyam 1994).

Ultrastructural and Molecular Characterization

Ultrastructural, biochemical and molecular studies in the nuclear gene controlled yellow stripe mutant of *Pennisetum glaucum* revealed the absence of chlorophyll (Subrahmanyam et *al* 1986), poor development of plastids and the absence of internal thylakoid membrane organization, plastid specific ribosomes and 23S and 16S ribosomal RNAs (Reddy et *al* 1988) indicating the lack of protein synthesizing machinery. Similar observations were made in *iojap* of maize (Walbot and Coe 1979) *albostrains* of barley (Borner et *al* 1976; Knoth and Hagemann 1977) and *Pelargonium zonale* (Borner et *al* 1972). Biochemical studies on pearl millet stripe mutant (Sujatha and Subrahmanyam 1991) revealed the presence of nuclear coded small subunit (SSU) and the absence of plastid coded large subunit (LSU)of the Rubisco in the yellow tissue and/or seedlings while no differences were observed with reference to PEPcase. Similar findings were reported in *iojap* mutants of maize (Siemenroth et *al* 1980), *albostrains* of barley (Borner et *al* 1976) and heat bleached ribosome-deficient plastids of rye (Fierabend and Wildner 1978) . Even in the absence of translational machinery in the plastid compartment, mutant plastids multiply during cell divisions indicating the presence of genetic material and utilization of the nuclear coded components required for its maintenance and continuity (Reddy and Subrahmanyam 1988a,b). These findings clearly establish the nuclear control over chloroplast

development. The lack of chlorophyll and the enzymes required for the Calvin cycle indicate the photosynthetic inability of these mutant plastids.

Chlorophyll synthesis **and plastid biogenesis**

Mutations that block chlorophyll synthesis have major effects on chloroplast biogenesis and thus are lethal. Moreover, these mutants have extensive pleiotropic effects in the expression of both nuclear and plastid genomes.

Von Wettstein and co-workers (1971) identified 20 different loci, all in the nuclear genome, at which mutations cause a yellow, chlorophyll-deficient phenotype in barley. Some mutants are blocked at specific steps in chlorophyll biosynthesis resulting in impaired chloroplast development at an early stage of internal membrane assembly. Mascia and Robertson (1978) described similar effects in maize mutants. The block in specific steps of chlorophyll synthesis is due to mutation leading to impairment in the synthesis and/or activity of an enzyme at a particular step. The lack of chloroplast membrane assembly may be a pleiotropic consequence of the absence of chlorophyll and/or the accumulation of chlorophyll precursors.

Several nuclear induced plastid mutants of higher plants and algae are known to block different steps in the chlorophyll biosynthesis (Kirk and Tilney Bassett 1978). Sager (1955) reported a Mendelian mutant of *Chlamydomonas reinhardtii* which had accumulated particularly protoporphyrin IX. A recessive Mendelian

mutant of *Arabidopsis thaliana* can form protochlorophyllide but is unable to photoreduce it to chlorophyll (Robbelen 1966). An albino mutant alb-f[17] and yellow mutant Xan-j[59] of barley were shown to photoreduce chlorophyllide, but were unable to phytylate the chlorophyllide to form chlorophyll-a (Von Wettstein et al 1971; Henningsen and Thorne 1973). Mutations which affect formation of the chlorophyll or carotenoids of the chloroplast also affect the synthesis of photosynthetic apparatus (Kirk and Tilney-Bassett 1978).

Plastid Genome Organisation

Most plastid genomes are circular and range in size from 120 to 217 kb (Palmer 1990). Variation in genome size is related primarily to the size of inverted repeat (IR) in the plastid genome and its coding capacity. Copy number per plastid ranges from 22 to 300 depending on the developmental stage. Complete sequence information in plastid genomes is available for tobacco (*Nicotiana tabacum*), liverwort (*Marchantia polymorpha*), rice (*Oryza sativa)* (Shinozaki *et al* 1986; Ohyama *et al* 1986, Hiratsuka et al 1989), maize and several other plants (Maier et *al* 1995). The unique feature of chloroplast genome is the presence of inverted repeat segments (IR and IR), separated by a large and small single copy regions (LSC and SSC) respectively (Sugita et *al* 1984). Studies on a wide range of species led to the establishment of consensus chloroplast gene order among vascular land plants identical to that found in tobacco (Palmer 1990). Data available for the cereals wheat, maize and rice show that their chloroplast genomes have diverged from

the consensus gene order through a series of overlapping inversions within the LSC Hiratsuka et *al* 1989). Plastid DNA encodes approximately 135 genes that fall into three major functional categories: genes encoding proteins and RNAs involved **in** transcription and translation of the plastid genome (RNA polymerase subunits, tRNAs, rRNAs, ribosomal proteins, initiation factor 1), genes encoding proteins of the photosynthetic apparatus (Rubisco, PSI, PSII, ATP synthase, cytochrome b_6/f complex) and genes encoding proteins of the NADH oxidoreductase complex. Most plastid genes are organized in complex operons that are conserved among plastid genomes (Sugiura 1992; Mullet 1993) and their transcription plays a central role in establishing the levels of many plastid mRNAs and proteins. Variations in plastid mRNA stability also significantly influences plastid gene expression (Rapp et *al* 1992).

Coordinated expression of nuclear and plastid genomes

Both the nuclear and chloroplast genomes encode components of the photosynthetic apparatus. Plants are able to accurately coordinate the accumulation of chloroplast and nuclear encoded subunits through a complex regulatory hierarchy including control of transcription rates and mRNA accumulation (Taylor 1989). Light mainly controls the expression of the nuclear encoded photosynthetic genes such as small subunit of Rubisco and chlorophyll a/b binding protein of PSII (Link 1988; Gilmartin et *al* 1990; Klein and Mullet 1990). The chloroplast genes are regulated by light, mRNA splicing and stability (Gruissem et

a21988; Link 1988). Study of mutants offers an alternate approach to separate the individual contribution of light and chloroplast development on gene expression.

Direct evidence for the role of nuclear genes in the regulation of plastid development comes from the processes in chloroplast biogenesis such as structural organization of thylakoid membranes (Martienssen et *al* 1987), assembly of multiprotein complexes (Leto et *al* 1985; Rochaix and Erickson 1988), plastid transcription (Jenssen et *al* 1986), plastid RNA processing and translation (jenssen et *al* 1986; Kuchka et *al* 1988; Taylor et *al* 1987) . An understanding of the control of plastid gene expression will be useful to decipher the mechanisms coordinating the expression of the plastid and nuclear compartments to developmental signals.

In leaf cells of plants having photooxidatively damaged plastids due to either bleaching by norflurazon (Oelmuller and Mohr 1986; Burgess and Taylor 1987) or mutations that block the biosynthesis of protective pigments (Mayfield and Taylor 1984; Giuliano and Scolnik 1988) the *rbcS* and *cab* gene families are not expressed. It was observed that the expression of *rbcS* and *cab* gene families was either repressed or not activated in *albino* mutants of *Hordeum vulgare* bearing undifferentiated plastids (Hess et *al* 1992 a & b) . Furthermore in experiments using transgenic plants a strong correlation was found between the presence of green differentiated chloroplasts and the expression of reporter genes controlled by promoters of nuclear encoded chloroplast

proteins (Herrea-Estrella et *al* 1984; Simpson et *al* 1986; Stockhaus et al 1989). A control mechanism was postulated that triggers the expression of nuclear genes encoding chloroplast proteins in accordance with the developmental status of the plastids (Borner and Sears 1986; Taylor 1989; Rajasekhar 1991; Susek and Chory 1992). Convincing evidence for the functionally normal plastid as the origin of the signal comes from the genetic studies of different variegation/chlorophyll-deficient mutants of various plants. A comparative analysis of progeny composition in crosses between variegated and normal inbred lines of pearl millet (Reddy and Subrahmanyam 1988b) suggest that in egg cells with exclusively defective plastids, the plastids do not revert back inspite of acquiring a dominant allele from the pollen parent while in egg cells with a mixture of green and mutant plastids, the mutant plastids could develop into functional plastids under the influence of a dominant allele. This was further substantiated by genetic analysis of stripe mutants from seven different accessions of pearl millet (Karunasri and Subrahmanyam 1994). Han et *al* (1993) and Hess et *al* (1994) showed evidence for the existence of a plastid-derived signal affecting the transcription of light induced nuclear genes in the ribosome-deficient plastids of *albostrains* of barley.

SCOPE

The development and differentiation of plastids is controlled temporally and spatially by co-ordinate expression of nuclear genes for plastid proteins. The diversity of plastid types is

controlled by the developmental program of the plant suggesting thereby a significant flow of information between the nuclear and plastid compartments. Nuclear mutations, which affect plastid gene expression are of immense value in dissecting out the mechanisms controlling plastid development. Many of such mutants display striped (variegated) phenotypes such as *iojap* (ij) and chloroplast modifier (cm) of maize (Rhoades 1943, Stroup 1970), chloroplast mutator (chm) of *Arabidopsis* (Knoth and Hagemann 1977), *albostrains (as)* of barley (Redei 1973) and stripe mutants of pearl millet (Ratnaswamy 1960; Krishna Rao and Koduru, 1978; Appa Rao and Mengesha 1984; Subrahmanyam et *al* 1986). Plastid defects in mutants of maize and barley are relatively well characterized at the genetic, ultrastructural and biochemical levels. Nuclear mutations in these plants are controlled by recessive gene (s) and the plastids are maternally transmitted.

In pearl millet, Ratnaswamy (1960) reported a white stripe phenotype controlled by single recessive gene. Gill et *al* (1969) reported foliage striping of two mutants (PYS 7 and GYS 8) controlled by 3 complementary recessive nuclear genes and another stripe mutant (GWS 14) was shown to be controlled by 3 different loci with duplicate, complementary, and inhibitory type of gene interaction. Subsequent studies of Reddy and Subrahmanyam (1988a) on the GWS 14 stripe mutant revealed expression of striping controlled by two recessive genes.

A single recessive gene control of yellow striping in a mutant of IP 5009 was reported by Appa Rao and Mengesha (1984) .

Intraplant, interspikelet crosses in the same mutant unequivocally established a pattern dependent maternal plastid inheritance (Subrahmanyam *et al* 1986). Results from reciprocal crosses of stripe mutant (IP 5009) with normal inbred lines provided the genetic basis of plastid alterations, their mode of transmission (Reddy and Subrahmanyam 1988b). A homozygous recessive genotype leads to defective plastid development with variable penetrance and expressivity (Reddy and Subrahmanyam, 1988b). On crossing of stripe plants with pollen from normal inbreds, green and yellow progeny were obtained. Selfing stripe plants or crossing with its green sib produced yellow, stripe and green progeny suggesting that in egg cells with exclusively defective plastids, the plastids do not revert back inspite of acquiring a dominant allele from the pollen parent, while in egg cells with a mixture of green (normal) and yellow (defective) plastids, the yellow plastids could develop into functional plastids under the influence of a dominant allele. Pearl millet is an amenable system to design and obtain the normal and chlorophyll-deficient phenotypes at will without altering the genotype under similar growth conditions. Using appropriate stripe mutants it is possible to produce yellow or white seedlings differing in their genotypes with respect to one or more independent loci controlling their phenotypes. The persistence of altered plastids in homoplastidic egg cells(in yellow spikelets) and the development of defective plastids to normal in the presence of normal plastid in the same cell as in heteroplastidic egg cells (in stripe spikelets) on acquiring a dominant allele

from the pollen parent established the requirement of a chloroplast coded factor (s) for the expression of the dominant allele in bringing about the normal development of otherwise defective plastids. Karunasri and Subrahmanyam (1994) determined the allelic composition of different stripe mutants based on segregation patterns in the progenies from crosses between yellow stripe and white stripe mutants.

Ultrastructural and molecular characterization of plastids in these mutants (Reddy *et al* 1988 and Sujatha and Subrahmanyam 1991) revealed the absence of : (i) chlorophyll, (ii) thylakoid membrane organization, (iii) plastid coded large subunit of Rubisco, and (iv) plastid specific ribosomes and ribosomal RNA (23s and 16s) vis *a vis* defective plastid protein synthesizing machinery. Chlorophyll is an essential component of the major light harvesting complexes of PS I and PS II thylakoid membranes. The poor organization of thylakoid membranes in the ribosome deficient plastids could be due to the lack of chlorophyll.

By deploying this genetically simple and amenable system, the present study was undertaken (i) to identify the nature of block(s) in chlorophyll biosynthetic pathway among the chlorophyll-deficient phenotypes derived from different genotypes, (ii) to study the regulation of gene expression in relation to nuclear-plastidic interactions in plastid development in green and yellow or green and white siblings from the respective stripe parent and (iii) to compare the plastid genome organization among different chlorophyll deficient mutants.

MATERIALS AND METHODS

2.1. PLANT MATERIAL

Genotypes of *Pennisetum glaucum* (L.) R.Br. stripe mutants and their breeding behaviour (Table 1) are well characterised (Karunasri and Subrahmanyam 1994). Selfed seeds from the yellow stripe mutants of IP 5009 and IP 9712 (Fig. 1) and the white stripe mutants of GWS 14 and VCM 36 (Fig. 2) were sown in trays (bottom of Figs. 1 and 2) under glass house conditions (14 h light). Seven day old green and non-green (yellow or white) seedlings were used.

2.2 **FLUORESCENCE SPECTROSCOPY:**

Six day old seedlings grown in plastic trays were harvested 2-3 h after sunrise and the fluorescence spectra were recorded immediately since the harvesting of seedlings in dark resulted in inconsistencies. Seedlings approximately equal in size were used. For each set of measurements five samples were taken from green and yellow siblings of IP 5009, IP 9712 accessions. Likewise they were repeated with green and white seedlings of VCM 36, GWS 14 and *albino.* Fluorescence emission spectra were recorded at 77K with Hitachi fluorescence spectrofluorometer (model 4010). The excitation wavelength was 438 nm, with 5 nm excitation slit and 4 nm emission slit. The integration time was 0.18s. The mean spectrum was smoothened before recording.

2.3. **RNA EXTRACTION AND PURIFICATION**

Total RNA from 7-day old seedlings was extracted according to

Table 1: Accessions of *Pennisetum glaucum* (L.) R.Br. used, their phenotype, genotype and source.

Accession	Phenotype (Genotype)	Source
IP 5009	Green yellow stripe (vi_1 vi_1)*	Dr. S. Appa Rao and Dr. M.H. Mengesha, Genetic Resources Division, ICRISAT, Patancheru, A.P., 502 324, India.
IP 9712	Green yellow stripe (vi_2 vi_2)*	
VCM 36	Green white stripe (vi_3 vi_3 vi_4 vi_4)*	
GWS 14	Green white stripe (vi_3 vi_3 vi_4 vi_4)*	Prof. J.L. Minocha Genetics Dept. Punjab Agric. Univ. Ludhiana, India.
Albino	Albino seedlings (*al al*)**	Prof. N.C.Subrahmanyam, School of Life Sciences, University of Hyderabad Hyderabad-500 046, India

according to Karunasri and Subrahmanyam (1994).

**according to Reddy (198 6) .

Figure 1: Yellow stripe mutants of IP 5009 and IP 9712 and their respective progenies given below.

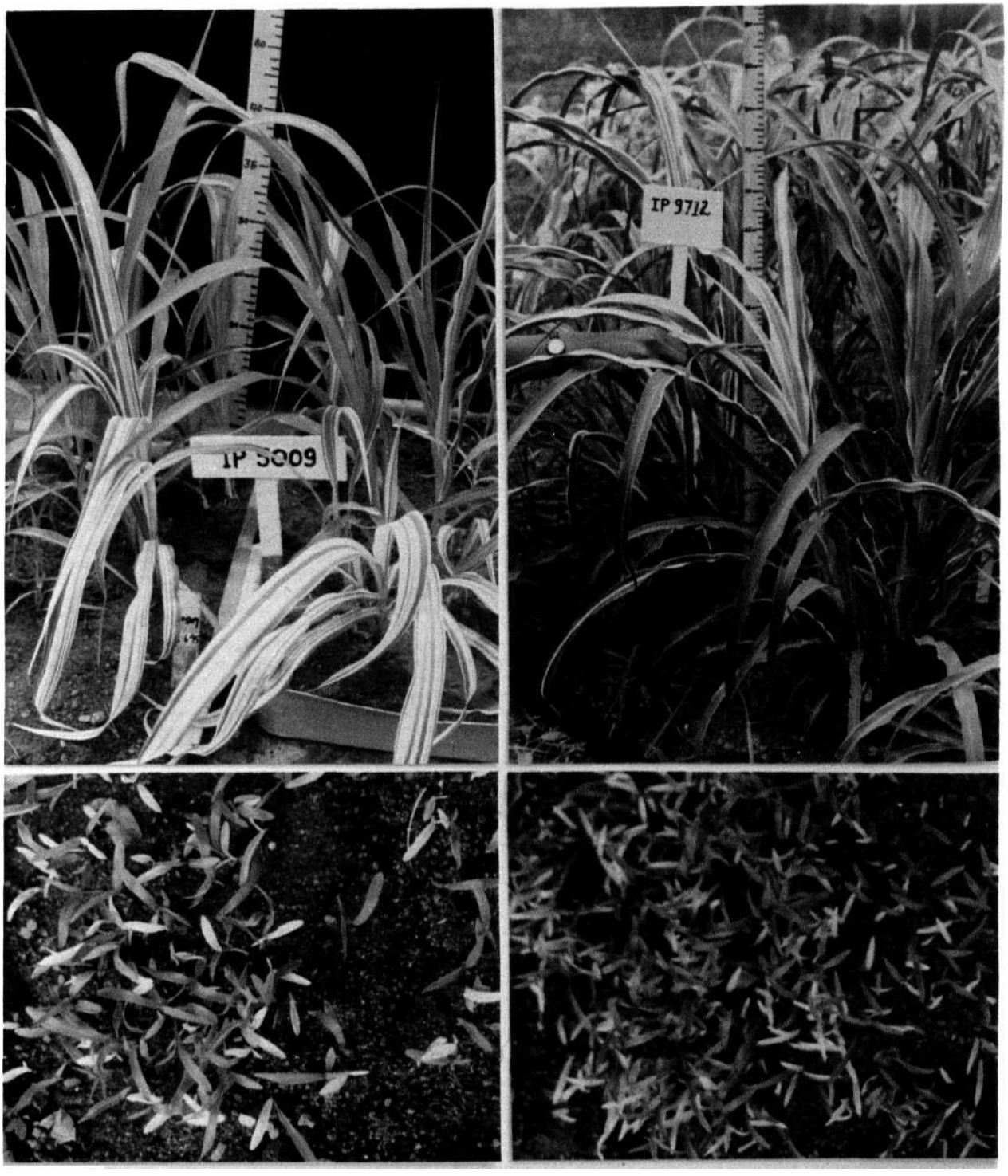


Fig. 1

Figure 2: White stripe mutants of VCM 36 and GWS 14 and their respective progenies given below.

Fig. 2

the STRATAGENE RNA isolation kit instruction manual. Care was taken to ensure that all glassware and solutions are free from nucleases by baking the glassware at 200°C for 6 h and preparing the solutions using DEPC (RNase inhibitor) treated water wherever permissible.

One gram of seedlings were quickly frozen in liquid N_2 and ground to a smooth powder using mortar and pestle. The contents were transferred immediately into a 50 mL polypropylene tube containing 10 mL denaturing solution (4M guanidinium isothiocyanate; 0.005M sodium citrate pH 7.0; 0.1M β-mercapto ethanol; 0.5% sarkosyl) and mixed well by shaking the tubes gently for 3 min at 25°C. To this 1 mL of 2M sodium acetate (pH 4.0) was added. After mixing thoroughly 10 mL of water saturated phenol was added and mixed thoroughly by inverting the tube. This was followed by the addition of 2 mL of CHISAM. The contents were shaken vigorously for 10 sec, chilled on ice for 15 min and centrifuged at 10,000 rpm in Sorvall SS-34 rotor for 20 min at 4 C. Aqueous phase was transferred to a fresh tube, mixed with an equal volume of isopropanol and was kept at -20°C for 1 h to precipitate RNA. The RNA was pelleted by centrifugation at 10,000 rpm in Sorvall SS-34 rotor for 20 min at 4°C. After decanting, RNA pellet was washed with chilled (-20 C) 70% ethanol, vacuum dried for 5 min and dissolved in DEPC water. After dissolving the RNA completely, an equal vol of 4M lithium chloride was added and kept on ice bath for 3 h to selectively precipitate high molecular weight RNA. Following centrifugation at 10,000 rpm in Sorvall

SS-34 rotor for 10 min at 4°C, RNA pellet was vacuum dried for 5 min and dissolved in DEPC water.

2.4 PLANT DNA EXTRACTION AND PURIFICATION

Total DNA from 7-day old seedlings (green and yellow/white) of stripe mutants IP 5009, IP 9712, VCM 36 and GWS 14 and an *albino* mutant were extracted according to the modified method of Sharp *et al* (1988). Seedlings were frozen and ground in liquid N_2 to a fine powder and 5 mL of lysis buffer (100mM Tris-HCl pH 8.5; 100mM NaCl; 50mM EDTA; 2% SDS) was added per gram of tissue and incubated at 37 C for 2 h with occasional stirring. An equal vol of phenol (buffer saturated with Tris-HCl pH 8.0): CHISAM (1:1) was added, stirred at low speed for 10 min and centrifuged at 10,000 rpm at 25°C in Sorvall SS-34 rotor for 20 min. To the supernatant 0.6 vol of isopropanol was added and gently mixed. DNA was spooled on to a glass rod and transferred to a fresh tube to which chilled (-20°C) 70% ethanol was added and recentrifuged at 10,000 rpm for 10 min. Ethanol was decanted, DNA pellet was vacuum dried for 30 min and was dissolved in 1X TE (pH 8.0) at 4°C overnight.

DNA was treated with RNase A (50 µg/mL) at 37°C for 2h followed by digestion with proteinase K (100 µg/mL) at 37°C for 3 h. SDS was added to a final concentration of 0.1%. DNA was further purified twice with equal vol of phenol: CHISAM (1:1) and once with CHISAM at 10,000 rpm in Sorvall SS-34 rotor for 10 min each at 4°C. To the aqueous phase, 0.6 vol of isopropanol was added and the DNA was allowed to precipitate at 25 C. DNA was

spooled and transferred to a fresh tube and washed once with chilled (-20°C) 70% ethanol. DNA was pelleted by centrifugation at 10,000 rpm in Sorvall SS-34 rotor at 4°C for 10 min, vacuum dried and dissolved in 1X TE pH 8.0.

2.5. QUANTIFICATION OF DNA AND RNA

DNA and RNA were quantified by their absorbance measurements at 260 nm (A_{260}) and 280 nm (A_{280}) using Beckman DU-65 spectrophotometer. An OD unit corresponds to approximately 50 µg/mL of double stranded DNA or 40 µg/mL of RNA. The purity of the nucleic acids was assessed by their A_{260}/A_{280} ratios. Preparations of DNA and RNA gave ratios of >1.8 and >2.0 respectively.

2.6. RECOMBINANT PLASMIDS

Recombinant plasmids (containing plastid specific genes) used in the present study are listed in Table 2.

2.6.1. Amplification and plasmid DNA extraction

A single bacterial colony carrying recombinant plasmid was transferred into 20 mL of LB medium (1% bactotryptone, 0.5% bactoyeast, 0.5% NaCl) containing ampicillin (100 µg/mL) and incubated overnight at 37°C on a platform shaker (250 rpm). Plasmid DNA from the clones was extracted by alkaline lysis method (mini preparation) according to Sambrook et *al* (1989). The cells were harvested at 5000 rpm (Sorvall SS-34 rotor) for 10 min. The pellet was suspended in 100 µL of ice-cold solution I (50 mM

Table 2: Gene specific probes used in the present study.

Gene(s)	(Coding for)	Size	Plasmid	Source
rpo A	(α-subunit of RNA polymerase)	1.0 kb	pRP4	Prof. M.Sugiura Nagoya Univ. Japan
rpo B	(β-subunit of RNA polymerase)	3.2 kb	pRP2	- do -
rbc L	(Rubisco larger subunit)	0.7 kb	pRP1	- do -
16s rDNA	(16s rRNA)	0.7 kb	AUM 1.6	Dr. Kunni Malayan ICGEB, India.
psb A	(D_1 reaction center protein (32 kD) of photo-system II)	1.0 kb	pCBgl II	Dr. Navin Khanna ICGEB, India.

glucose; 25 mM Tris-Cl pH 8.0; 10 mM EDTA pH 8.0) and transferred to a 1.5 mL eppendorf tube, vortexed well and 200 µL of solution II (0.2 N NaOH, 1% SDS) was added and mixed by inverting the tube rapidly five times. Finally 150 µL of solution III (5 M potassium acetate) was added, vortexed and centrifuged at 12,000 x g for 5 min (Sorvall 24S microfuge). The supernatant was collected and extracted with equal vol of phenol:CHISAM (1:1) by centrifugation at 12,000 x g for 2 min in the microfuge. The aqueous phase was collected into a fresh eppendorf tube and plasmid DNA was precipitated by adding 2 vol of chilled ethanol (-20°C). DNA was pelleted by centrifugation in the microfuge at 12,000 x g for 5 min at 4 C and washed with 70% ethanol, dried under vacuum (450 mm Hg) for 2 min and dissolved in 25-50 µL TE (pH 8.0). The plasmid DNA was treated with RNase A (100 µg/mL final concentration) at 37°C for 30 min, its quality and quantity were checked on 0.8% agarose gels.

2.6.2. PCR Amplification

Oligonucleotide primers (20 bp long were selected based on prior DNA sequence information on rice chloroplast genome (Hiratsuka *et al* 1989; as presented below:

Gene	Forward primer	Reverse primer
rpo A	5' TAGAACTTAT TCTTAGGTAA3'	5′ CAAGCTCTCC TCCATCGTCC 3′
rpo B	5' ATGCTCCGGA ATGGAAAT3′	5' GTTGAAGAAG GGATAATTTGG 3′
rbc L	5′ AGAGCTGTAT TTGCGAGGGA3'	5' GTGTTAAGGA TTATAAATTG 3′

Amplification of gene specific fragments from rice chloroplast genome were carried out by polymerase chain reaction (PCR) 18-20 bp in 0.5 mL microfuge tubes containing the following:

10 x Taq poly buffer (STRATAGENE)		5 µL
dNTPS (1.25 mM)	-	8 µL
H_2O		34 µL
Forward primer (150 ng/µL)	-	1 µL
Reverse Primer (150 ng/µL)	-	1 µL
Taq polymerase (STRATAGENE)		0.5 µL
Template DNA (100 ng)		1 µL
		50 µL

The components were mixed gently and 30 µL of mineral oil (SIGMA) was layered on top of the mixture to limit evaporation during high temperature regimes. PCR amplification was performed using Perkin Elmer Cetus thermocycler with cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 4 min for 30 cycles.

DNA fragments were separated on 0.8% agarose gel in 1X TBE to confirm their sizes using BI0RAD horizontal electrophoresis unit at 3 V/cm for 8 h at 25°C.

2.6.3. Elution of DNA fragments

Gel piece containing specific DNA fragment was cut and transferred into a 1 mL sterile syringe and was passed through the nozzle into an eppendorf tube with the piston. An equal vol of buffer saturated phenol (pH 8.0) was added, mixed vigorously and

kept at -80°C for 15 min. The tubes were then centrifuged at 14,000 rpm for 15 min at 25°C in a microfuge (Beckman). The aqueous phase was mixed with an equal vol of phenol: CHISAM (1:1) and spun at 10,000 rpm for 10 min at 25°C. DNA was precipitated by adding 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of chilled (-20°C) ethanol and kept overnight at -20°C. DNA was pelleted at 14,000 rpm for 15 min at 4°C, washed once with chilled (-20°C) 70% ethanol, vacuum dried and dissolved in 50 μL of 1X TE (pH 8.0) at 4°C.

The size, purity and concentration of PCR amplified DNA fragments were rechecked on 0.8% agarose gels with 1 Kb DNA ladder (FIBCO-BRL) as markers.

2.7. RADIOLABELLING OF PROBES

DNA fragments to be used as probes were labelled by nick translation according to the manufacturer's (GIBCO-BRL, Maryland, USA) instructions making use of the unique property of E. coli DNA polymerase I which adds nucleotide residues to 3'OH terminus when one strand of the DNA helix was nicked by DNase. This enzyme also has 5'⟶3' exonuclease activity and removes nucleotides from 5' direction of the nick. The simultaneous removal of nucleotides from 5' end and addition at 3' end ensures specificity of translation process. Reaction mixture consisted of the following:

Solution A - 5 μL (0.2 mM each of dATP, dGTP, dTTP, dCTP in 500 mM Tris-HCl, pH7.8; 500 mM MgCl ; 100 mM 2-mercaptoethanol; 100 μg/mL BSA)

DNA (50 ng) - x μL.

H_2O	-	y μL.
α^{32}P dCTP	-	10 μL 100 μCi (3000 Ci/mL specific activity)
Solution C	-	5 μL (0.4 U/μL DNA polymerase I; 40 pg/μL DNase I; 50 mM Tris-HCl, pH 7.5; 5 mM magnesium acetate; 1 mM 2-mercaptoethanol; 0.1 mM PMSF; 50% (w/v) glycerol; 100 μg/mL BSA
		50 μL

The contents were gently mixed by vortexing and spinning in a microfuge and were incubated at 15 C for 90 min. 5 μL of stop buffer (0.3 M EDTA, pH 5.2) was added to terminate the reaction and volume was made upto 100 μL with STE. To remove the unincorporated dNTPs and α-^{32}P dCTP, labelled DNA solution was loaded onto a spun column according to Sambrook et *al*. (1989) and centrifuged at 1600 x g (3800 rpm) in Sorvall SS-34 at 25°C for 4 min. Flowthrough was collected and labelling efficiency was determined by using Cerenkov counting. The radioactivity of the probe was measured using liquid scintillation counter (Beckman) and specific activity (dpm/ng DNA) was determined from cpm per μL sample and the counting efficiency (3.33)

2.8 ISOLATION OF THE TRANSCRIPTIONALLY ACTIVE CHROMOSOME (TAC):

Transcriptionally active chromosomes (TAC) of plastids were prepared as described by Tewari and Goel (1983). Triton X-100 lysates of plastids were prepared from 20 g each of green and yellow siblings separately. The lysates were clarified by centrifugation at 12000 rpm for 30 min in a Sorvall SS-34 rotor. Plastid DNA and associated proteins were pelleted at 1,00,000 x g

for 3.5 h in Beckman SW-41 rotor. The pellet was dissolved in 50 mM Tris-HCl pH 8.0, 25% glycerol, 100 mM KCl, 10 mM BME and 1 mM PMSF. Reaction mixture for RNA polymerase assay consisted of 50 mM Tris-HCl pH 8.0, 10 mM MgCl , 10 mM $MnCl_2$, 0.25 mM DTT, 25 mM KCl, 100 μM each of CTP, GTP, UTP, 20 μCi *a* ^{32}P ATP (300 Ci/mmol) and 15 μL of dissolved TAC in a final volume of 50 μL. The reaction was carried out at 30°C and terminated at different intervals using 5% cold TCA. The precipitable radioactivity was collected on to GF/C Whatman filter and measured in liquid scintillation counter (Beckman).

2.9. NORTHERN BLOT HYBRIDIZATION:

2.9.1 Electrophoresis of RNA:

RNA was separated on formaldehyde agarose gels according to Sambrook *et al* (1989). The gel was prepared by melting the appropriate amount of agarose in water, cooled at/to 60°C. The gel was cast and allowed to set for 1 h at room temperature. 5X formaldehyde gel running buffer (0.1 M MOPS pH 7.0; 4 0 mM sodium acetate, 15 mM EDTA pH 8.0) and formaldehyde (SIGMA) were mixed to give final concentrations of 1X and 2.2 M respectively. RNA samples were prepared by the following method in sterile microfuge tubes:

RNA (upto 3 0 μg)	variable volume
5X formaldehyde gel running buffer	3.5 μL
3 7% (w/v) formaldehyde	6.0 μL
deionized formamide	17.5 μL.
	35.0 μL

The samples were incubated at 65°C for 15 min and chilled on ice bath. 2 µL of sterile DEPC treated formaldehyde gel loading buffer (50% glycerol; 1 mM EDTA pH 8.0; 0.25% bromophenol blue 0.25% xylene cyanol FF) was mixed with RNA sample and loaded onto the gel which was prerun for 5 min at 5 V/cm. Samples were loaded in two parallel sets in a single gel and run at 25 C for 5 h in 1X formaldehyde electrode buffer at 2 V/cm. One set was stained to check the extent of separation of RNA while the other was used for Northern blotting.

2.9.2. Northern Blotting:

Soon after the fractionation, the gel was soaked in 5 vol of water for 5 min to remove traces of formaldehyde. RNA in the gel was partially denatured in alkaline solution (50 mM NaOH, 10 mM NaCl) for 15 min and neutralized in 0.1 M Tris-HCl (pH 7.4) solution for 15 min. Nylon membrane (Gene Screen Plus®) was soaked in sterile water for a few seconds and then in 20 x SSC for 15 min. The gel was carefully placed on nylon membrane. Several blotting papers and stacks of tissue paper were placed above the membrane. A glass plate and a 500 g weight were placed over the top of the stack. The transfer of RNA was carried out in 20X SSC by semi-dry capillary blotting method (Sambrook *et al* 1989) for 16 h. The membrane was rinsed in 2X SSC to remove residual agarose and placed on wet filter paper to prevent drying of membrane.

2.9.3. Immobilization:

UV cross linking of RNA to the membrane was done twice to

minimize loss of RNA during hybridization at 1200 μJoules for 30 sec using Stratalinker (STRATAGENE). The membrane was baked at 80°C for 3h to reverse the formaldehyde reaction.

2.9.4 Blot Hybridization:

The membrane was placed in a heat sealable polythene bag to which 20 mL of prewarm prehybridizatior? solution (5X SSC, 5% dextran sulphate, 50 mM sodium phosphate pH 7.2, 5X Denhardt's solution, 2.5 mM EDTA, 0.5% SDS and 100 μg/mL denatured salmon sperm DNA) were added. Prehybridization was carried out overnight in a shaker water bath at 65°C.

Denatured radiolabelled probe (sp. activity > 1 x 10 dpm/ng) was added to the prehybridization mixture and the bag was resealed. Hybridization was carried out in the shaker water bath at 65°C for 18h.

2.9.5. Post hybridization processing:

The membrane from the hybridization bag was carefully taken out and washed for 10 min in each of the following solutions (i) 3X SSC and 0.1% SDS, (ii) 2X SSC and 0.1% SDS, (iii) 0.5X SSC and 0.1% SDS to remove non-specifically bound probe. The membrane was air dried and covered in saran wrap. Autoradiography was done for varying periods depending on the signal (sec. 2.14).

2.10 PROTEIN FRACTIONATION AND DETECTION:

2.10.1 Soluble Proteins

Total soluble proteins were prepared according to Han *et al*

(1993). Seven day old green, yellow/white seedlings from each accession were separately ground using mortar and pestle in (2 mL/g tissue) the homogenizing buffer (0.1 M 2-(N-Morpholino) ethane sulfonic acid,Na-MOPS pH 7.5, 10 mM NaCl, 1 mM EDTA, 10% sucrose, 5% β-mercaptoethanol (BME) and 4% SDS). Extracts were denatured at 70°C for 5 min, microfuged at 10,000 rpm for 10 min. Supernatants containing total cellular proteins were retained.

2.10.2 Thylakoid membranes:

Thylakoid membranes were isolated by the procedure of Vainstein et *al* (1989). Green and yellow or white siblings from each accession were ground in suspension buffer (0.6 M sorbitol, 10 mM HEPES-KOH (pH 7.5) and 2 mM CaCl) . The suspension was filtered through four layers of cheese cloth and subsequently through four layers of miracloth and centrifuged at 3,000 rpm (Sorvall SS-34 rotor). The supernatant was recentrifuged at 7,000 rpm (Sorvall SS-34) for 20 min at 4°C and the pellet (thylakoid membranes) was suspended in 10 mM HEPES-KOH (pH 7.5) buffer.

2.10.3 Polyacrylamide gel electrophoresis (PAGE):

Denaturing SDS-PAGE of soluble and thylakoid membrane proteins was performed using Laemmli (1970) system. Based on visual assessment of protein concentrations on gels stained with Coomassie Brilliant Blue, equal amounts of protein samples were loaded onto the gel and separated on 12% resolving gel (pH 8.5) with a 5% stacking gel (pH 6.8) at 70 V constant voltage for 16 h

at 15°C,

2.10.4 Immunological detection of proteins:

Proteins from polyacrylamide gels were transferred onto nitrocellulose membrane in a transfer buffer (Tris-glycine-methanol) according to Towbin *et al* (1979) using BIORAD electroblotting apparatus at 600 mA constant current for 3 h at 4°C. The blot was transferred to blocking solution (1 x PBS; Tween-20; milk protein) and incubated for 1 h at 25°C and was washed in 1 x PBS for 15 min. For the detection of the Rubisco large subunit (rbcL), light harvesting complex of photosystem II (LHCP-II) and ferredoxin oxidoreductase (FNR) proteins, separate antisera raised against each of these proteins from maize were used (kind gift from Dr. M.K. Reddy, ICGEB, India). Following the incubation in the primary antibody solution at 4 C overnight, the blot was washed in PBST solution 5 times for 5 min and rinsed once in 1 x PBS solution. The blot was further incubated with anti-rabbit IgG conjugated to peroxidase. For the colour reaction 4-chloronaphthol was the substrate.

2.11 DNA POLYMERASE PURIFICATION AND ASSAY:

Plastid DNA polymerase was purified and assayed for its activity by the method of McKown and Tewari (1984).

2.11.1 Preparation of Plastid Lysate:

30 grams of 5 day old (green and yellow) seedlings were chopped in buffer (50 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$, 10 mM BME

and 1 mM PMSF) and filtered through four layers of cheese cloth and miracloth. The filtrate was centrifuged at 1000 x g for 10 min. The pellet (of plastids and nuclei) was suspended in the same buffer and Triton X-100 was added to a final concentration of 2.5%. The mixture was kept on ice for 30 min to lyse plastid membranes. The plastid lysate was clarified and separated from nuclei by centrifugation at 5000 x g for 30 min.

2.11.2 Column Chromatography:

To remove contaminating nucleic acids and nucleases, the plastid lysate was loaded on to 15 mL (settled volume) DEAE-cellulose column pre-equilibrated with buffer comprising 50 mM Tris-HCl pH 8.0, 25 mM KCl and 25% glycerol. The column was washed with 4-5 bed vol of a buffer containing 10 mM BME and 1 mM PMSF. The bound proteins were eluted with equilibration buffer including 500 mM KC1 (at this salt concentration nucleic acids were bound to the column while the proteins were eluted out).

The eluted proteins (approximately 30 mL) were dialysed against buffer (50 mM Tris pH 8, 100 mM KCl, 10 mM BME, 1 mM PMSF, 25% (w/v) glycerol, 0.1% Triton X-100, 0.1 mM EDTA) and then loaded on to a 5 mL (settled volume) heparin sepharose column pre-equilibrated with dialysis buffer. The column was washed extensively with the same buffer to elute unbound proteins whereas the bound proteins were eluted with 500 mM KC1 (same buffer except 0.1% Triton X-100 and 0.1 mM EDTA) as 1.0 mL fractions. The fractions were assayed for DNA polymerase.

2.11.3. DNA polymerase assay:

Reaction mixture consisted of 50 mM Tris-HCl pH 7.5, 10 mM MgCl , 100 mM KCl, 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 1 μCi ^{3}H dTTP, 1-2 μg of nicked calf thymus DNA and 10 μL of partially purified enzyme in a total volume of 100 μL. The reaction mixture was kept at 37°C for 30 min. The reaction was terminated in samples taken out at different time intervals by adding 5% TCA. The precipitate was collected on to GF/e glass fiber filter and the radioactivity was measured in a liquid scintillation counter.

2.12 STAINING AND VISUALIZATION OF DNA IN PLASTIDS AND NUCLEUS:

Protoplasts were isolated from green and yellow siblings. 4-5 young expanding leaves (green and yellow) were taken and cut into narrow strips and incubated in protoplast isolation solution (given below) for 60 min. Wash solution was replaced by protoplast isolation solution and the contents were incubated at 28°C for 12 h in dark. Leaf tissues were squeezed with forceps to remove major debris.

DAPI (1 μg/mL) solution was prepared in the protoplast wash buffer. A drop of DAPI solution was taken on the slide and mixed gently with a drop of solution containing protoplasts and coverslip was placed. The protoplasts were examined under a microscope with a UV filter attachment. Plastids stained orange red while the nucleus stained blue.

COMPOSITION OF PROTOPLAST ISOLATION SOLUTION

Ingredient		Stock Solution	Final Conc. in Medium
1.	KH_2PO_4	Added as dry powder	27.2 mg/L
2.	KNO_3	Added as dry powder	101 mg/L
3.	$CaCl_2.2H_2O$	Added as dry powder	1.48 g/L
4.	$MgSO_4.7H_2O$	Added as dry powder	246 mg/L
5.	KI Stock	0.1 mg/ml	0.16 mg/L
6.	$CuSO_4.5H_2O$ Stock	0.01 mg/ml	0.025 mg/L
7.	MES Buffer Stock (20X)	100 mM	5 mM
8.	Mannitol	Added as dry powder	130 g/L
9.	Cellulase RS	Added as dry powder	1.5%
10.	Macerozyme	Added as dry powder	0.05%

* The medium was prepared and autoclaved.

* pH was adjusted to 5.8.

* Enzymes were dissolved in the medium at room temperature and

* The solution was filter sterilized (0.2 μm pore size).

2.13 SOUTHERN BLOT HYBRIDIZATION

2.13.1 Restriction Digestion of Plant DNA:

Bgl I and Bam H I restriction enzymes were used for digesting the DNA samples from the siblings of each accession according to the manufacturer's instructions (Genei, Banglore, India). Single and double digestions were carried out at 37 C in a circulating water bath for 3h for single and 5h for double digestions

respectively. Reaction was terminated by heat inactivating the enzymes at 65°C for 15 min.

2.13.2. Southern Blotting

DNA fragments were size fractionated on 1.1% agarose gel in 1X TAE. The gel was submerged in 1X TAE and electrophoresis was performed at 2 V/cm for 18 or 20 h (specified in figure legend) at 25°C in Pharmacia LKB unit. DNA fragments from the gel were vacublotted according to the suppliers manual (LKB 2016 Vacu Gene) onto nylon membrane (Genescreen hybridization transfer membrane, DuPont, NEN research products, USA) preincubated in 3 X SSC for 10 min. DNA in the gel was denatured in alkaline solution (1.5 M NaCl, 0.5 M NaOH) for 3 min followed by treatment with neutralizing solution (1M Tris, 2M NaCl pH 5) for 3 min under suction (60 cm H_2O). DNA transfer was carried out in 20 X SSC for 3 h to ensure complete transfer of high molecular weight DNA. The membrane was rinsed in 3 X SSC, air dried and baked at 80°C for 2 h in a vacuum oven.

2.13.3 Blot hybridization

Membrane containing immobilized DNA was placed in a heat sealable polythene bag. Preheated (at 65 C) prehybridization solution (1 mM EDTA, 7 0% SDS, 0.5M $NaHPO_4$ pH 7.2) mixed with denatured salmon sperm DNA (100 μg/mL) was added into the bag ($10\ mL/dm^2$ of membrane) and heat sealed. Prehybridization was carried out for 18h at 65°C in a shaker water bath (Haake).

Radio-labelled probe was denatured at 100°C for 5 min and chilled on ice bath for 10 min. Probe was delivered into the bag by cutting one corner. The bag was resealed after removing trapped air. Hybridization was carried out in the shaker water bath at 65°C for 18 h.

2.13.4 Post hybridization Processing

The membrane was carefully removed from the bag and washed twice in 500 mL each of the following solutions: (i) 1 mM EDTA, 40 mM Na HPO (pH 7.2), 5% SDS, (ii) 1 mM EDTA, 40 mM Na HPO (pH 7.2), 1% SDS at 65°C for 30 min to remove nonspecifically bound of the probe.

2.14 AUTORADIOGRAPHY

The washed membrane was air dried, covered in saran wrap and exposed to X-ray film (Kodak) at -70 C in casette (SIGMA) with intensifying screen (Cronex). The exposure times varied depending upon the intensity of the radioactive signal monitored with a mini GM counter. Exposed film was developed in: a) Kodak developer at 20 C for 2 min, b) water bath for 1 min c) rapid fixer (Kodak) for 10 min and running water for 5 min.

3. ACCUMULATION OF INTERMEDIATES IN CHLOROPHYLL BIOSYNTHESIS CONTROLLED BY DIFFERENT GENES

3.1. INTRODUCTION

In higher plants, chlorophyll synthesis is light-dependent although most of the steps in the pathway are light-independent Castelfranco and Beale (1983). Abundance and the activity of the enzymes catalyzing different steps in the pathway, however, are subject to modulation by both light and cell type (Boese et *al* 1991; Spano and Timko 1991; Witty et *al* 1993). The first intermediate in the formation of chlorophylls and other tetrapyrroles is 5-aminolevulinic acid (ALA) and protoporphyrin IX is the last intermediate common to chlorophyll biosynthesis (Fig. 3) and heme formation (Rüdiger and Schoch 1988).

Formation of Mg-protoporphyrin-IX (Fig.3) is the first step in the chlorophyll branch of the pathway followed by its esterification, protochlorophyllide (Pchlide) formation and its reduction to chlorophyllide (Chlide) and to chlorophyll(s). In most angiosperms Pchlide reduction is a light-dependent reaction catalyzed by NADPH: PCOR (Griffiths, 1978). Both NADPH: PCOR and its substrate Pchlide accumulate to high levels in the leaves of dark-grown angiosperms. The transient formation of Chlide, its conversion and concomitant integration into the newly forming light harvesting complexes of thylakoid membrane appear to be closely tied to chloroplast development (He et *al* 1994). Information on chlorophyll biosynthetic pathway mostly depended

Figure 3: The chlorophyll biosynthetic pathway

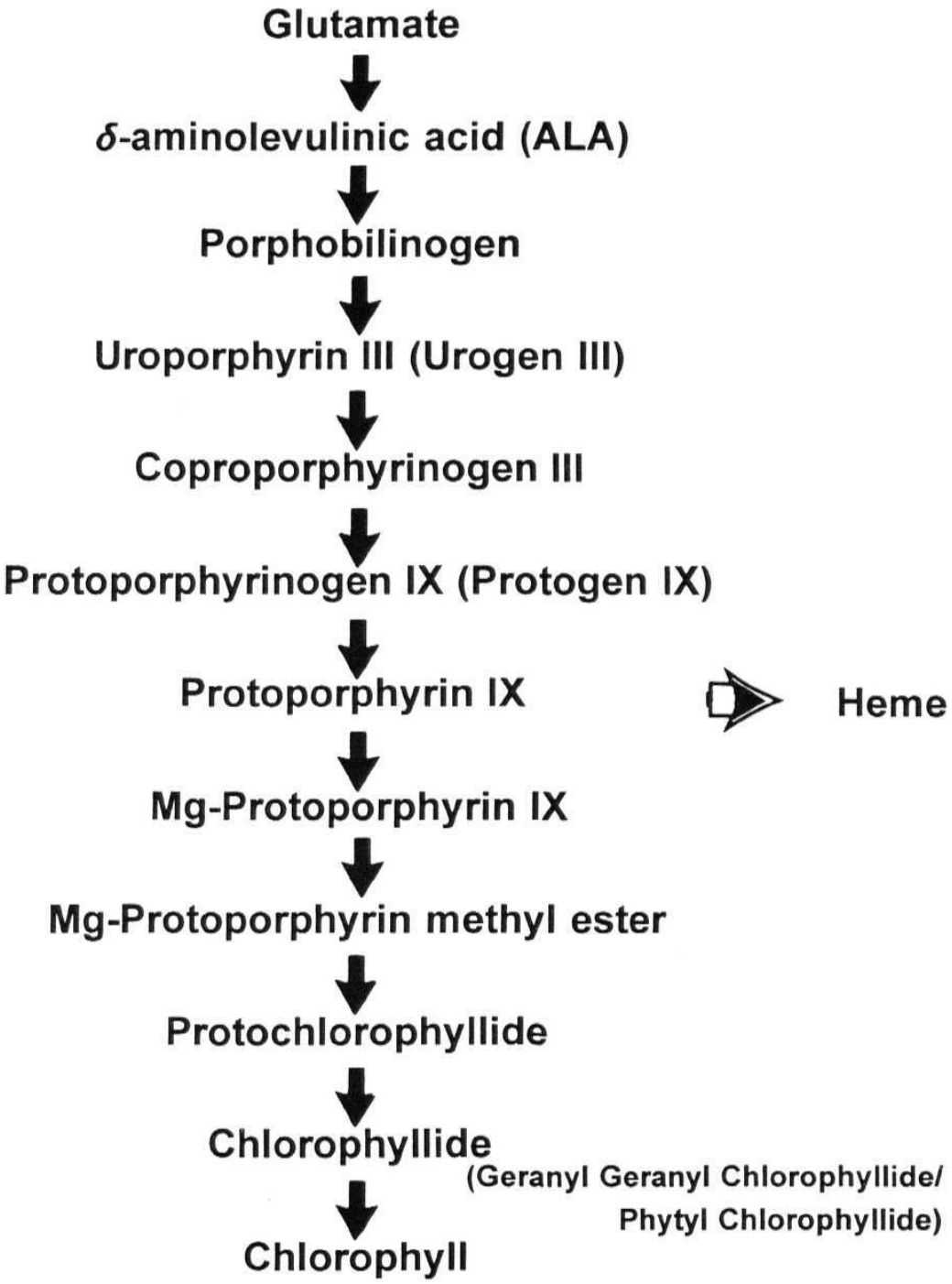


Fig. 3 The chlorophyll biosynthetic pathway

on the use of green, etiolated or dark grown plants (Castelfranco and Beale 1983; Rüdiger and Schoch 1988; Senge 1993).

Work in seemingly unrelated areas using different plant systems has provided insight into the regulatory communication between plastid and nuclear genomes (Taylor 1989). Genes that play important role(s) in the process of interest can be identified using mutants as tools in dissecting a complex mechanism into smaller parts. Studies on chlorophyll-deficient (stripe) mutants in pearl millet provided genetic evidence for plastid-nuclear gene interactions leading to the development of chlorophyll (Reddy and Subrahmanyam 1988a,b).

Genes that regulate the development of chloroplasts from proplastids are known. In barley 20 different nuclear mutants which cause yellow, chlorophyll-deficient phenotype were identified (von Wettstein et *al* 1971). Some mutants had blocks at specific points in chlorophyll biosynthesis and in all cases, chloroplast development was arrested at an early stage of internal membrane assembly (Taylor et *al* 1987). Similar defects were found in *iojap (ij)* and chloroplast modifier *(cm)* of maize (Thompson *et al* 1983), *albostrains* of barley (Knoth and Hagemann 1977) and variegation mutants of pearl millet (Reddy et *al* 1988).

Low-temperature (77K) fluorescence spectroscopy is a sensitive and powerful tool frequently used to study Pchlide forms and its transformation in dark-grown leaves (Boddi et *al* 1992; Hendrich and Bereza 1993). Biosynthesis of Pchlide, the structure, localization and properties of NADPH-PCOR and

reduction of Pchlide to Chlide are extensively reviewed (Hendrich and Bereza 1993). Pchlide in leaves of dark-grown plants show a spectral heterogeneity *in vivo* which probably reflects their molecular arrangement within the etioplast membranes. The main form, Pchlide (absorption maximum at 650 nm and a fluorescence emission maximum at 657 nm), has a basic role in the greening of etiolated leaves (Gassman et *al* 1968; Granick and Gassman 1970; Oliver and Griffiths 1982) and has been described as a photoactive complex of PCOR - Pchlide (Boddi et *al* 1989; Boddi et *al* 1990). On irradiation of etiolated plants or leaves, Pchlide is transformed into Chlide (680 nm form). Then the newly synthesized chlorophyll is built in a short time into different Chl-protein complexes of PS I and II which can be measured by their fluorescence emission at 733 nm and 685 nm respectively (Hendrich and Bereza 1993).

Several stripe mutants of pearl millet have been genetically characterized (Subrahmanyam et *al* 1986; Reddy and Subrahmanyam 1988a,b). So far four independent loci have been identified based on the segregation patterns in the progenies of intercrosses of different stripe mutants (Karunasri and Subrahmanyam 1994) mutants. Any one locus in homozygous recessive condition leads to the mutant phenotype while development of chlorophyll is accomplished by the complementary interaction of dominant genes at these loci (Karunasri and Subrahmanyam 1994).

In the present study, in *vivo* low-temperature (77 K) fluorescence emission spectra of five different chlorophyll deficient (yellow/white) phenotypes of pearl millet (Table 1) and

their respective green siblings among different genotypes and phenotypes were recorded to detect differences and/or similarities in the accumulation of intermediates in the chlorophyll biosynthetic pathway. Comparison of the accumulation of intermediate(s) of chlorophyll biosynthesis among different chlorophyll-deficient (yellow and white) phenotypes, their normal siblings derived from different genotypes and stripe phenotypes enabled the identification of blocks at specific step(s) and their control by different loci.

3.2 MATERIALS AND METHODS

Six day old seedlings grown in plastic trays were harvested 2-3 hours after sunrise and the fluorescence spectra were recorded immediately since the harvesting of seedlings in dark resulted in inconsistencies. Seedlings approximately equal in size were used. For each set of measurements five samples were taken from green and yellow siblings of IP 5009, IP 9712 accessions. Likewise, they were repeated with green and white seedlings of VCM 36, GWS 14 and *albino.* Fluorescence emission spectra were recorded at 77K with Hitachi fluorescence spectrofluorimeter (model 4010). The excitation wavelength was 438 nm, with 5 nm excitation slit and 4 nm emission slit. The integration time was 0.18. The mean spectrum was smoothed before recording.

3.3 RESULTS

The *in vivo* low temperature fluorescence emission spectra of green seedlings from different genotypes are presented in Fig. 4(a

to e) while the spectra from the corresponding yellow or white siblings from each mutant are presented in Fig.5. The emission spectra were recorded at 438 nm excitation wavelength which preferentially detects Pchlide (Lebdev *et al* 1985). The excitation at 440 nm which preferentially detects chlorophyll from PS II also showed similar results (data not presented). The excitation at 470 nm detected only two peaks with emission maxima at 685 and 733 corresponding to chlorophyll in PS II and I respectively abolishing the Pchlide peak at 657 nm. Hence 438 nm excitation wavelength was used to detect different forms of compounds in the leaf sample emitting fluorescence.

The fluorescence emission maxima in green seedlings were at 657 nm, 685 nm and 733 nm (Fig. 4 a to e). The relative levels of the main peaks varied depending on the genetic background. To obtain sharper and better resolution of spectral bands, derivatives of the spectra were obtained. The second and fourth derivatives of the emission spectra did not resolve any additional peaks (data not presented) but revealed the invariable fluorescence emission peak positions in accordance with the elegant demonstration by Boddi et *al* (1992) on the repeatability of the technique. The emission spectrum from green seedlings showed a broader peak in the 720-733 nm range (Fig. 4, a to e).

Yellow seedlings from IP 5009 and IP 9712 exhibited fluorescence emission maxima at 657 nm and 678 nm (Fig. 5 a,b). The 678 nm peak was found to be sensitive to the light conditions at the time of harvesting. Freshly harvested leaves showed 678 nm

Figure 4: Low temperature (77K) fluorescence emission spectra of green seedlings from different genotypes (a,b,c,d,e) at 438 nm excitation wavelength: a -IP 5009; b -IP 9712; c –VCM 36; d –GWS 14; e -Albino).

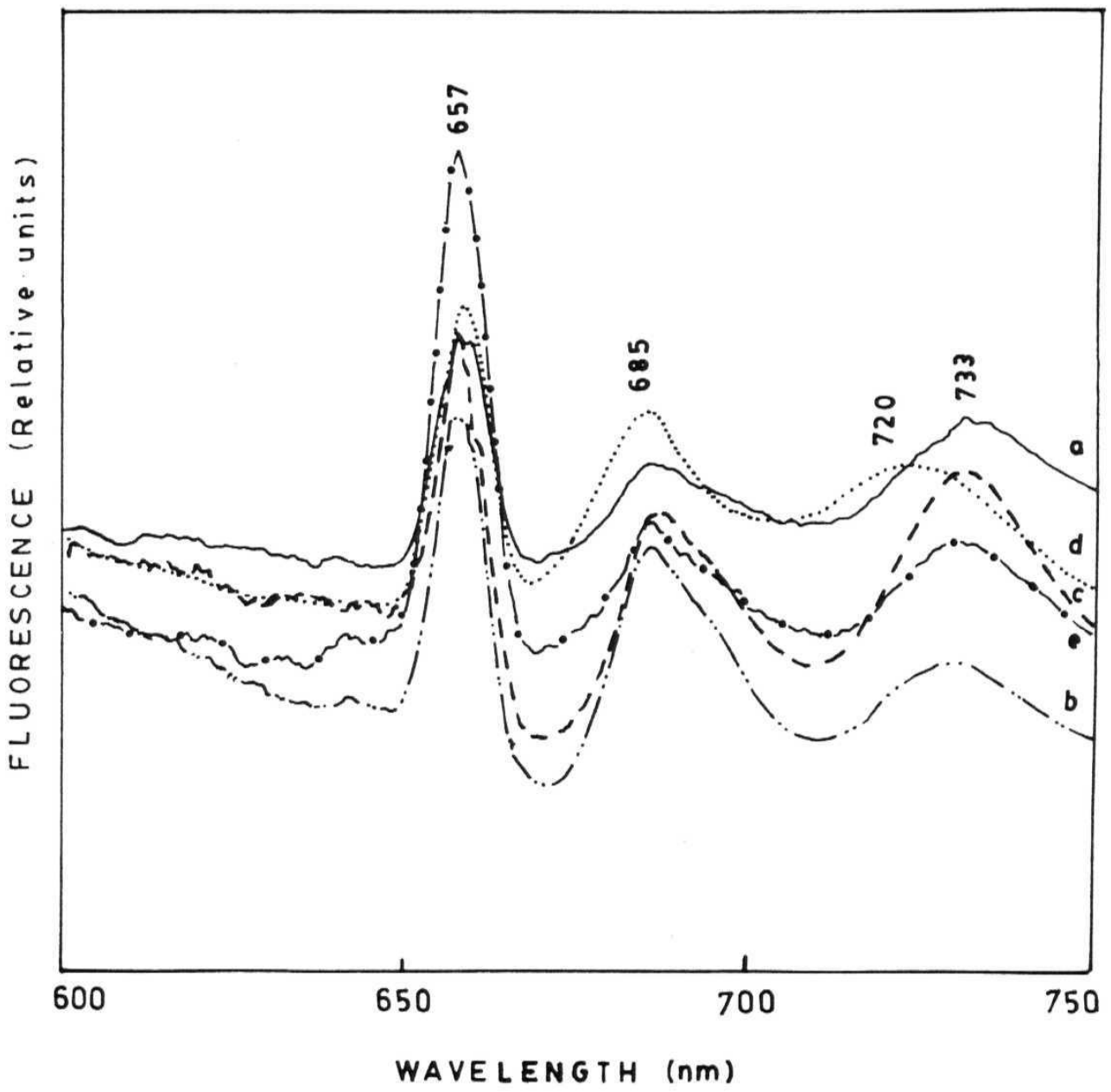

FLUORESCENCE (Relative units)
657
685
720
733
a
d
c
b
600
650
700
750
WAVELENGTH (nm)

Fig. 4

Figure 5: Low temperature (77K) fluorescence emission spectra from seedlings at 438 nm excitation wavelength. (a - IP 5009 Yellow seedlings; b - IP 9712 Yellow seedlings; c - VCM 36 White seedlings; d - GWS 14 White seedlings; e - *al al* White seedlings)

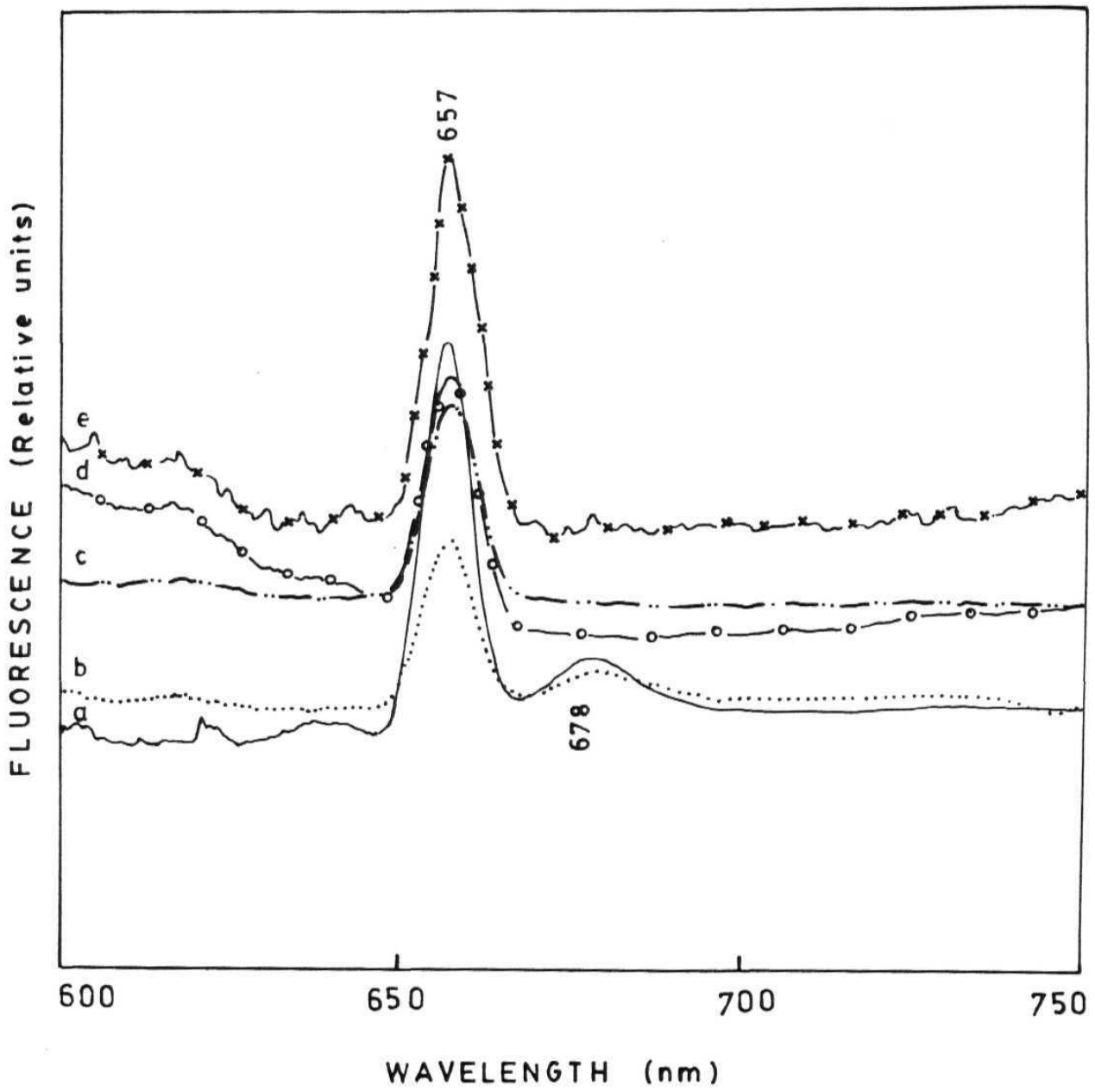

FLUORESCENCE (Relative units)
657
678
e
d
c
b
a
600
650
700
750
WAVELENGTH (nm)

Fig. 5

peak while few hours of storage in dark resulted in the loss of this peak. The second derivatives of the spectra did not alter the number and position(s) of the peaks (data not presented). White seedlings from GWS 14 (c) and VCM 36 (d) and *albino* (e) showed fluorescence emission maxima at 657 nm while an additional broad and less sharper emission spectrum in the 613-620 nm range was detectable in the white seedlings of the white stripe mutants.

3.4 DISCUSSION

Fluorescence emission spectra of green progeny irrespective of the source (Fig. 4a to e) showed emission maxima at 657 nm, 685 nm and 733 nm corresponding to Pchlide, PS II and PS I respectively at 438 nm excitation wavelength (Lebdev et *al* 1985; Böddi et *al* 1992; Hendrich and Bereza 1993). Emission band of 678 nm corresponding to Chlide was not detectable in green seedlings confirming the transient presence of Chlide,its rapid conversion to chlorophyll and its concomitant integration into membrane protein complexes of PS I and II.

A comparison of emission spectra of white seedlings (Fig. 5 c,d,e) with those of green seedlings (Fig. 4c,d,e) from three different mutants (GWS 14, VCM 36 and *albino)* revealed a common emission peak at 657 nm corresponding to Pchlide and emission peaks at 685 nm and 733 nm corresponding to the chlorophyll of PS II and I respectively were absent. Furthermore, a second peak at 678 nm corresponding to Chlide detected in yellow seedlings (Fig. 5a,b) is absent in the white seedlings. This clearly suggests that

white seedlings accumulate Pchlide and are thus defective in the conversion of Pchlide to Chlide. The reduction of Pchlide to Chlide is catalyzed by plastid specific NADPH-PCOR which accumulates in the plastids of dark-grown plants and eventually becomes the dominating polypeptide of the chloroplast inner membranes (Apel *et al* 1980; Oliver and Griffiths 1980). The white stripe mutants (GWS 14, and VCM 36) are homozygous recessive at two loci (vi_2 vi_3 vi_4 vi_4)while the yellow stripe mutants (IP 5009 and IP 9712) carry dominant alleles at these loci (Karunasri and Subrahmanyam 1994). It is, thus, likely that the conversion of PChlide to Chlide is controlled by one or both the loci in pearl millet. Further confirmation about the nature of the gene products is awaited.

Pchlide reduction to Chlide is catalysed by NADP dependent PCOR. NADPH production is plastid specific and is catalysed by Ferredoxin oxidoreductase (FNR). Immunoblot analysis revealed comparable FNR levels in green and yellow sibling of IP 5009 but was absent in white seedlings (presented in the next chapter Fig. 9).

The presence of an additional emission peak at 678 nm (Fig. 5 a,b) in the yellow seedlings signifies the accumulation of Chlide in these mutants and the absence of PS I and II complexes in the yellow seedlings indicate a block in the conversion of Chlide to chlorophyll. The two yellow stripe mutants (IP 5009 and IP 9712) are homozygous recessive at *vi* and *vi* loci respectively, and dominant alleles at these two loci (*Vi* and Vi_2) are complementary

in the production of chlorophyll (Karunasri and Subrahmanyam 1994). It is thus proposed that, either of these loci in homozygous recessive condition is adequate to block the conversion of Chlide to chlorophyll and control the production and/or the activity of chlorophyll synthetase (Table 3).

When the leaves are illuminated, Pchlide is immediately reduced to Chlide which is thereafter esterified. In the present study, a major Pchlide and a minor Pchlide forms were found in the white seedlings (GWS 14, VCM 36 and *albino]* whereas three spectral forms with fluorescence emission maxima at 632 nm, 645 nm and 657 nm were reported in etioplasts (Hendrich and Bereza 1993). Emission peak at 678 nm in the yellow seedlings from IP 5009 and IP 9712 of pearl millet represents single Chlide form (an esterified form - phytyl Chlide) unlike different Chlide forms in etiolated (dark-grown)seedlings (Rudiger and Schoch 1988).

Henningsen and Thorne (1973) found that the newly formed Chlide in barley mutants directly emit fluorescence at 678 nm and the rate of esterification was more rapid than in the wild type. Rudiger et *al* (1980) suggested that such esterification of Chlide in etioplasts is catalyzed by an enzyme or a group of enzymes, termed chlorophyll synthetase. The occurrence of a single emission spectral peak at 678 nm and the absence of chlorophyll (685 and 733 nm emission peaks) in the yellow seedlings of pearl millet in the present study suggests that the esterification of Chlide is catalysed by an enzyme different from that catalysing the conversion of Chlide to chlorophyll. Since the green and yellow

siblings of each accession carry the same genotype, the Chlide in the yellow seedlings is likely phytyl Chlide. Each of these mutants are homozygous recessive at one of the loci (vi_1vi_1 or vi_2vi_2) and block the conversion of phytyl Chlide to chlorophyll but dominant alleles at these loci are complementary in restoring the chlorophyll synthesis. This emphasizes that both these loci control the conversion of Chlide to chlorophyll. Further experiments are needed to establish the nature of the gene product(s) and their regulation..

Table 3: Accumulation of protochlorophyllide/chlorophyllide and proposed block(s) in chlorophyll biosynthetic pathway in chlorophyll-deficient phenotypes of *Pennisetum glaucum* (L.) R. Br.

Phenotype Accession)	Genotype	Accumulation of		Block in chlorophyll biosynthesis
		Pchlide	Chlide	
Yellow (IP 5009)	vi_1vi_1 Vi_2Vi_2 Vi_3Vi_3 Vi_4Vi_4	+	+	Chlide $\xrightarrow{CS}$ Chl
Yellow (IP 9712)	Vi_1Vi_1 vi_2vi_2 Vi_3Vi_3 Vi_4Vi_4	+	+	Chlide $\xrightarrow{CS}$ Chl
White (VCM 36)	Vi_1Vi_1 Vi_2Vi_2 vi_3vi_3 vi_4vi_4	+	-	Pchlide $\xrightarrow{PCOR}$ Chlide
White (GWS 14)	Vi_1Vi_1 Vi_2Vi_2 vi_3vi_3 vi_4vi_4	+	-	Pchlide $\xrightarrow{PCOR}$ Chlide
White (*albino*)	*al al*	+	-	Pchlide $\xrightarrow{PCOR}$ Chlide

Note: Green siblings irrespective of the accession and genotype had normal chlorophyll synthesis (Figure 4).

Pchlide-protochlorophyllide; Chlide - Chlorophyllide; Chl - Chlorophyll CS - chlorophyll synthetase; PCOR - protochlorophyllide oxidoreductase (+) accumulation; (-) absent.

4. REGULATION OF GENE EXPRESSION DURING PLASTID DEVELOPMENT

4.1 INTRODUCTION

Chloroplast development requires coordinated expression of plastid and nuclear genes and activation of plastid gene transcription must be therefore coordinated with the activation of nuclear gene expression (Taylor 1989). Development and differentiation of photosynthetically competent chloroplasts and other plastid types present challenging opportunities to decipher how plastid gene expression is controlled temporally and spatially in coordination with the expression of nuclear genes coding for chloroplast proteins. Nuclear genes encoding light regulated photosynthetic proteins appear to be regulated primarily at the level of transcription and to some extent at the post-transcriptional and translational levels (Deng and Gruissem, 1987; Mullet and Klein 1987; Klein and Mullet 1990; Schruber et *al,* 1990).

The expression of Rubisco small subunit *(rbcS)* and chlorophyll a/b binding protein *(cab)* gene families in *albostrains* of barley are either repressed or not activated (Borner et *al* 1976; Batschauer et *al* 1986; Hess et *al* 1991, 1994). Similar observations were made in *iojap* mutants of maize (Han et *al* 1993) in photooxidatively damaged plastids following treatment with a bleaching herbicide, norflurazon (Oelmüller and Mohr 1986; Ernst and Schefbeck 1988; Burgess and Taylor 1987) or mutations that block the biosynthesis of protective pigments (Mayfield and

Taylor, 1984; Simpson et *al* 1986; Stockhaus et *al* 1989). It was postulated that the expression of nuclear genes encoding chloroplast proteins is triggered in accordance with the developmental status of the plastids (Borner and Sears 1986; Taylor 1989; Rajasekhar 1991; Susek and Chory 1992).

A large increase in plastid transcription occurs during development although basal levels of plastid gene expression are common to all plastid types suggesting a central regulatory point in chloroplast differentiation and activation of plastid transcription (Mullet 1993). A differential transcription was suggested as the likely mechanism involved in the regulation of chloroplast gene expression based on the differential expression of *rbcL* found in mesophyll versus bundle sheath cells of maize (Link et *al* 1978) and selective modulation of *psbA* mRNA in illuminated maize leaves (Bedbrook *et al* 1978). Development of run-on transcription assays led to the direct analysis of the transcriptional regulation of plastid genes (Deng et *al* 1987; Mullet and Klein 1987). Furthermore, differential transcription of genes encoding the transcriptional/translational apparatus occurs early in barley chloroplast development (Baumgartner et *al* 1993). Rate of transcription varies among the plastid genes. Transcriptional activity varies in parallel with mRNA level and protein abundance, with the Rubisco large subunit *(rbcL)* being the most abundant protein, followed by proteins of the electron transport, ribosomes and subunits of the plastid encoded RNA polymerase (Rapp and Mullet 1991; Rapp et *al* 1992).

Nuclear mutants affecting plastid development are powerful tools in studying nuclear-plastidic interactions to understand regulation of gene expression. In the ribosome-deficient plastids of *iojap* mutants of maize (Han et *al* 1993) and in *albostrains* of barley (Hess *et al* 1993; 1994), transcription of several nuclear genes and plastid genes involved in photosynthesis are affected although the plastid encoded RNA polymerase *(rpo)* genes are transcribed in the *albostrains* of barley (Hess *et al*, 1993). By screening norflurazon treated *Arabidopsis thaliana* mutants, Susek and Chory (1992) proposed a plastid derived signal involved in coordinating nuclear and plastid genome expression. Recently, Hess et *al* (1994) have provided genetic evidence for the plastid derived signal necessary for the transcription of several nuclear genes in the ribosome-deficient plastids of *albostrains* of barley.

Nuclear induced ribosome-deficient altered plastids in variegation mutants of pearl millet lack plastid specific ribosomes, ribosomal RNAs, Rubisco large subunit (LSU)and thylakoid membrane organization (Reddy et *al* 1988; Sujatha and Subrahmanyam 1991). Genetic analysis of intercrosses between different white stripe and yellow stripe plants revealed at least 4 independent loci, any one of which in homozygous recessive condition leads to mutant phenotype(s) while the development of chlorophyll/chloroplasts is accomplished by the complementary interaction of dominant alleles at these loci (Karunasri and Subrahmanyam, 1994). These distinct mutants are deployed to study the expression of: (i) the plastid genes for 16s rRNA and RNA polymerase subunits necessary for plastid transcriptional

apparatus and for Rubisco LSU (*rbc* L), ii) D_1 protein gene *(psb A)* of thylakoid membranes and (iii) nuclear genes encoding components of chlorophyll a/b binding protein *(cab)* and FNR.

4.2. MATERIALS AND METHODS (covered in chapter *2)*.

4.3 RESULTS

4.3.1 Capacity for **RNA Synthesis**

Transcriptional ability of the plastids was checked *in vitro* by incorporation of (α-^{32}p) ATP into newly synthesized transcripts in transcriptionally active chromosomes (TACs) from both green and yellow plastids of IP 5009. RNA synthesis by TAC isolated from green plastids showed maximum activity within 20 min whereas over 2-fold less activity was found in TAC isolated from yellow plastids (Fig. 6).

4.3.2 Transcription of **Plastid** genes

Total RNAs from green and non-green siblings of five different genotypes were size fractionated on denaturing gels blotted and immobilized onto nylon membranes and hybridized with radio-labelled fragments of *16rDNA, rbcL* and *psbA* genes. Autoradiograms of northern blot hybrids are presented in Fig. 7. Close examination of the transcripts hybridizing to 16s *rDNA* probe revealed single size transcript in all the green seedlings (Fig. 7A, lanes 1,3,5,7,9) and no such transcript in yellow or white siblings (Fig.7a, lanes 2,4,6,8,10) derived from the genotypes *vi.vi* (IP 5009), vi_2vi_2 (IP 9712), $vi_3vi_3vi_4vi_4$ (VCM 36 and GWS 14), and *al al (albino)* respectively. Although all green

Figure 6: Plastid DNA bound RNA polymerase activity in green (•) and yellow (D) plastids from an yellow stripe mutant (IP 5009) of *Pennisetum glaucum* as measured by the incorporation of a- p ATP in their transcripts.

Figure 7: Northern blot hybridization of *16s rDNA* (A), *rbcL* (B), and *psb A* (C) transcripts in green (G) and yellow (Y)/white (W) seedlings from different genotypes of *Pennisetum glaucum*. Total RNA was hybridized with radio-labelled plastid specific *16s rDNA* from tobacco, a fragment of *rbcL* from rice and *psb A* from pea.

IP 5009 IP 9712 VCM 36 GWS 14 Albino

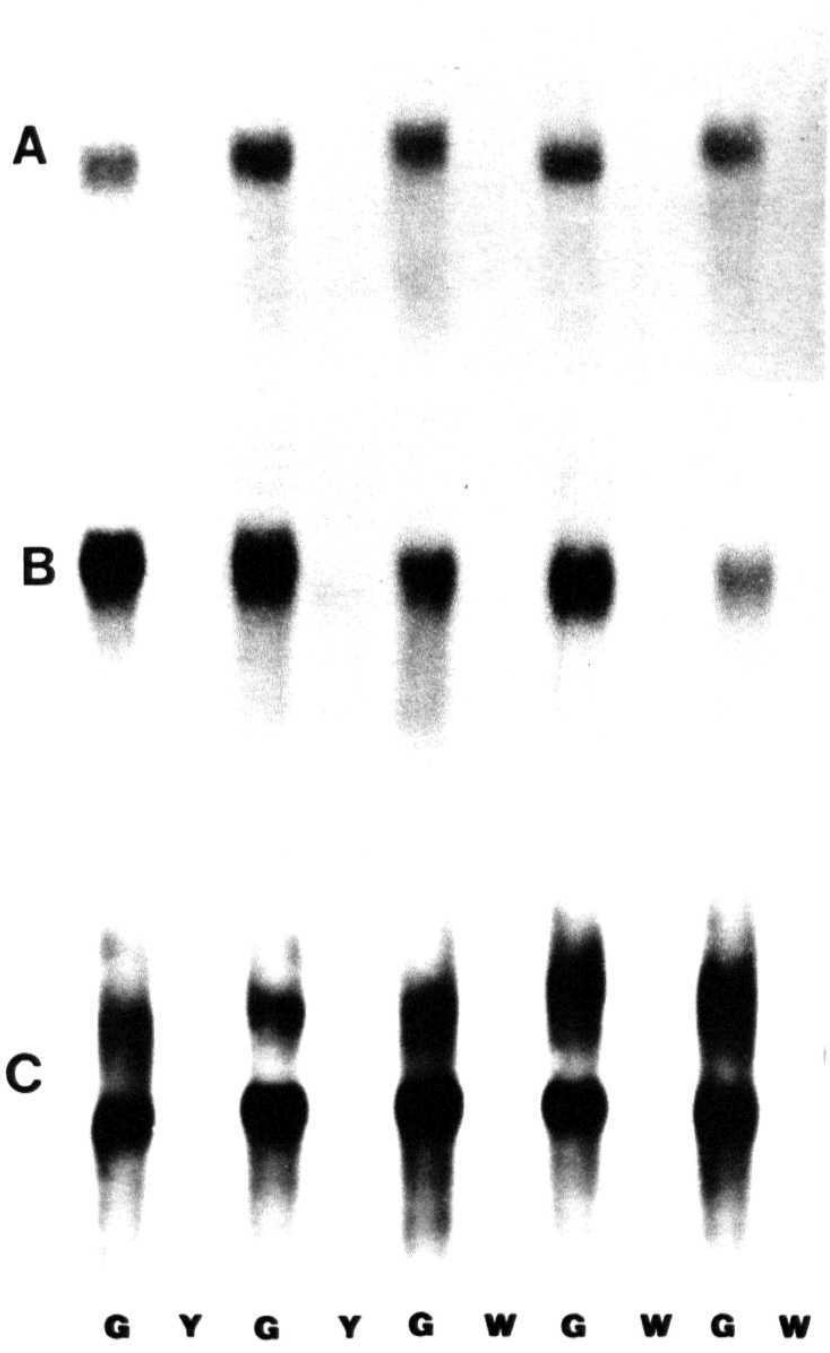


Fig. 7

seedlings, irrespective of their genotype had similar level of transcripts, there were differences in their transcript sizes.

When *rbcL,* a plastid gene encoding larger subunit of Rubisco was used as probe, uniform size transcripts were found in all green (G) seedlings (Fig. 7B) while their yellow (Y) or white (W) siblings lacked them (Fig. 7B).

Probing for the monocistronic plastid gene, *psbA* encoding the 32 kD herbicide binding D polypeptide of photosystem II (Barkan et *al* 1986) revealed transcripts of two sizes in all green (G) seedlings (Fig. 7C). These were absent in all the yellow (Y) and white (W) seedlings (Fig. 7C).

4.3.3 Plastid encoded RNA polymerase (RNAP-p)

Transcripts for plastid encoded RNA polymerase (RNAP-P) genes (*rpoA, rpoB)* in green (G) and non-green (yellow, Y/white, W) seedlings were checked by northern hybridization with PCR amplified gene probes from rice chloroplast genome. Green seedlings in general displayed comparable *rpo A* transcript size and abundance. However, the transcripts of *rpoA* were aberrant in all the chlorophyll deficient seedlings but differing in their size range and/or abundance among different genotypes (Fig. 8A). Yellow (Y) seedlings from IP 5009 *(vi vi)* displayed three-fold higher transcript level compared to their normal (G) siblings (Fig. 8A). In yellow (Y) seedlings from IP 9712 $(vi_2 vi)$ transcripts were at least 4-fold more abundant than their green (G) siblings (Fig. 8A). In VCM 36 $(vi_3 vi_4)$, the transcript size

Figure 8: Transcripts for plastid specific RNA polymerase subunit genes, *rpo A* (A) and rpo B (B) in green (G) and yellow (Y)/white (W) seedlings from different genotypes of *Pennisetum glaucum.* Northern blots (A and B) were hybridized with PCR amplified *rpo A* and *rpo B* gene probes from rice chloroplast clone Bank (A kind gift of Prof. M. Sugiura).

IP 5009 IP 9712 VCM 36 GWS 14 Albino

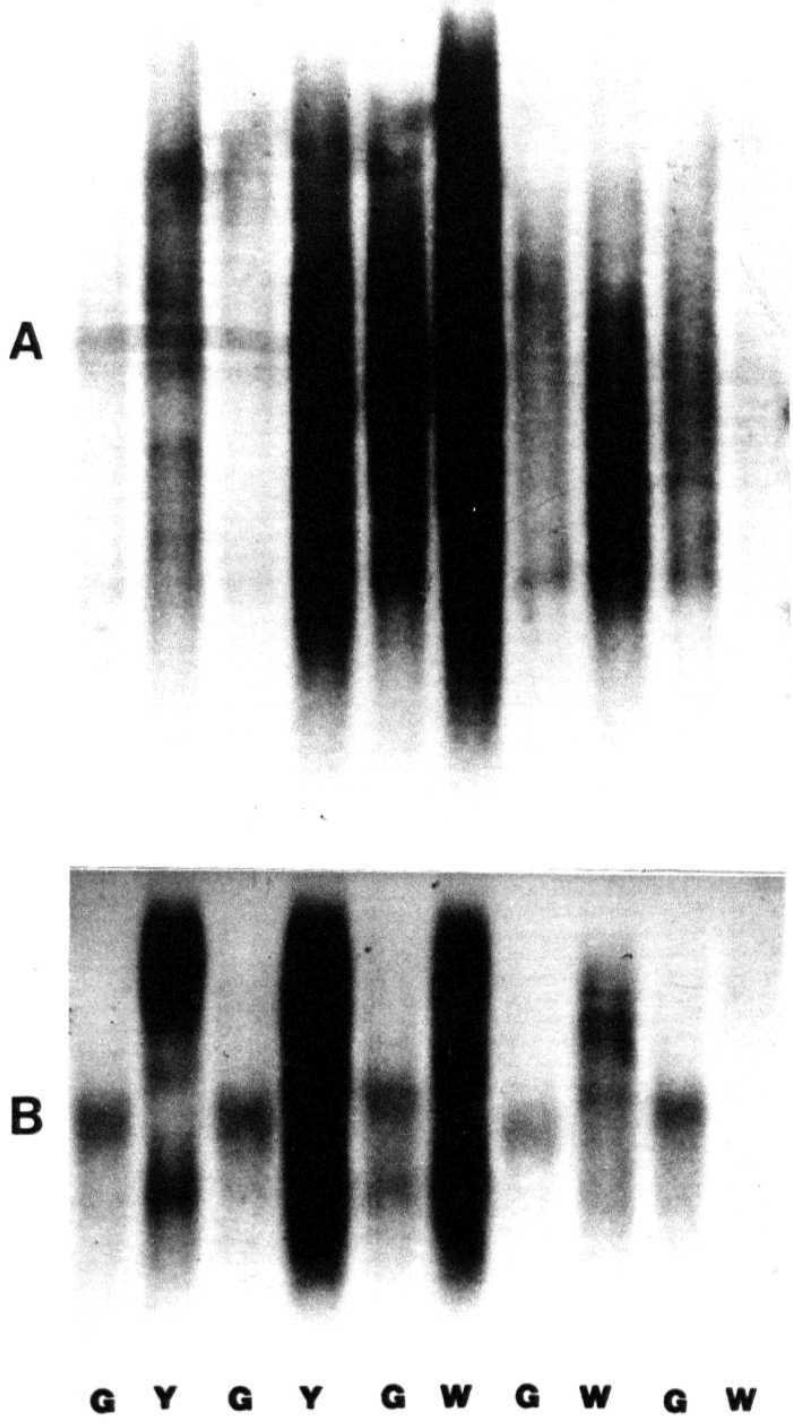


Fig. 8

range was narrow in green (G) seedlings as compared to the wide size range and 4-fold higher abundance found in their white (W) siblings (Fig. 8A). In green (G) and white (W) siblings from the white stripe mutant GWS 14 *(vi vi.)*, the size range of *rpoA* transcripts were similar to that of VCM 36 but the transcript abundance in white seedlings was 3-fold higher compared to their green siblings (Fig. 8A). In the *albino (al al)* mutant, mRNA for *rpoA* was 50% less than in their green siblings (Fig. 8A) with a narrow range of transcript sizes. Transcripts of *rpoA* among green (G) seedlings from the five different accessions can be placed in order of their abundance (VCM 36 > albino > GWS 14 > IP 9712 > IP 5009). Variation in size and abundance of transcripts among chlorophyll deficient (yellow, Y or white, W) seedlings from these genotypes displayed a different order for *rpoA* transcripts (VCM 36 > IP 9712 > GWS 14 >IP 5009 > *albino,* Fig. 8A).

RNA hybridized to PCR amplified radio-labelled 3.2 kb *rpo B* gene among different green and non-green seedlings are presented in Fig. 8B. In green (G) seedlings of the genotype *vi vi.* (IP 5009), a single processed transcript was found whereas transcripts of higher and lower molecular sizes were predominant and abundant in yellow (Y) siblings (Fig. 8B). Green (G) seedlings from IP 9712 and IP 5009 showed transcript which was similar in size and abundance. However, *rpoB* transcript levels were found to be at least 4-fold higher in yellow (Y) seedlings of IP 9712 compared to their green siblings (Fig. 8B). Transcripts of *rpoB* were of two different sizes in green (G) seedlings from VCM 36 whereas their white (W) siblings had an aberrant transcript

of high (3-fold) abundance (Fig. 8B). Transcripts of higher molecular size and abundance were also found in white (W) seedlings of GWS14 compared to that of their green (G) siblings (Fig. 8B). A single transcript was detectable in green (G) seedlings of *albino* mutant while white (W) siblings showed both aberrant transcript size and 50% less abundance (Fig. 8B).

A comparison of *rpoB* transcripts among green (G) seedlings of different genotypes revealed a single processed transcript in IP 5009 (vi_1), IP 9712 (vi_2), GWS 14 ($vi_3 vi_4$) and *albino* (*al*) and two transcripts in VCM 36 (*vi vi*). Aberrant transcripts were detectable in all the non-green (yellow (Y) or white (W)) seedlings but differed in their relative abundance (IP 9712 > VCM 36 > IP 5009 > GWS 14 > *albino).*

4.3.4 Plastid and nuclear encoded proteins

Immunoblots of total soluble proteins from green (G) and yellow (Y) or white (W) seedlings from different genotypes probed with polyclonal antibodies to LSU of Rubisco from maize are presented in Fig. 9A. A polypeptide of 55 kD corresponding to LSU of Rubisco was found in green siblings while it was absent in their corresponding non-green siblings (Fig. 9A, LanesY and W, Table 4).

Immunoblots of thylakoid membranes following separation on SDS-PAGE probed with LHCP-II antibodies enabled the detection of four subunits of molecular sizes 30 kD (a), 28 kD (b), 27 kD (c) and 24 kD (d) in their green (G) seedlings (Fig. 9B, all Gs).

Figure 9: Immunoblot analysis of plastid encoded rbc L nuclear encoded LHCP II complex and FNR in green (G) and yellow (Y)/white (W) seedlings from different genotypes of *Pennisetum glaucum*. Total soluble proteins (A) and thylakoid membrane proteins (B,C) from 7-day old seedlings were separated on SDS-PAGE and transferred to nitrocellulose membrane and probed with antibodies raised against LSU (A), whole complex of LHCP-II (B) and FNR (C) from maize.

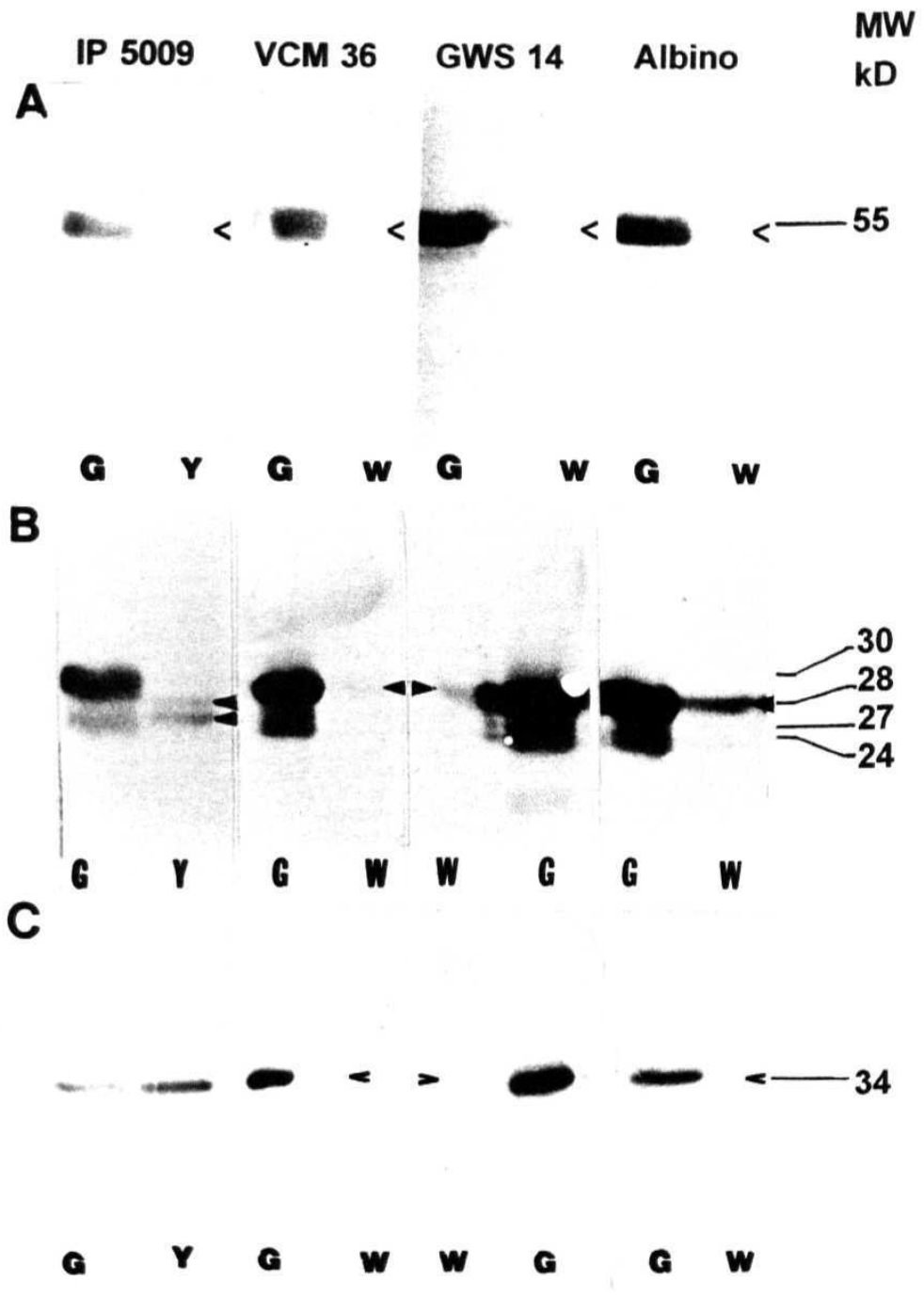

IP 5009
VCM 36
GWS 14
Albino
MW
kD
A
55
G Y G W G W G W
B
30
28
27
24
G Y G W W G G W
C
34
G Y G W W G G W

Fig. 9

Table 4: Plastid and nuclear gene specific products in green (G) and yellow (Y)/white (W) siblings of chlorophyll-deficient mutants of *Pennisetum glaucum* (L.) R. Br.

Source		Plastid genes[1]					Nuclear genes[2]				
		16s rRNA	rbcL	psbA	rpoB	rpoA	LHCP-II (kD) 30	28	27	24	FNR
IP 5009	G	+	+	+	+	+	+	+	+	+	+
	Y	-	-	-	+++	+++	-	-	+	+	+
IP 9712	G	+	+	+	+	+			nd		nd
	Y	-	-	-	++++	+++			nd		nd
VCM 36	G	+	+	+	+	+	+	+	+	+	+
	W	-	-	-	+++	++++	-	+	-	-	-
GWS 14	G	+	+	+	+	+	+	+	+	+	+
	W	-	-	-	++	+++	-	+	-	-	-
Albino	G	+	+	+	++	+++	+	+	+	+	+
	W	-	-	-	+	+	-	+	-	-	-

1-Transcriptional products; 2-Translational products; (+) present; (-) absent; nd-not determined

Yellow siblings of IP 5009 showed 27 kD (c) and 24 kD (d) subunits (Fig. 9B), while the white (W) seedlings from VCM 36, GWS 14 and *albino,* displayed 28 kD subunit only (Fig. 9B, all Ws, Table 4).

Western blot analysis of nuclear encoded FNR revealed a 34 kD polypeptide in the green (G) and yellow (W) siblings of IP 5009 and in the green seedlings of other accessions (Fig. 9C, all,Gs). The white seedlings from VCM 36, GWS 14 and *albino* lacked FNR (Fig. 9C, all Ws, Table 4).

4.4. DISCUSSION

Transcriptional activity in yellow plastids as ingreen plastids indicates plastid localized RNA polymerase activity inspite of the absence of plastid translational machinery (Fig. 6). This would imply that, nuclear encoded RNA polymerase (RNAP-N) transcribes plastid genes in the mutant plastids of pearl millet. However, the low level of transcription in the yellow seedlings (Fig. , all Ys and Ws) is either due to a slow rate of transcription or selective/preferential transcription of a few genes. The absence of transcripts of 16s *rDNA, rbcL* and *pbsA* genes (Fig. 7, Y and W) and abundant levels of *rpoA* and *rpoB* (Fig. 8A & B) demonstrate selective transcription of plastid coded RNA polymerase (RNAP-P) genes in the chlorophyll deficient seedlings.

The absence of 16s rRNA in plastids of yellow or white seedlings are consistent with earlier reports on pearl millet (Reddy *et al* 1988), ribosome deficient plastids of *iojap* mutant

of maize (Walbot and Coe 1979) and *albostrains* of barley (Hess et *al* 1992b) substantiating the absence of the plastid translational machinery in these mutants. Abundance of transcripts for RNAP-P subunit genes (*rpoA*, *rpoB)* in the absence of translational machinery in these ribosome-deficient mutants, implies that the transcription is carried out by the RNA polymerase of nuclear origin (RNAP-N). Abundance of aberrant transcripts could be a consequence of altered transcriptional levels and impaired post-transcriptional processing.

In chloroplasts of *Euglena gracilis* and higher plants, there are at least two RNA polymerase activities that are each capable of selective transcription of different classes of genes (Greenberg et *al* 1985). To explain the inconsistent results on the properties of the transcriptional apparatus in plastids (Gruissem 1989), it has been suggested that plastids might contain more than one kind of RNA polymerase (Bogorad 1992). Evidence for a nuclear-encoded plastid-localized RNA polymerase came from the study of *Epifagus*, a non-photosynthetic plant (de Pamphilis and Palmer 1990; Morden et *al* 1991). *Epifagus* plastids deficient for *rpoB, rpoC* and *rpoC*, genes accumulate rRNA. In ribosome-deficient plastids of *albostrains* of barley, accumulation of mRNA for *rpoB-rpoC -rpoC* was reported (Hess et *al*, 1993) suggesting that the *rpoB* operon is transcribed by the nuclear encoded plastid localized RNA polymerase. Our results further demonstrate that the RNAP-N transcribes genes for plastid encoded RNA polymerase (RNAP-P) subunits and suggests that RNAP-P is required for the transcription of other plastid encoded genes such as 16s *rDNA,*

rbcL and *psbA.*

Griffiths (1978) has shown that plastid provides NADPH essential for a ternary complex PCOR- NADPH - PClide, a prerequisite for the photoconversion of Pchlide to Chlide. In chloroplasts of higher plants, FNR binds to thylakoid membranes and participates in the photoreduction of NADP by mediating electron transfer from reduced Fd to NADP of the PS I (Nakataki and Shin 1991) thereby provides NADP essential for formation of ternary complex for photoconversion of Pchlide to Chlide. The absence of photosynthetic complexes and the presence of FNR in yellow seedlings (Fig. 9 B, C) in which Chlide accumulates (Chapter 3, Fig. 3, Table 3) suggests that the reducing power (NADPH) for PCOR activity (conversion of Pchlide to Chlide) might have been imported into the plastids.

From the results presented in the preceding chapter (Chapter 3), the two white stripe mutants (GWS14 and VCM36) are homozygous recessive for two loci *(vi vi vi vi)* controlling chlorophyll synthesis (Chapter 3, Table 3). As discussed earlier, one of these two loci likely codes for PCOR. It is now proposed that the other locus may code for FNR which reduces NADP to NADPH and the production of FNR and PCOR are coregulated.

Block(s) in the chlorophyll biosynthetic pathway (Chapter 3) resulting in the absence of chlorophyll suggests that the enzymes catalyzing specific steps in the pathway are controlled by nuclear genes (Table 3, Chapter 3). Thus the expression of the nuclear genes encoding plastid proteins was examined in ribosome deficient plastid mutants of pearl millet. Chlorophyll a/b binding proteins *(Cab)* form the light harvesting complexes of PS I and II (LHCP I

and II). These are the most abundant thylakoid membrane proteins synthesized on cytosolic ribosomes as precursor polypeptides, transported into the plastid compartment and are further processed and integrated into chlorophyll-protein complexes. Absence of 30 kD polypeptide in all these chlorophyll deficient mutants of pearl millet suggests that chlorophyll synthesis triggers the production of the LHCP-II proteins. The presence of 27 kD and 24 kD polypeptides (subunits c and d) of LHCP-II in the yellow seedlings (Fig. 9B) in which Chlide is synthesized (Chapter 3, Fig. 3, Table 3) and the absence in the white seedlings (Fig. 9B) in which Chlide is not synthesised indicate that Chlide is the likely signal required for the synthesis of these two polypeptides. However, the present data are insufficient to explain the presence of 28 kD polypeptide in white as well as in normal seedlings and its absence in yellow seedlings. These findings are consistent with the observations on *Chlamydomonas reinhardtii* (Johanningmeier 1988) where an accumulation of intermediates of the later steps of chlorophyll biosynthesis was proposed to be involved in the regulation of the transcription of nuclear genes. Thus it is also proposed from the present study that the signal molecules for different nuclear encoded LHCP-II polypeptides are different.

5. PLASTID GENOME ORGANIZATION

INTRODUCTION 5.1

The first physical (restriction) map of chloroplast genome was established by Bedbrook and Bogorad (1976) and cloning of the first rRNA gene was achieved by Bedbrook et *al* (1978). The unique feature of chloroplast genome is the presence of inverted repeat segments (IR_A and IR_B), separated by large and small single copy regions (LSC and SSC) respectively (Sugita et *al* 1984). Most plastid genomes are circular and range in size from 120 to 217 kb (Palmer 1990). Variation in genome size is related primarily to the size of inverted repeat (IR) in the plastid genome and its coding capacity. Complete sequence information of plastid genomes is available for tobacco (Shinozaki et *al* 1986), liverwort (Ohyama et *al* 1986) rice (*Oryza sativa)* (Hiratsuka et *al* 1989), maize and several other plants (see Maier et *al* 1995). Copy number per plastid ranges from 22 to 300 depending on the developmental stage (Bendich 1987). Studies on a wide range of species led to the establishment of consensus chloroplast gene order among vascular land plants identical to that found in tobacco. Data available for the cereals (wheat, maize and rice) show that their chloroplast genomes have diverged from the consensus gene order through a series of overlapping inversions within the LSC (Hiratsuka et *al* 1989). Plastid DNA encodes approximately 135 genes that fall into three major functional categories: genes encoding proteins and RNAs involved in transcription and translation of the plastid genome (4 RNA polymerase subunits, 30-31 tRNAs, 4 rRNAs, 20 ribosomal proteins,

initiation factor 1), genes encoding proteins of the photosynthetic apparatus (Rubisco, PS I, PS II, ATP synthase, cytochrome b /f complex) and genes encoding protein of the NADH oxidoreductase complex. Most plastid genes are organized in complex operons that are conserved among plastid genomes (Sugiura, 1992; Mullet, 1993; Maier et *al* 1995) and their transcription plays a central role in establishing the levels of many plastid mRNAs and proteins. Variations in plastid mRNA stability also significantly influences plastid gene expression (Rapp et *al* 1992).

In pearl millet, nuclear gene induced plastid mutants are known (Subrahmanyam et *al* 1986), Genetic analysis following intercrosses among different stripe mutants revealed at least 4 independent loci controlling the development of chlorophyll in otherwise defective plastids (Karunasri and Subrahmanyam 1994). Ultrastructural and molecular studies of these mutant plastids revealed the absence of (i) chlorophyll (Subrahmanyam et *al* 1986), (ii) Plastid coded *psbA*, *16srDNA* and *rbcL* transcripts (iii) thylakoid membrane organisation (iv) plastid specific ribosomes (Reddy and Subrahmanyam 1988a), (v) Rubisco LSU (Sujatha and Subrahmanyam 1991), (vi) the nuclear encoded polypeptides of LHCP-II (vii) nuclear encoded FNR and (viii) presence of aberrant and abundant transcripts of plastid encoded RNA polymerase genes *rpoA* and *rpoB* (Chapter 4).

To examine of there transcriptional deficiencies related to any replicational alterations in the plastomes, DNA specific

staining and plastid specific DNA polymerase activity were examined. Southern blot hybridizations of genomic DNA were carried out using plastid specific genes (*16srDNA*, *rbcl*,*rpoA* and *rpoB)* as probes to identify plastome structural rearrangements in the green and yellow/white seedlings derived from different stripe mutants.

5.2 **MATERIALS AND METHODS**

(covered in sections 2.11 to 2.14)

5.3. RESULTS

5.3.1 **DAPI** staining and visualization of plastid **DNA**

Intact protoplasts were isolated from green and yellow seedlings from the yellow stripe mutant (IP 5009) of *Pennisetum glaucum*, stained with DNA fluorochrome DAPI (4',6-diamidino-2-phenylindole) according to Coleman *et al* (1981) for the visual quantification of plastid DNA using a microscope with UV filter. Photomicrographs of DAPI stained intact and broken protoplasts from both green and yellow seedlings are presented in Fig. 10. Plastid nucleoids are visualised after DAPI staining due to their compactness. The plastids from green and yellow seedlings showed differences in the relative DNA content in the two plastid types. The plastids from yellow seedlings (Fig. 10 E,F) were smaller than those from their green siblings (Fig. 10 C,D).

5.3.2 **DNA** Polymerase activity

Plastid specific DNA polymerase was partially purified from plastid lysates of green and yellow seedlings of the yellow

Figure 10: DAPI (4,6-diamidino-2-phenyl indole) staining of protoplasts isolated from green and yellow seedlings of IP 5009. Note plastid DNA (orange colour) and nuclear DNA (blue colour). **(Panel A** - Protoplasts from immature cells; B - Intact protoplasts from green seedlings; C - Partially disrupted protoplasts from seedlings; D -Plastids from green seedlings; **E-**Protoplasts from yellow seedlings; **F-**Plastids from yellow seedlings).

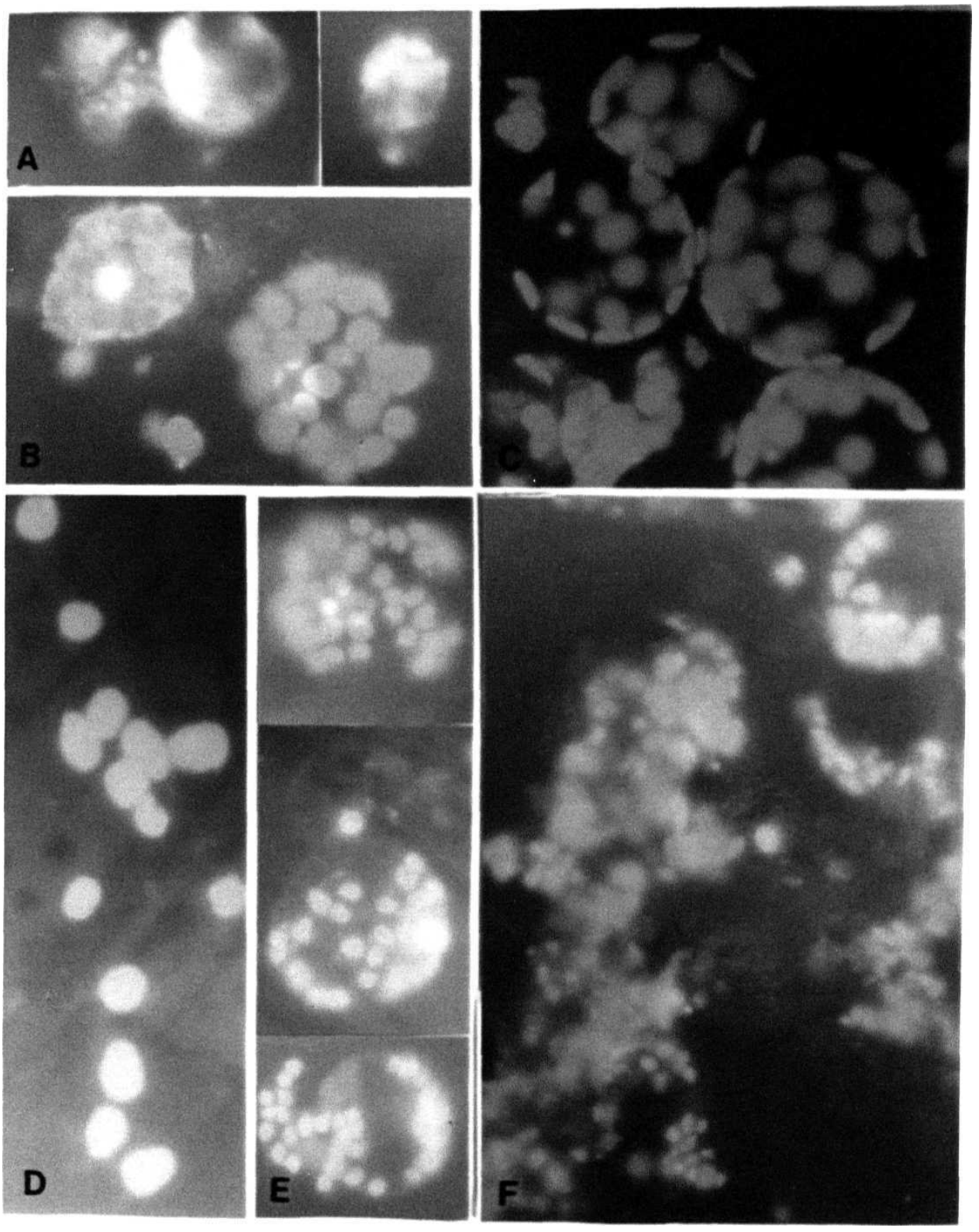


Fig. 10

stripe mutant of *Pennisetum glaucum* (Sec. 2.11). **DNA** polymerase activity was assayed *in vitro* by the incorporation of H dTTP **into the** newly synthesized DNA/replicating DNA. DNA polymerase activity was found in both green and yellow plastids (Fig. 11). In plastids from yellow seedlings the DNA polymerase activity was over 2 folds higher than in plastids from their **green siblings (Fig. 11).**

5.3.3 Southern Blot Hybridizations

DNAs from green and yellow siblings from IP 5009 & IP 9712 and green and white siblings from GWS 14 and VCM 36 variegation mutants were digested with Bgl I and/or Bam HI, size fractionated on 1.1% agarose gels and blotted onto nylon membranes. Southern blots were hybridized with α ^{32}P dCTP labelled plastid specific *16 s rDNA, rbcL*, *rpoA* and *rpoB* probes from rice. Autoradiograms were used to examine restriction fragment length polymorphisms between green and chlorophyll deficient siblings within each genotype among different accessions/genotypes with respect to each of the plastid gene specific probes.

Autoradiograms of Southern hybridizations of genomic DNAs from green and yellow/white siblings from different mutants with labelled plastid specific 16s rDNA probe are shown in Fig. 12. Results are summarized in Table 5. Hybridization with Bgl I digests revealed a 2.9 kb fragment with high signal intensity in all the genotypes irrespective of the phenotypes (Fig. 12 A,B). Bgl I digests of GWS14 white seedlings showed additional bands of 1.5 kb and 1.2 kb (Fig. 12 A, lanes 7 & 10) whereas VCM 36 white

Figure 11: Activity of partially purified DNA polymerase in green (•) and yellow (D) plastids of IP 5009 as measured by the incorporation of H-TTP into the newly synthesized DNA.

Partially purified DNA polymerase activity from green and yellow plastids

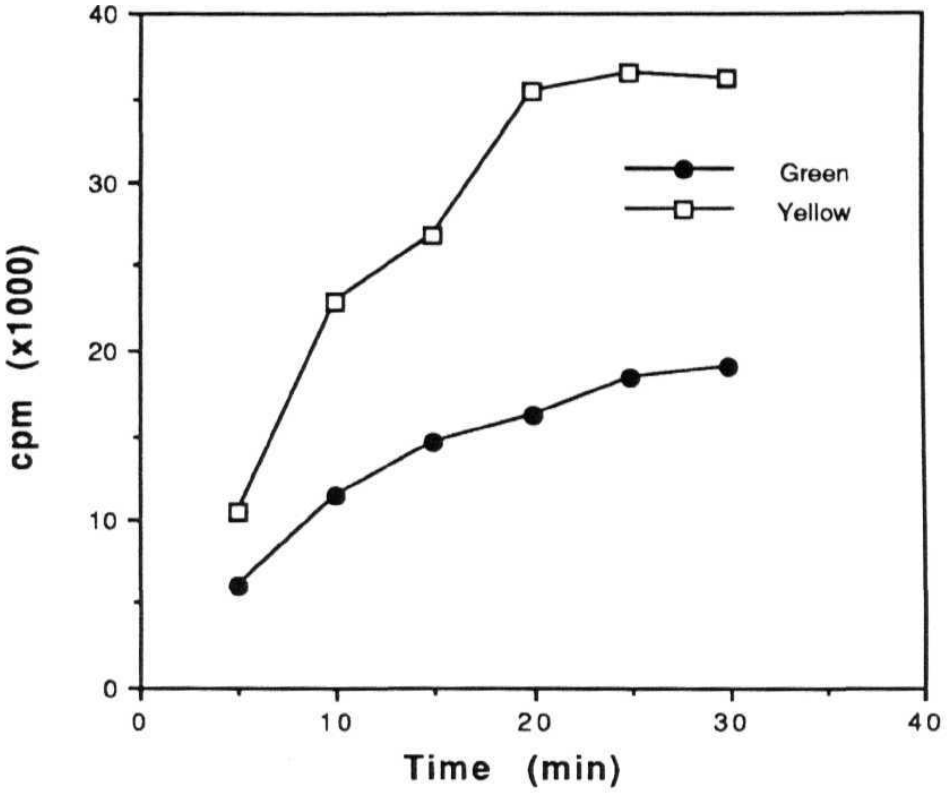
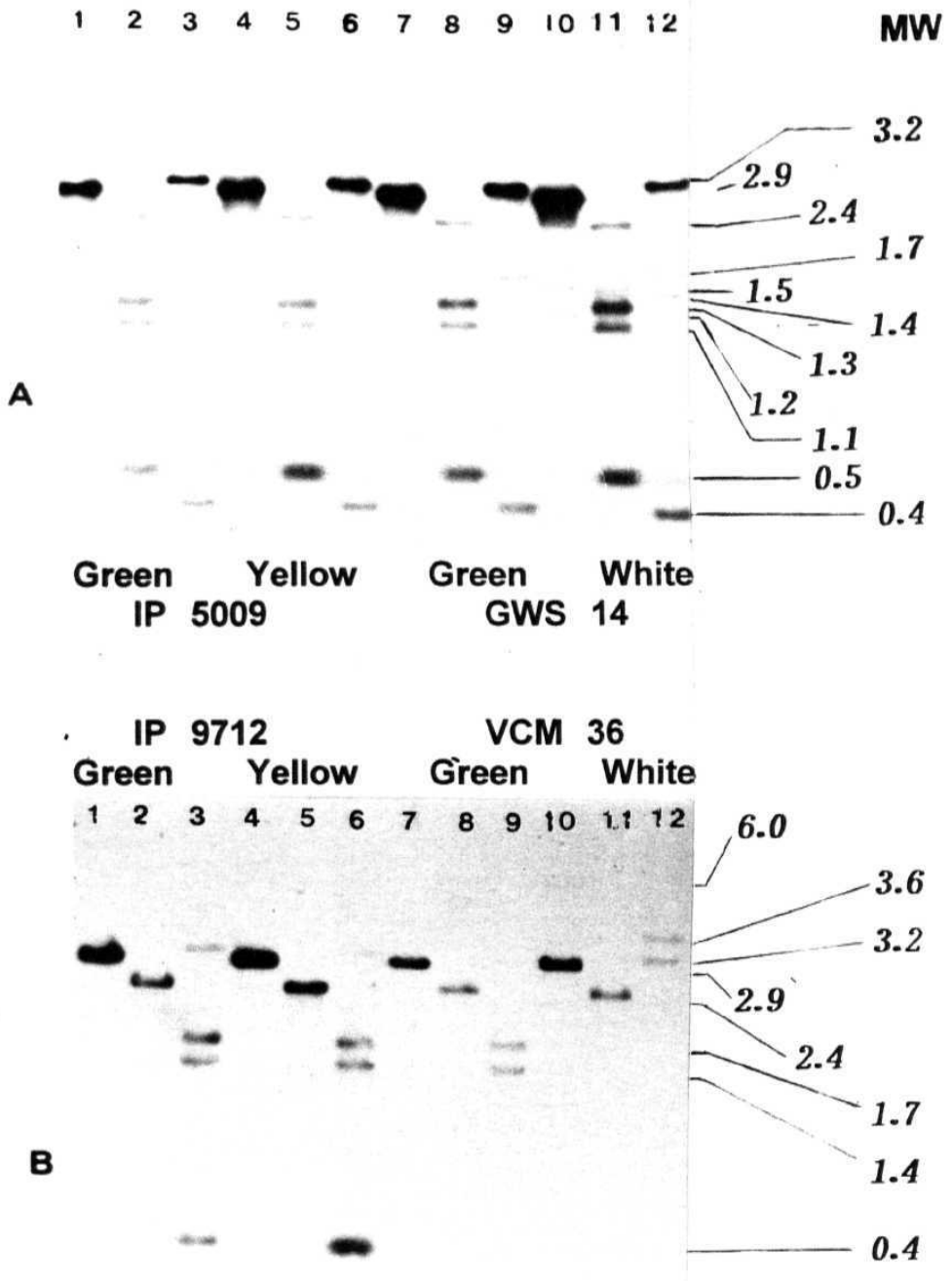


Fig. 11

Figure 12: Autoradiograms of Southern blots of genomic DNA from green and yellow/white seedlings from IP 5009 and GWS14 (A) and IP 9712 and VCM36 (B), hybridized to plastid encoded 16s rDNA probe from tobacco. (Lanes 1, 4, 7, 10 - Bgl I digests; Lanes 3, 6, 9, 12 - Bam HI digests; Lanes 2, 5, 8, 11 - Double digests).


1 2 3 4 5 6 7 8 9 10 11 12
MW
3.2
2.9
2.4
1.7
1.5
1.4
1.3
1.2
1.1
0.5
0.4
A
Green Yellow Green White
IP 5009 GWS 14
IP 9712 VCM 36
Green Yellow Green White
1 2 3 4 5 6 7 8 9 10 11 12
6.0
3.6
3.2
2.9
2.4
1.7
1.4
0.4
B

. Fig. 12

Table 5: Restriction fragments from total genomic DNA from different accessories of *Pennisetum glaucum* hybridized to the 0.7 kb 16s rDNA probe from tobacco

Fragment			Accession/phenotype							
			IP 5009		IP 9712		VCM-36		GWS-14	
Bgl I	Bgl I Bam HI	Bam HI (kb)	G	Y	G	Y	G	W	G	W
6.0								+		
		3.6						+		
		3.2	++	++	+	+		+	++	++
2.9			++	++	++	++	++	++	++	++
	2.4		+	+	++	++	++	++	++	++
		1.7			+	+	+			
1.5										+
		1.4			+	+				+
	1.3		++	++					++	++
1.2										+
	1.1		++	++					++	++
	0.5		+	++					++	++
		0.4	++	++	+	++			++	++

(+) Present; (++) intensity; G- Green; Y-yellow; W-white.

seedlings revealed a band corresponding to 6.0 kb Bgl I fragment (Fig. 12 B, lane 10). No differences in hybridization pattern were found between green and yellow siblings of yellow stripe mutants (Figs. 12 A, B lanes 1 & 4).

Hybridization of 16s *rDNA* probe to Bam HI digests of DNAs from different genotypes are presented in Fig. 12A & B. In IP 5009 green and yellow siblings two Bgl I fragments (3.2 kb and 0.4 kb) hybridized to the probe (Fig. 12A, lanes 3 & 6). The signal intensity of the 3.2 kb was found to be higher. Green and yellow seedlings from IP 9712 revealed two more (1.7 and 1.4 kb) fragments (Fig. 12 B, lanes 3 & 6). Bam HI digests of VCM 36 green revealed 1.7kb and 1.4 kb fragments hybridizing to the probe whereas VCM 36 white seedlings showed 3.6 kb and 3.2 kb bands (Fig. 12 B, lanes 9 & 12). Bam HI digests of both green and white siblings of GWS 14 showed 3.2 kb, 1.7 kb, 1.4 kb and 0.4 kb bands (Fig. 12 A, lanes 9 & 12).

Bgl I and Bam HI double digests of IP 5009 and GWS 14 showed bands corresponding to 2.4 kb 1.3 kb, 1.1 kb and 0.5kb (Fig. 12A, lanes 2,5,8,11). Moreover an additional fragment of 1.5 kb was found in GWS 14 white while IP 9712, and VCM 36 showed a single band corresponding to 2.4 kb (Fig. 12B, lanes 2,5,8,11). No discernible differences were observed between green and yellow/white siblings of each accession.

Results of the hybridizations with *rbcL* gene probe are presented in Figs. 10 A and B. Bgl I digests of green and yellow siblings from IP 5009 and green and white siblings from GWS 14

showed bands corresponding to 12.3 kb and 10.1 kb (Fig. 13A, lanes 1,3,7,10), whereas band corresponding to 10.1 kb Bgl I fragment was absent in IP 9712 and VCM 36 (Fig. 13B, lanes 1,4,7,10). Bam HI fragments of 6.2 kb and 3.3 kb were found to hybridize to rbcL probe in IP 5009, IP 9712 and GWS 14 (Fig 13A & B, lanes 3,6,9,12). VCM36 green and white siblings showed only the 3.3 kb band (Fig. 13B, lanes 9 & 12). The signal intensity of 3.3 kb band was at least twice that of the 6.2 kb band. Double digests of IP 5009 and GWS 14 revealed 3 bands of 6.2 kb, 3.8 kb and 3.3 kb (Fig. 13A lanes 2,5,8,11) whereas in the double digests of the IP 9712 and VCM 36 3.8 kb fragment did not light up. The signal intensity of 3.3 kb band was found to be higher than 3.8 kb & 6.0 kb bands in IP 5009, GWS 14 and IP 9712 (Fig. 13B lanes 2,5,8,11). In VCM 36 white, the intensity of the 3.3 kb and 6.2 kb fragments was more than 3.3 kb fragment (Fig. 13B, lanes 8,11).

Southern blots of restricted DNAs from green and chlorophyll-deficient seedlings from different accessions / genotypes were hybridized with labelled plastid specific, PCR amplified *rpoA* (1.0 kb) and *rpoB* (3.2 kb) gene sequences from rice chloroplast genome.

Southern blot hybrids of genomic DNA from green (G) and Yellow (Y) / white (W) seedlings from different stripe mutants probed with plastid specific *rpoA* gene are shown in Fig. 14 A & B. Bgl I digests of DNA from all the accessions showed 18.2 kb fragment hybridizing to *rpoA* (Figs. 14 A & B, lanes 1,4,7,10),

Figure 13: Southern blot hybridization of genomic DNA from green and yellow/white seedlings from IP 5009 and GWS14 (A) and IP 9712 and VCM36 (B) to plastid encoded rbc L probe from rice. (Lanes 1, 4, 7, 10 - Bgl I digests; Lanes 3, 6, 9, 12 - Bam HI digests; Lanes 2, 5, 8, 11 - Double digests).

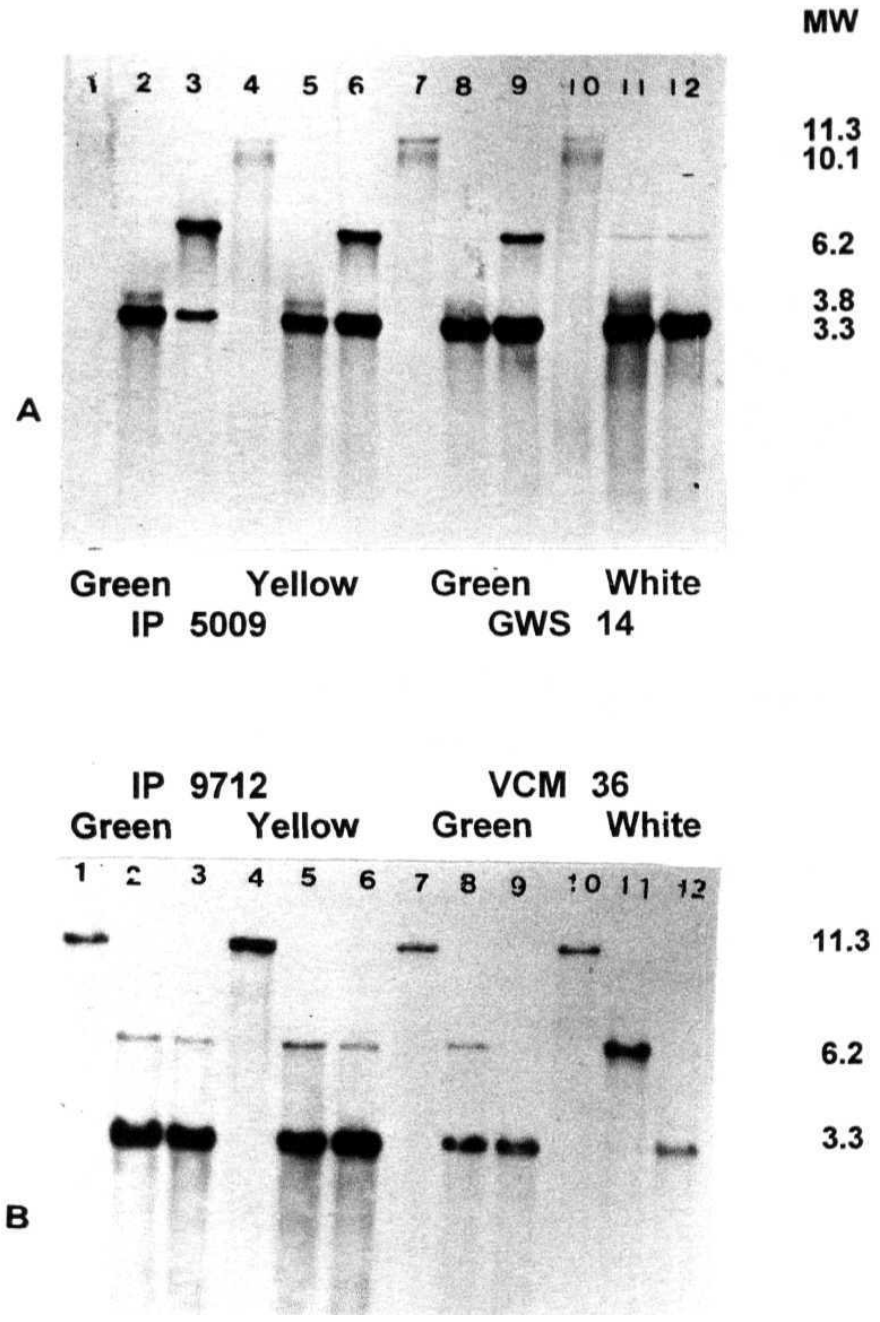

MW
1 2 3 4 5 6 7 8 9 10 11 12
11.3
10.1
6.2
3.8
3.3
A
Green Yellow Green White
IP 5009 GWS 14
IP 9712 VCM 36
Green Yellow Green White
1 2 3 4 5 6 7 8 9 10 11 12
11.3
6.2
3.3
B

Fig. 13

whereas **Bam** HI digests revealed two (1.5 kb and 1.3 kb) fragments hybridizing to the probe (Figs. 14 A & B, lanes 3,6,9,12). Double digests displayed hybridizations similar to **Bam** HI digests with 1.5 kb and 1.4 kb bands (Figs. 14 A & B, lanes 2,5,8,11). In both Bam HI digests and double digests, the signal intensity of the 1.3 kb fragment was higher than the other fragments.

Autoradiograms of Southern blots of genomic DNA from different genotypes hybridized to plastid specific *rpoB* probe **are** presented in Fig. 15 A & B. The results are summarised in Table 6.

Bgl I digests of DNA from both green and yellow seedlings of IP 5009 revealed similar hybridization patterns with *rpoB* showing bands corresponding to 12.3 kb, 11.0 kb, 5.0 kb, 3.0 kb (Fig 15 A, lanes 1 & 4). In green and white siblings of GWS 14, additional Bgl I fragments (1.9 kb, and 0.7 kb) hybridized to the probe (Fig. 15 A, lanes 7 & 10). Bgl I digests of green and yellow siblings of IP 9712 revealed similar hybridizing patterns as that of GWS 14 (Fig 15 B, lanes 1 & 4). The intensities of 1.9 kb and 0.7 kb fragments were 2 fold higher in green seedlings as compared to yellow seedlings from IP 9712 (Fig 15 B, lanes 1 & 4). In green and white siblings of VCM 36, the 1.7 kb and 0.7 kb bands were absent (Fig. 15 B, lanes 7 & 10). The intensity of 12.3 kb and 5.0 kb bands showed higher intensity compared to other bands. No differences were found between green and white siblings of VCM36. Comparison of hybridization patterns among the four genotypes revealed differences between different genotypes / accessions (Fig. 15 A & B; lanes 1,4,7,10, Table 6). A comparison

Figure 14: Southern blot hybrids of genomic DNA from green and yellow/white seedlings from IP 5009 and GWS 14 (A) and IP 9712 and VCM-36 (B) probed with plastid encoded rpo A gene from rice. (Lanes 1, 4, 7, 10 - Bgl I digests; Lanes 3, 6, 9, 12 - Bam HI digests; Lanes 2, 5, 8, 11 - Double digests).

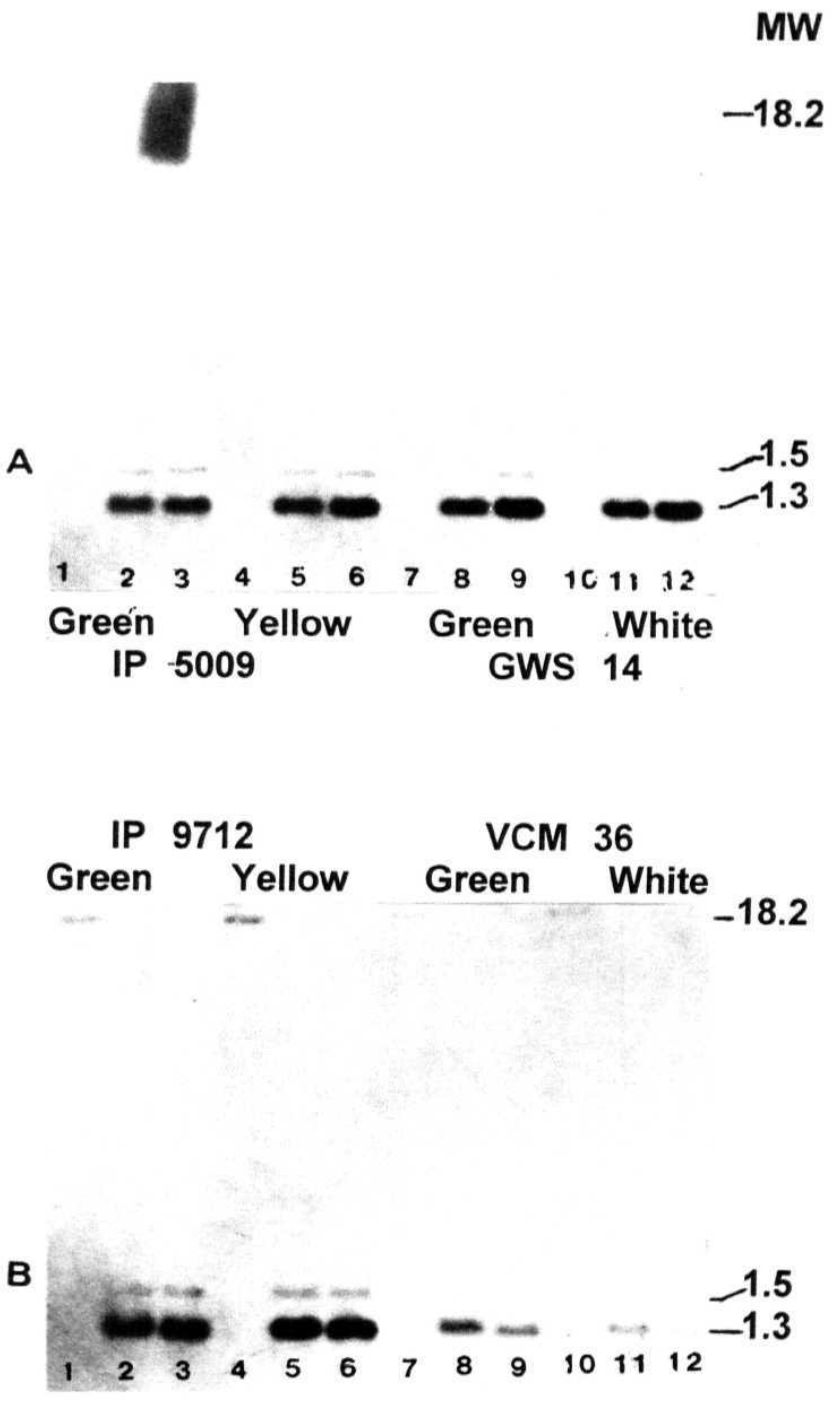

MW
18.2
A
1.5
1.3
1 2 3 4 5 6 7 8 9 10 11 12
Green Yellow Green White
IP 5009 GWS 14
IP 9712 VCM 36
Green Yellow Green White
18.2
B
1.5
1.3
1 2 3 4 5 6 7 8 9 10 11 12

Fig. 14

Figure 15: Autoradiograms of Southern blots of genomic DNA from green and yellow/white seedlings from IP 5009 and GWS14 (A) and IP 9712 and VCM36 (B) hybridized with plastid encoded rpo B probe from rice: (Lanes 1, 4, 7, 10 - Bgl I digests; Lanes 3, 6, 9, 12 - Bam HI digests; Lanes 2, 5, 8, 11 - Double digests).

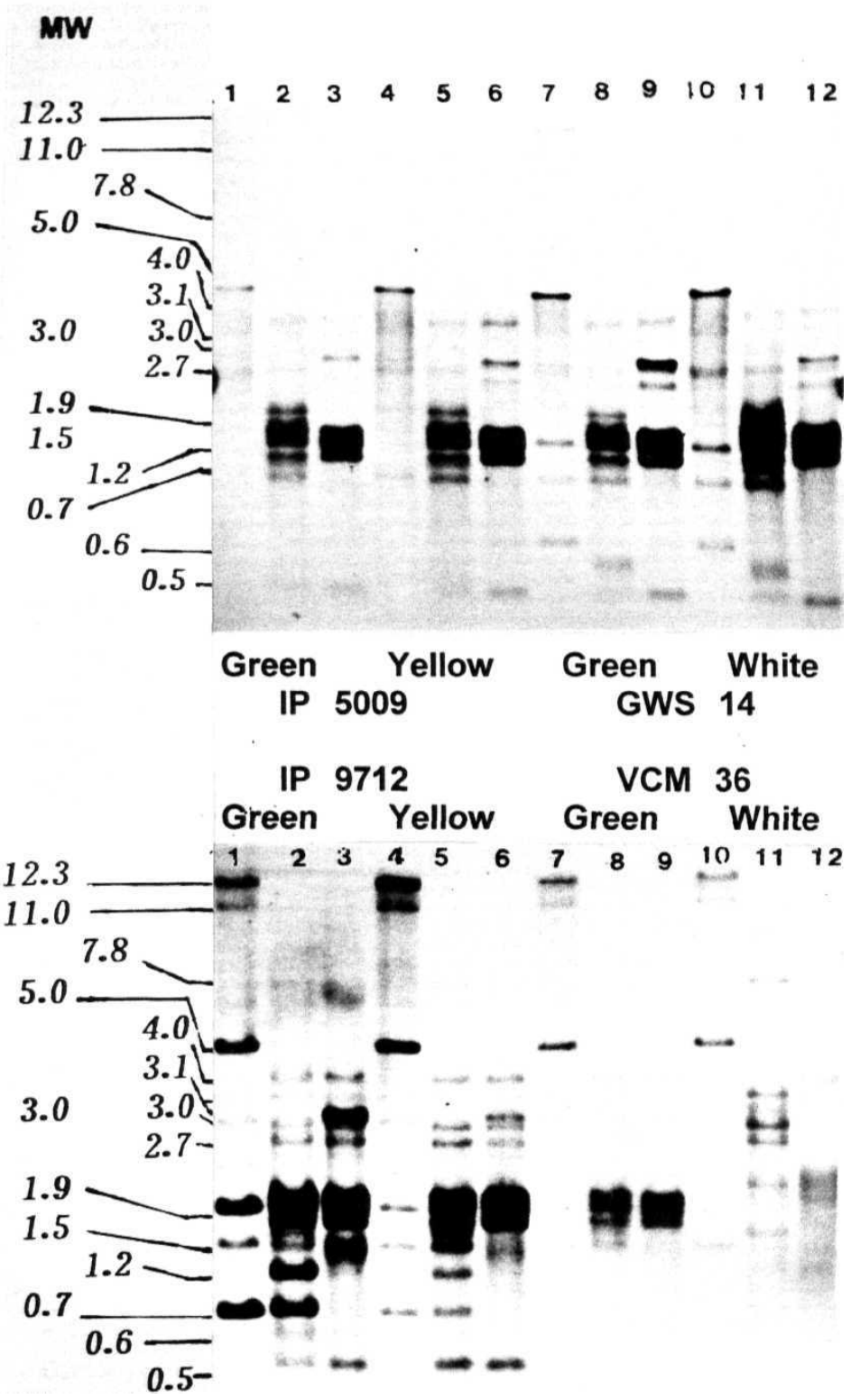

MW
1 2 3 4 5 6 7 8 9 10 11 12
12.3
11.0
7.8
5.0
4.0
3.1
3.0
3.0
2.7
1.9
1.5
1.2
0.7
0.6
0.5
Green Yellow Green White
IP 5009 GWS 14
IP 9712 VCM 36
Green Yellow Green White
1 2 3 4 5 6 7 8 9 10 11 12
12.3
11.0
7.8
5.0
4.0
3.1
3.0
3.0
2.7
1.9
1.5
1.2
0.7
0.6
0.5

Fig. 15

Capacity of RNA synthesis by TAC isolated from green and yellow plastids

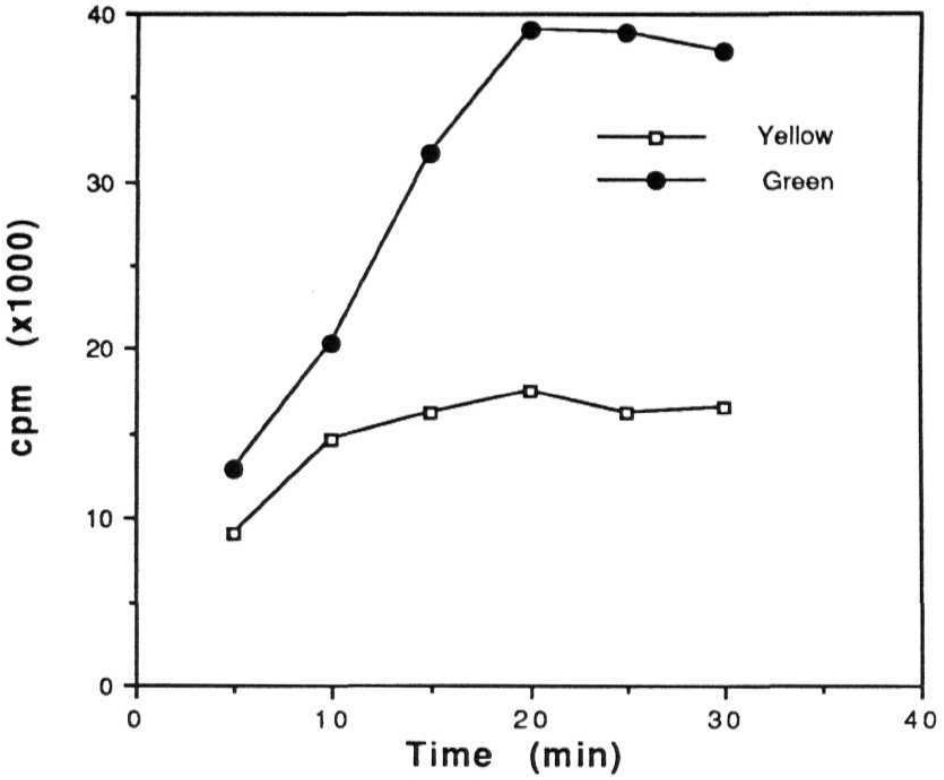


Fig. 6

Table 6: Restriction fragments from total genomic DNA from different accessions of *Pennisetum glaucum* hybridized to the 3.2 kb rpoB probe from rice

Fragment			Accession/phenotype							
			IP 5009		IP 9712		VCM-36		GWS-14	
Bgl I	Bgl I Bam HI	Bam HI (kb)	G	Y	G	Y	G	W	G	W
12.3			+	+	++	++	++	++	+	+
11.0			+	+	+	+	+	+	+	+
	7.8		+					+		
5.0			++	++	++	++	++	++	++	++
		4.0	+	+	+	+	+	+	+	+
		3.1	++	++	++	++	++		++	++
3.0		3.0	+	+	+	+	+	+	+	+
		2.7			+	+			+	+
1.9					+++	+			++	++
1.5			+	+	+	+	+	+	+	+
		1.4	+++	+++	+++	+			+++	+++
	1.2		+	+	+++	+			+	+
0.7					+++	+			+	+
	0.6				+	+			+	+
		0.5	+	+	++	++	+		+	+

(+) Present; (++) intensity; G- Green; Y-yellow; W-white.

of hybridization pattern between yellow stripe mutants IP 5009 and IP 9712 revealed the presence of additional bands of 1.7 kb and 0.7 kb in IP 9712 and GWS 14 differed from VCM 36 in having 1.7 kb and 0.7 kb fragments.

Bam HI digests of different genotypes hybridizing to the probe are presented in Fig. 15 A & B, lanes 3,6,9,12. In IP 5009 green and yellow, Bam HI fragments of 4.0 kb, 3.1 kb, 3.0 kb, 2.7 kb, 1.4 kb and 0.5 kb hybridized with the probe. The 3.1 kb band showed 2 fold higher intensity than the other bands (Fig. 15 A & B, lanes 3 & 6). No differences were discernible between green and yellow siblings of IP 5009. IP 9712 showed an additional band of 1.3 kb, which was absent in IP 5009 (Fig 15 B, lanes 3 & 6). In VCM 36 green seedlings , Bam HI fragments of 4.0 kb, 3.1 kb, and 0.5 kb were found whereas white seedlings were characterized by the absence of 0.5 kb fragment (Fig. 15 B, lanes 9 & 12) and by the presence of fragments in the size range of 2.3 to 1.7 kb.

Double digests revealed additional bands hybridizing to *rpoB* probe. IP 9712 showed 2 additional bands of 1.2 kb and 0.6 kb (Fig. 15 B, lanes 2 & 5). The signal intensity of 1.2 kb and 0.7 kb bands in IP 9712 green were at least 2-3 fold higher than in their yellow siblings (Fig 15 B, lanes 2 & 5; Table 6). In GWS14 white, an additional band of 1.2 kb was detected in comparison with their green siblings (Fig. 15 A, lanes 8 & 11). In VCM 36 green, bands corresponding to 4.0 kb 3.0 kb, 2.0 kb, 1.9 kb, 1.8 kb, 1.7 kb, 1.5 kb, 1.3 kb and 0.5 kb hybridized to the probe.

The intensity of 2.0 kb to 1.5 kb range fragments were higher than other bands (Fig 15 B, lane 8) . VCM 36 white revealed the presence of additional bands of 10.3 kb, 7.8 kb, 3.7 kb, 3.4 kb and 1.4 kb (Fig 15B, lane 11) **with the** 3.0 kb **fragment** showing higher signal intensity.

5.3.4. DISCUSSION

Detection of DNA in both green and yellow plastids following DAPI staining (Fig. 11; B,C,D,E,F) is consistent **with earlier** observations in the stripe mutant IP 5009 (Reddy et *al* 1988). DAPI is a DNA fluorochrome used for the detection and measurement of nuclear and organellar DNA in plants (Coleman et *al* 1981; Galbraith 1990; Sodmergen et *al* 1992; Nakamura et *al* 1992; Suzuki et *al* 1992; Zhu et *al* 1992; Izumi et *al* 1993; Sodmergen et *al* 1994) . The DNA - DAPI fluorescence was shown to have 20 fold increase as compared to DAPI alone (Lin et *al* 1977; Kapuscinski and Skwozylas 1977) and the fluorescence emitted is directly proportional to the DNA content.

The relatively low DNA content in the yellow plastids (Fig. 10, E,F) as compared to green plastids could be a reflection of differences in the genome copy number. These results are consistent with the observations in w2 mutant of maize where the relative plastid DNA content in the mutant was found to be 20 fold lower than in normal seedlings (Han et *al* 1993).

DNA polymerase activity in the ribosome deficient yellow plastids (Fig. 11) is indicative of a functional replication

machinery of cytosolic origin. However, the > 2 fold increase in the DNA polymerase activity in yellow plastids as compared to that of green plastids (Fig. 11) does not correspond well with the plastid DNA content (Fig. 10 E,F).

Following Southern hybridization of genomic DNAs from different stripe accessions of P. *glaucum* with plastid specific probes from rice, it is evident that there are considerable differences in plastid DNA organization between different genotypes and between phenotypes to a lesser extent.

Hybridization of 0.7 kb 16s rDNA probe only to 2.9 kb Bgl I fragment in IP 5009, IP 9712, VCM 36 (Fig. 12 A & B, lanes 1,4,7,10) and to three Bgl I fragments of 2.9 kb, 1.5 kb, and 1.2 kb in GWS 14 (Fig. 12 A lanes 7 & 10) suggests that there are no Bgl I sites available in 26s *rDNA* in IP 5009, IP 9712, VCM 36 and that there are at least two Bgl I sites in the *16s rDNA* of GWS 14. No differences were detected in Bam HI pattern between green and yellow/white seedlings of IP 5009, IP 9712, GWS 14 (Fig. 12 A & B lanes 3,6,9,12), while green and white siblings of VCM 36 showed differences in the Bam HI restriction pattern (Fig. 12 B, lanes 9 & 12) . Since the Bam HI restriction pattern of all four genotypes were found to be different, it is suggested that there are variations in the number of Bam HI sites within 16s rDNA in these genotypes. From the Bgl I and /or Bam HI restriction patterns of different genotypes, it is evident that the organization of 16s rDNA in green and yellow/white siblings within a genotype is same but differs from genotype to genotype.

Bgl I and / or Bam HI restriction patterns of the genotypes IP 5009, IP 9712, VCM 36 and GWS 14 showed no differences with respect to the organization of *rbcL* gene (Fig. 13 A & B). No differences were found in the *rpoA* restriction pattern among different accessions / genotypes (Fig. 14 A & B). Thus it is considered that the *rbcL* and *rpoA* genes which are monocistronic are unaltered in different accessions or genotypes studied.

Comparison of hybridization of *rpoB* probe with Bgl I digests of green and yellow/white siblings from same genotypes did not differ in their but all four genotypes differed from each other. Similar conclusions can be drawn from Bam HI restriction pattern. This suggests that Bgl I and Bam HI restriction sites within *rpoB* gene vary considerably between different stripe mutants/genotypes. Differences in the number and/or signal intensity of the Bgl I and/or Bam HI fragments of plastid specific *rpoB* gene (RNAP-P) indicates variation in the number of sites or accessible sites among plastid genomes in pearl millet accessions.

Restriction analysis revealed considerable differences in plastid DNA organization of 16s ribosomal RNA gene and *rpoB* gene, encoding RNA polymerase subunit between different genotypes/accessions. These differences are indicative of structural rearrangements in the plastomes arising due to loss or gain of recognition site(s) for a given restriction enzyme. Furthermore there are differences between the normal plastomes from different accessions which may represent hot spot regions

similar to those found in *Aegilops* species (Ogihara et *al* 1991; **Maier** et *al* 1995). Nevertheless, such rearrangements within plastid genomes of green progenies from different variegation mutants so far studied, did not reveal any functional defect.

Nuclear genes inducing **plastome** mutations are known in several higher plants (Tilney - Bassett 1978; Borner and Sears, 1986), but the mode of their action in plastids is mostly unknown. Association of chloroplast DNA polymorphisms to the plastome mutator activity in *Oenothera* has been shown recently (Chiu *et al* 1990). But no mutations of plastid DNA have been detected in heat bleached rye plants (Herrmann and Fierabend 1980), *iojap* mutant of maize (Han et *al* 1992) and *albostrains* of barley (Hess et *al* 1993). In the stripe mutants of pearl millet *(Pennisetum glaucum)* information in plastid DNA polymorphisms is limited to a small region and requires screening of entire genome to understand its significance.

GENERAL DISCUSSION AND CONCLUSIONS

Accumulation **of only** Pchilde in white seedlings from white stripe (VCM 36, GWS 14) and albino mutants, both Pchlide and Chlide in yellow seedlings (Fig. 5) derived from yellow stripe mutants (IP 5009, IP 9712) of pearl millet (P. *glaucum*) and the absence of chlorophyll or chlorophyll protein complexes of PS I and PS II in these chlorophyll deficient phenotypes reflects the impairment at the penultimate and final steps in chlorophyll biosynthesis contrary to the *albostrainsof* barley which lacks chloroplast glutamate tRNA, the precursor for chlorophyll biosynthesis (Borner and Meister 1980; Hess et *al* 1992 a & b). Presence of Pchlide and/or Chlide but not chlorophyll in white/yellow seedlings clearly demonstrates that the enzyme PCOR, catalyzing the conversion of Pchlide to Chlide and CS, catalyzing the conversion of Chlide to chlorophyll are either non-functional or absent in these mutants while the enzymes catalyzing the synthesis of Pchlide from delta-ALA are active in these plastid mutants of pearl millet. Comparison of yellow and white seedlings with their corresponding green siblings showed the absence of Chlide and chlorophyll components of PS I & PS II.

Genetic analysis following intercrosses between yellow stripe and white stripe mutants of pearl millet revealed 4 independent loci controlling the development of chlorophyll (Karunasri and Subrahmanyam 1994) while the development of chlorophyll is controlled by a single recessive gene al/al in *albino* mutant (Reddy 1986) and in white stripe mutants GWS 14 and VCM 36, it is controlled by two recessive genes ($vi_3 vi_3 vi_4 vi_4$).

The accumulation of Chlide and the absence of PS I & PS II complexes in yellow seedlings (Fig. 5) from yellow stripe mutants (IP 5009 and IP 9712) is indicative of a block in the conversion of Chlide to chlorophyll. Thus it is conceivable that the recessive genotypes *vi vi* (IP 5009) and vi_1 vi_2 (IP 9712) block the conversion of Chlide thereby leading to its accumulation in these mutants in which vi_1 and *vi* are complementary to each other (Karunasri and Subrahmanyam 1994). It is now evident that *vi* or vi_2 in homozygous recessive condition is adequate to block the conversion of Chlide to chlorophyll. In higher plants Pchlide gets accumulated in the dark (Boddi *et al* 1992) gets reduced immediately to Chlide by PCOR on illumination and is thereafter esterified with geranyl gerenol to form geranyl geranyl Chlide (ChlGG). ChlGG is hydrogenated in three steps to become chlorophyll (Schoch 1978).

The enzymatic esterification of Chlide in etioplasts is catalysed by an enzyme or a group of enzymes, chlorophyll synthetase (Rudiger et *al* 1980). In barley mutants with disturbed pigment biosynthesis, the esterification of Chlide was more rapid in chlorophyll-deficient mutants than in the wild type (Henningsen & Thorne 1973). Thus the Chlide in yellow seedlings of pearl millet could be the esterified form and that *CS* is not needed for the esterif ication of Chlide but is required for its conversion to chlorophyll. Much of this information available was from dark grown or etiolated seedlings (Hendrich and Bereza 1993). Since the green and mutant seedlings within each accession carry the same genotype, the Chlide in the yellow

seedlings is likely to be phytyl Chlide. Each of these yellow mutants (IP 5009, IP 9712) are homozygous recessive for one of the two loci (*vi* or vi_2) which control the conversion of phytyl chlorophyllide to chlorophyll.

Mutants that block the development of chlorophyll also have effects on the expression of plastid and nuclear genes (Taylor, 1989). RNA synthesis by transcriptionally active chromosome (TAC) provides a plastid run-on transcription system in which transcriptions initiated *in vivo* will be extended *in vitro* to monitor all three classes of chloroplast genes (Igloi and Kossel 1992). The rate of transcription in yellow plastids decreased as compared to green plastids. Transcription in the ribosome deficient plastids implies the presence of nuclear encoded RNA polymerase (RNAP-N) in these plastids.

Analysis of the transcripts from northern hybridizations showed the absence of 16s rRNA, *rbcL* and *psbA* RNA and the accumulation of aberrant transcripts for RNA polymerase genes *rpoA* and *rpoB* in both yellow and white seedlings from different genotypes/accessions. This suggests that plastid encoded RNA polymerase (RNAP-P) is necessary for the transcription of 16s *rDNA*, *rbcL* and *psbA* genes. Accumulation of transcripts for the RNA polymerase genes even in the absence of ribosomes implies that RNA polymerase transcribing these genes is of nuclear origin and is transported from the cytoplasm into the plastid compartment. Abundance of aberrant transcripts could be a consequence of altered transcriptional rates and post

transcriptional processing. Indirect evidence for the existence of a nuclear encoded chloroplast RNA polymerase has been presented based on incorporation of UTP in purified fractions of ribosome-deficient plastids (Siemenroth et *al* 1980) or using immunodetection of proteins (resulting from the translation of poly (A)+ mRNA (Lerbs et *al* 1985). Direct evidence for a nuclear-encoded plastid localized RNA polymerase came from the study of plastid gene expression in *Epifagus* in which rRNA gets accumulated even in the absence of *rpoB*, *rpoC* and $rpoC_2$ genes. In chlorophyll-deficient mutants of pearl millet, inspite of the presence of *rpoA* and *rpoB* transcripts, 16s rRNA and transcripts for *rbcL, psbA* were absent. Thus RNAP-N transcribes *rpo* genes in these mutant plastids of pearl millet similar to those of *albostrains* of barley (Hess et *al* 1993) and *iojap* mutants of maize (Han et *al* 1993).

The state of chloroplast development significantly effects the expression of nuclear genes coding for plastid proteins (Taylor 1989; Susek and Chory 1992). It has been proposed that coordination of nuclear and plastid gene expression is achieved, at least in part, by the activity of a plastid-derived factor (referred to as chloroplast/ plastid factor) that is required for the optimal or continued expression of nuclear genes encoding proteins destined for the chloroplasts of the developing and mature leaves. Nuclear encoded chlorophyll a/b binding *(cab)* proteins form the light harvesting complexes of PS I & II (LHCP I & II) along with chlorophyll molecules. Absence of 30 kD polypeptide of LHCP II in the chlorophyll-deficient yellow and

white seedlings of pearl millet suggests that chlorophyll is required for its synthesis. It has been documented that the other components fail to accumulate when one component is missing in a thylakoid protein complex, especially in PS II (Leto *et al* 1985; Rochaix and Erickson 1988). The presence of 27kD and 24kD polypeptides of LHCP II in the yellow seedlings in which Chlide is accumulated may indicate that PCOR and *cab* genes are coregulated. These findings are consistent with the observations made in *Chlamydomonas* (Johanningmeier 1988) where an accumulation of intermediates of the later steps of chlorophyll biosynthesis was proposed to be involved in the regulation of transcription of nuclear genes.

In chloroplasts of higher plants, plastid specific enzyme, FNR catalyzes the photoreduction of NADP by mediating the electron transfer from reduced ferridoxin (FD) to NADP of the PS I (Nakataki and Shin 1991). For the conversion of Pchlide to Chlide during chlorophyll biosynthesis PCOR forms a ternary complex with plastid specific NADPH and Pchlide for its catalytic activity (Griffith 1978). Accumulation of Chlide in the absence of photosynthetic complexes and the presencee of FNR in yellow seedlings suggests that the activity of PCOR is dependent on reducing power imported into the plastid. The white seedlings (GWS 14 and VCM 36) are homozygous recessive for two loci controlling chlorophyll biosynthesis. One of these loci was already assigned to PCOR, it is likely that the other locus codes for FNR.

Detection of DNA in both green and yellow plastids was consistent with earlier observations (Reddy et *al* 1988) . However the apparent low DNA content in yellow plastids as compared to green plastids could be due to low genome copy number. In **W2** mutant of maize, 20-fold decrease in relative plastid DNA content was observed as compared to **normal** seedlings (Han et *al* 1993). DNA **polymerase** activity in the **ribosome-deficient** plastids is indicative of functional replication machinery in these **chlorophyll-deficient** plastids and that the DNA polymerase is of cytosolic origin.

Differences in the expression of different plastid genes were observed between normal and mutant seedlings. There are also considerable differences in the organization of *16S rDNA* and *rpoB* genes among different genotypes/accessions. These differences are indicative of structural rearrangements in their **plastomes** arising from loss or gain of recognition site(s) for a given restriction enzyme.

Chloroplast DNA polymorphisms associated with **plastome mutator** activity was found in *Oenothera* (Chiu et *al*1990) whereas no detectable differences were found in the restriction pattern of mutant plastids of heat bleached rye plants (Herrmann and Fierabend 1980) and *albostrains* of barley (Hess et *al* 1993).

Nuclear genes that regulate chloroplast gene expression and chloroplast development contribute to part of the coordinate control. However, **information** also flows in the opposite

direction, from chloroplast to nucleus. Signal transduction pathway functions to coordinate the expression of nuclear genes encoding components of the photosynthetic apparatus with the functional state of the chlorophyll as the primary signal molecule in a complex regulatory circuit of nuclear plastidic interactions.

An analysis of nuclear mutations that cause chloroplast deficiencies provided the first suggestion of signal transduction pathway. Using one such mutant Bradbreer et *al* (1979) excluded proteins synthesized on plastid ribosomes as signal molecules. According to the data published *hitherto*, the putative signal would act on those genes which are regulated by light and expressed in green tissues. Association of chlorophyll synthesis with nuclear gene expression has been hypothesised (Batschauer et *al* 1980; Johanningmeier 1988). In *albostrains* of barley, chloroplast tRNA glutamate was postulated as the signal initiating molecule in the signal chain and was suggested that it was the absence of and not the accumulation of one or several products of chlorophyll biosynthetic pathway which control the nuclear gene expression (Hess et *al* 1992 a & b). An accumulation of intermediates in the later steps of chlorophyll biosynthesis, *ie* between magnesium-protoporphyrin methyl ester and Pchlide was proposed to be involved in the regulation of the nuclear gene transcription in *Chlamydomonas* (Johanningmeier 1988). Present results show selective accumulation of intermediates of chlorophyll biosynthesis in different plastid mutants and concurrently the nuclear encoded products.

References

Anderson, E.G. 1923. Maternal inheritance of chlorophyll in maize. Bot. Gaz. 76: 411-418.

Apel, K., Santel, H.J., Redlinger, T.E. and Falk, H. 1980. The protochlorophyllide holochrome of barley (*Hordeum vulgare* L.). Isolation and characterization of the NADPH: protochlorophyllide oxidoreductase. Eur. J. Biochem. 111: 251-258.

Appa Rao, S. and Mengesha, M.H. 1984. Inheritance of stripe in pearl millet. J. Hered. 75: 314-316.

Ayyangar, G.N.R., Hariharan, P.V. and Ramakrishnan, R. 1935. Basal branching in the ear heads of the pearl millet *Pennisetum typhoides*. Stapf and Hubbard. Curr. Sci. 4: 237-238.

Barkan,A., Miles,D. and Taylor,W.C. 1986. Chloroplast gene expression in nuclear, photosynthetic mutants of maize. EMBO J. 5: 1421-1427.

Batschauer, A., Mosinger, E., Kreuz., K., Dorr, I. and Apel, K. 1986. The implication of a plastid derived factor in the transcriptional control of nuclear genes encoding the light - harvesting chlorophyll a/b protein. Eur. J. Biochem. 154: 625-634.

Baumgartner, B.J., Rapp, J.C. and Mullet, J.E. 199 3. Plastid genes encoding the transcription/translation apparatus are differentially transcribed early in barley chloroplast development. Evidence for selective stabilization of *psbA,* mRNA. Plant. Physiol. 101: 781-791.

Baur, E. 1909. Das **Wasen und** die **Erblichkeitsverhaltnisse** der. **"varietates albomarginatae hort"** von *Pelargonium jalapa, Urtica pilulifera* und ***Lunaria*** *annua.* Z. Vererbungslehre 1: 291-329.

Bedbrook, J.R. and Bogorad, L. 1976. Endonuclease recognition sites mapped in Zea mays. **Proc. Natl.** Acad. Sci. USA. 73:4309-4319.

Bedbrook, J.R., Link, G., Coen, **D.M.,** Bogorad, L. and Rich, A. 1978. Maize plastid gene expressed during photoregulated development. Proc. Natl. Acad. Sci. USA 75: 3060-3064.

Bendich, A.J. 1987. Why do chloroplasts and mitochondria contain so many copies of their genome? Bio Essays 6: 279-282.

Boddi, B., Lindsten, A., Ryberg, M. and **Sundquist,** C. 1989. The aggregational state of protochlorophyllide in isolated **prolamellar** bodies. Physiol. Plant 76: 135-143.

Boddi, B., Lindsten, A., Ryberg, M. and Sundquist, C. 1990. **Phototransformation** of aggregated forms of **protochlorophyllide** in isolated etioplast inner membranes. **Photochem.** Photobiol. 52: 83-87.

Boddi, B., Ryberg, M. and Sundquist, C. 1992. **Identification** of four universal protochlorophyllide forms in dark-grown leaves by analysis of the 77 K fluorescence emission spectra, J. Photochem. Photobiol. B: **Biol.** 12: 389-401.

Boese, **Q.F.,** Spano, **A.J.,** Li, J. and **Timko,** M.P. 1991. **Aminolevulinic** acid dehydratase in pea (***Pisum sativum*** L.). **Identification** of an unusual metal-binding domain in the plant enzyme. J. Biol. **Chem.** 266: 17060-17066.

Bogorad, L. 1992. Replication and transcription of plastid DNA, in cell culture and somatic cell. Genetics of plants. Vol. 7,93. Bogorad, L. and Vasil, I.K., (eds.) Academic Press NY.

Borner, T. Knoth, R., Herrmann, F. and Hagemann, R. 1972. Struktur und Funktion der genetischen Information in den plastiden. V. Das Feblen von ribosomaler RNS in den plastiden der plastommutante 'Mrs. Parker' von *Pelargonium zonale* Ait. Theor. Appl. Genet. 42: 3-11.

Borner, T., Herrmann, F. and Hagemann, R. 1973. Plastid ribosome deficient mutants of *Pelargonium zonale.* FEBS Lett. 37: 117-119.

Borner, T., Schumann, B. and Hagemann, R. 1976. Biochemical studies on a plastid ribosome deficient mutant of ***Hordeum vulgare.*** In Genetics and biogenesis of chloroplasts and mitochondria. pp. 41-48. Bucher, Th., Neupert, W., Sebald, W. and Werner, S. (eds) Elsevier/North Holland Biomedical Press, Amsterdam.

Borner, T.and Meister, A. 1980. Chlorophyll and carotenoid content of ribosome deficient plastids. Photosynthetica 14: 589-593.

Borner, T. and Sears, B.B. 1986. Plastome mutants. Plant Mol. Biol. Rep. 4: 69-92.

Bradbeer, J.W., Atkinscri, Y.E., Borner, T. and Hergemann, R. 1979. Cytoplasmic synthesis of plastid polypeptides may be controlled by plastid synthesized RNA. Nature 279: 816-817.

Burgess, D.G. and Taylor, W.C. 1987. Chloroplast Photooxidation affects the accumulation of cytosolic messenger RNA encoding chloroplast protein in maize. Planta 170: 520-527.

Burton, G.W. and Powell, J.B. 1965. Six chlorophyll deficient seedlings of pearl millet, ***Pennisetum typhoides*** and a suggested **system** for their nomenclature. Crop. Sci. 5: 1-3.

Castelfranco, **P.A.** and **Beale,** S.I. 1983. Chlorophyll biosynthesis: Recent advances and areas of current interest. Ann. Rev. Plant Physiol. 34: 241-278.

Chiu, W. L., Johnson, E. **M.**, Kaplan, S.A., **Blasko,** K., Sokalki, M.V., **Wolfson,** R. And Sears, B.B 1990. ***Oenothera*** chloroplast **DNA** polymorphism associated with **plastome** mutator activity. **Mol.** Gen. Genet. 221: 590-64.

Coe, E.H. Jr., Thompson, D.L. and **Walbot,** V. 1982. Nuclear genes and chloroplast **modifications** in maize. Stadler **Symp.** 14: 29-46.

Coleman, A.W., Maguire, **M.J.** and Coleman, J.R. 1981. **Mithramycin** - and 4 - 6 - **diamidino** - 2 - phenylindole **(DAPI)** DNA staining for fluorescence microspectrophotometric measurement of DNA in nuclei, plastids and virus particles. J. **Histochem Cytochem** 29: 959-968.

Correns, C., 1909. Verebungsversuche **mit blass (gelb)** grunen und buntblattrigen Sippen bei *Mirabills jalapa, Utrica* ***pilulifera*** und ***Lunaria*** *annua.* Z. Verebungs. 1:291-329.

Demerec, M. 1927. A second case of maternal inheritance of chlorophyll in maize. Bot. Gaz. 84: 139-155.

Deng, **X.W.** and **Gruissem,** W. 1987. Control of plastid gene expression during development: the limited role of transcriptional regulation. Cell. 49: 379-387.

Deng, X.W., Stern, D.B., **Tonkyn, J.C.** and Gruissem, W. 1987.

Plastid run-on transcription. Application to determine the transcriptional regulation of spinach plastid genes. J. Biol. Chem. 262: 9641-9648.

de Pamphilis, C.W. and Palmer, J.D. 1990. Loss of photosynthetic and chlororespiratory genes from the plastid genome of a parasitic flowering plant. Nature 348: 337-339.

Epp, M.D. 1973. Nuclear gene induced plastome mutations in *Oenothera hookeri* I. Genetic analysis. Genetics 75: 467-483.

Ernst, D. and Schefbeck, K. 1988. Photooxidation of plastids inhibits transctription of nuclear encoded genes in rye *(Secale cereale)*. Plant physiol. 880: 255-258.

Feierabend, J. and Wildner, G. 1978. Formation of the small subunit in the absence of the large subunit of ribulose 1,5-bisphosphate carboxylase in 70's ribosome deficient rye leaves. Arch. Biochem. Biophys. 186: 283-291.

Frost, S., Viver, L. and Carlbom, C. 1970. Reciprocal extra chromosomal inheritance in rye *(Secale cereale* L.). Hereditas. 65: 251-260.

Galbraith, D.W. 1990. Flow cytometric analysis of plant genomes. Methods cell biol. 33: 549-562.

Gassman, M., Granick, S. and Mauzerall, D. 1968. A rapid spectral change in etiolated red kidney bean leaves following phototransformation of protochlorophyllide. Biochem. Biophys. Res. Commun. 32: 295-300.

Gill, B.S., Virmani, S.S. and Phul, P.S. 1969. Inheritance of foliage striping in pearl millet. Indian. J. Genet. and Plant Breed. 29: 473-477.

Gilmartin, P.M., Sarokin, L., Memelink, J. and Chua, N.H. 1990. Molecular light switches for plant genes. The Plant Cell. 2: 369-378.

Giuliano, G. and Scolink, P.A. 1988. Transcription of two photosynthesis-associated nuclear gene families correlates with the presence of chloroplasts in leaves of variegated tomato *ghost* mutant. Plant Physiol. 86: 7-9.

Granick, S. and Gassman, M. 1970. Rapid regeneration of protochlorophyllide$_{65}$. Plant Physiol. 45: 201-205.'

Greenberg, B.M., Narita, J.O., DeLuca-Flaherty, C.R. and Hallick, R.B. 1985. Properties of chloroplast RNA polymerase. In: Molecular biology of the photosynthetic apparatus, pp. 303-309. Steinback, K.E., Bontita, S., Arntzen, C.J. and Bogoard, L. (eds.). Cold Spring Harbor Laboratory, Cold Spring Harbor NY.

Griffiths, W.T. 1978. Reconstitution of chlorophyllide formation by isolated etioplast membranes. Biochem. J. 174: 681-692.

Gruissem, W., Barkan, A., Deng, X.W. and Stern, D. 1988. Transcriptional and post-transcriptional control of plastid mRNA levels in higher plants. Trends Genet. 4(9): 258-263.

Gruissem, W. 1989. Chloroplast RNA transcription and processing. In: The biochemistry of plants vol. 15: 151-192. Marcus, A. (ed.). Academic Press. New York.

Han C. D., Coe. E. H. Jr and Maatriensen R. A., 1992. Molecular cloning and characterization of *iojap* (ii) a pattern striping gene of maize. The EMBO. J. 11: 4037-4046.

Han, C.D., Patrie, W., Polacco, M., Edward, C.D. and Coe, Jr.

1993. Aberrations in plastid transcription and deficiency of plastid DNA in striped and *albino* mutants in maize. Planta 191: 552-563.

He, Z.H., Li, J., Sundquist, C. and Timko, M.P. 1994. Leaf developmental age controls expression of genes encoding enzymes of chlorophyll and heme biosynthesis in pea (*Pisum sativum* L.). Plant Physiol. 106: 537-546.

Hendrich, W. and Bereza, B. 1993. Spectroscopic characterization of protochlorophyllide and its transformation. Photosynthetica. 28(1): 1-16.

Henningsen, K.W. and Thorne, S.W. 1973. Esterification and spectral shifts of chlorophyllide in wild type and mutant seedlings developed in darkness. Physiol. Plant 30: 82-89.

Henny, R.J. 1982. Inheritance of foliar variegation in two *Dieffenbachia* cultivars. J. Hered. 73: 384-386.

Herrera-Estrella, L., van den Broeck, G., Maenhaut, R., Van Montagu, M., Schell, J., Timko, M. and Cashmore, A. 1984. Light-inducible and chloroplast-associated expression of a chimeric gene introduced into *Nicotiana tabacum* using a Ti-plasmid vector. Nature 310: 115-120.

Herrmann, R.G. and Fierabend J. 1980. The presence of DNA in ribosome-deficient plastids of heat-bleached rye leaves. Eur. J. Biochem. 104: 603-609.

Hess, W.R., Schendel, R., Borner, T. and Rediger, W. 1991. Reduction of mRNA level for two nuclear encoded light regulated genes in the barley mutant albostrains is not correlated with phytochrome content and activity. J. plant

physiol. 138 : 292-298.

Hess, W.R., Schendel, R., Rüdiger, W., Fieder, B. and Borner, T. 1992a. Components of chlorophyll biosynthesis in a barley albino mutant unable to synthesize δ-aminolevulinic acid by utilizing the transfer RNA for glutamic acid. Planta. 188: 19-27.

Hess, W.R., Blank-Hubber, M., Fieder, B. and Rudiger, W. 1992b. Chlorophyll synthetase and chloroplast tRNA glu are present in heat-induced, ribosome-deficient plastids. J. Plant Physiol.139: 427-430.

Hess, W.R., Prombona, A., Fieder, B., Subramanian, A.R. and Borner, T. 1993. Chloroplast rps 15 and the rpo B/C /C_2 gene cluster are strongly transcribed in ribosome-deficient plastids: Evidence for a functioning non-chloroplast encoded RNA polymerase EMBO J. 12: 563-571.

Hess, W.R., Müller, A., Nagy, F. and Borner, T. 1994. Ribosome - deficient plastids affect transcription of light - induced nuclear genes: genetic evidence for a plastid derived signal. Mol. Gen. Gent. 242: 305-312.

Hiratsuka, J., Shimada, H., Whittler, R., Ishibashi, T., Sakamoto, M., Mori, M., Kondo, C., Honji, Y., Sun, C.R., Meng, B.Y., Li, Y.Q., Kanno, A., Nishizawa, Y., Hirai, A., Shinozaki, K. and Sugiura, M. 1989. The complete sequence of the rice *(Oryza sativa)* chloroplast genome: Intermolecular recombination between distinct tRNA genes accounts for a major plastid DNA inversion during the evolution of the cereals. Mol. Gen. Genet. 217: 185-194.

Igloi, G.L. and Kossel, H. (1992). The transcriptional apparatus of chloroplasts. Crit. Rev. Plant Sci. 10: 525-558.

Imai, Y. 1928. A consideration of variegation. Genetics. 13: 544-562.

Imai, Y. 1935. Variation in the rate of recurring plastid mutations *Hordeum vulgare* caused by differences in the sowing times. Genetica, 20: 36-41.

Imai, Y. 1936a. Chlorophyll variegation due to mutable genes and plastids. Z. vererbungslehre. 71: 61-83.

Imai, Y. 1936b. Recurrent auto and exo mutation of plastids resulting in tricolored variegation of *Hordeum vulgare.* Genetics. 21: 754-757.

Izumi, Y., Ono, K, Takamiya, M and Fukui, K. 1993. Chloroplast division in cultured cells of the hornwort *Anthoceros punctatus*. J. Plant Res. 106: 319-325.

Jenkins, M.T. 1924. Heritable characters of maize. XX. Iojap striping, a chlorophyll defect. J. Hered. 5: 467-472.

Jenssen, K.H., Herrin, D.L., Plumley, G. and Schmidt, G.W. 1986. Biogenesis of photosystem II complexes: transcriptional, translational, and post-translational regulation. J. Cell. Biol. 103: 1315-1325.

Johanningmeier, U. 1988. Possible control of transcript levels by chlorophyll precursors in chlamydomonas. Eur. J. Biochem. 177: 417-424.

Kadam, B.S., Patil, S.M. and Kulkarni, P.K. 1940. Consequences of inbreeding in bajra. J. Hered. 31: 3 01-3 07.

Kapuscinski, J. and Skowzylas, B. 1977 Simple and rapid

fluorimetro method for DNA **microassay.** Anal. **Biochem** 88: 252.

Karper, **R.E.** and **Conner,** A.B. 1931. Inheritance of chlorophyll characters in *Sorghum.* Genetics, 16: 291-308.

Karper, R.E. 1934. Maternal inheritance of chlorophyll in *Sorghum.* J. Hered. 25: 49-54.

Karunasri, K. and **Subrahmanyam, N.C.** 1994. Genetic analysis of variegation mutants of pearl millet. ***Pennisetum*** *glaucum.* R. Br. Hereditas 120: 203-209.

Kirk, J.T.O. and Tilney-Bassett, R.A.E. 1978. The plastids. Elsevier North-Holland **Biomedical** Press, Amsterdam, New York, Oxford pp. 960.

Klein, R.R. and Mullet, J.E. 1990. Light-induced transcription of chloroplast genes. *psbA* transcription is **differentially** enhanced in illuminated barley. J. **Biol.** Chem. 265 (4): 1895-1902.

Knoth, R. and **Hagemann,** R. 1977. Struktur und Funktion der genetischen Information in den Plastiden. XVI. Die Feinstruktur der Plastiden und der elektronenmikroskopiche Nachweis enter Mischzellen in Blattern der **Plastom mutationen** auslosenden Genmutante albostrains von Hordeum vulgare L. Biol. Zentralbl. 96: 141-150.

Koduru, P.R.K. and Rao, M.K. 1980. Spontaneous chlorophyll mutants of *Pennisetum* ***americanum*****:** Genetics and chlorophyll quantities. Theor. **Appl.** Genet. 56: 137-143.

Koduru, P.R.K. and Rao, M.K. 1983. Genetics of qualitative trails and linkage studies in pearl millet. Z. Pflanzenzuchtg. 90: 1-22.

Krishna Rao, M. and Koduru, R.K. 1978. Biparental plastid inheritance in *Pennisetum americanum J.* Hered. 69: 327-330.

Kuchka, M.R., Mayfield, S.P. and Rochaix, J.D. 1988. Nuclear mutations specifically affect the synthesis and/or degradation of the chloroplast-encoded D2 polypeptide of photosystem II in *Chlamydomonas reinhardtii.* EMBO J. 7: 319-324.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of bacteriophage T.. Nature 227: 680-685.

Lebedev, N.N., Šiffel, P. and Krasnovskii, A.A. 1985. Detection of protochlorophyllide forms in irradiated green leaves and chloroplasts by difference fluorescence spectroscopy at 77 K. Photosynthetica 19: 183-187.

Lerbes, S., Bräutigam, E. and Parthier, B. 1985. Polypeptides of DNA - dependent RNA polymerase of spinach chloroplasts. Characterization by antibody linked polymerase assay and determination of sites of synthesis. EMBO J. 4: 1661-1666.

Leto, K.J., Bell, B. and Mclntosh, L. 1985. Nuclear mutation leads to an accelerated turnover of chloroplast-encoded 48 kd and 34.5 kd polypeptides in thylakoids lacking photosystem II. EMBO J. 4: 1645-1653.

Lin, M.S., Comings, D.E and Alli, O.S. 1977. Optical studies of the interaction of 4,6 - diamidino - 2 - phenylindole with DNA and metaphase chromosomes. Chromosoma 60: 15.

Link, G., Coen, D.M. and Bogorad, L. 1978. Differential expression of the gene for large subunit of ribulose bisphosphate carboxylase in maize leaf cell types. Cell. 15: 725-731.

Link, G. 1988. Photocontrol of plastid gene expression. Plant Cell and Environ. 11: 329-38.

Maier, R.M., Neckermann, K., Igloi, G.L. and Kossel, H. 1995. Complete sequence of the maize chloroplast genome: Gene content, hotspots of divergence and fine tuning of genetic information by transcript editing. J. Mol. Biol. 251: 614-628.

Martienssen, R.A., Barkan, A., Scriven, A. and Taylor, W.C. 1987. Identification of a nuclear gene involved in thylakoid structure. pp. 181-192. In"Plant Membrane: Structures, Function, Biogenesis (UCLA Symposia on Molecular and Cellular Biology)." Leaver C, Sze H (eds.). New York: Alan R. Liss, Inc.,

Masica, P. and Robertson,D.S. 1978. Studies of chloroplast development in four maize mutants defective in chlorophyll biosynthesis. Planta Med. 143 : 207-211.

Mayfield, S.P. and Taylor, W.C. 1984. Carotenoid-deficient maize seedlings fail to accumulate light-harvesting chlorophyll a/b binding protein (LHCP) mRNA. Eur J. Biochem. 144: 79-84.

McKown, R.L. and Tewari, K.K. 1984. Purification and properties of a pea chloroplast DNA polymerase Proc. Natl. Acad. Sci. (USA) 81: 2354-2358.

Michaelis, P. 1965. Uber Kerninduzeirte plasma und plastidenaban derungen. Z. Naturforsch. 20b: 264-267.

Miller, E.R. 1968. Inheritance of white stripe and rolled-leaf

characteristics in *Sorghum bicolor* (L., Moench). Crop Sci. 8: 769-771.

Morden, C.W., Wolfe, K.H., de Pamphilis, C.W. and Palmer, J.D. 1991. Plastid translation and transcription genes in a non-photosynthetic plant. Intact, missing and pseudo genes. EMBO J. 10: 3281-3288.

Mullet, J.E. and Klein, R.R. 1987. Transcription and RNA stability are important determinants of higher plant chloroplast RNA levels. EMBO J. 6: 1571-1579.

Mullet. J.E. 1993. Dynamic regulation of chloroplast transcription. Plant Physiol. 103: 309-313.

Nakamura, S., Ikehara, T., Uchida, H., Suzuki, T. and Sodmergen, T.S. 1992. Fluorescence microscopy of plastid nucleoids and a survey of nuclease C in higher plants with respect to mode of plastid inheritance. Protoplasma 169: 68-74.

Nakataki, S. and Shin, M. 1991. The reconstituted NADP photoreducing system by rebinding of the large form of ferredoxin-NADP reductase to depleted thylakoid membranes. Arch. Biochem. Biophys. 291: 390-394.

Oelmüller, R. and Mohr, H. 1986. Photo-oxidative destruction of chloroplasts and its consequences for expression of nuclear genes. Planta 167: 106-113.

Ogihara, Y., Terachi, T. and Sasakuma, T. 1991. Molecular analysis of the hot spot region related to length mutations in wheat chloroplast DNAs. I. Nucleotide divergence of genes and intergenic spacer regions located in the hot spot region. Genetics, 129: 873-884.

Ohyama, K., Fukuzawa, H., Shirai, T., Shirai, H., Sano, T., Sano, S., Umesono, K., Shiki, Y., Takeuchi, M., Chang, Z., Aota, S., Inokuchi, H. and Ozeki, H. 1986. Chloroplast gene organization deduced from complete sequence of liverwort *Marchantia polymorpha* chloroplast DNA. Nature 322: 572-574.

Oliver, R.P. and Griffiths, W.T. 1980. Identification of the polypeptides of NADPH-protochlorophyllide oxidoreductase. Biochem. J. 191: 277-280.

Oliver, R.P. and Griffiths, W.T. 1982. Pigment-protein complexes of illuminated etiolated leaves. Plant Physiol. 70: 1019-1025.

Pal, B.P. and Ramanujam, S. 1941. A new type of variegation in rice. Indian. J. Agric Sci. 11: 170-176.

Palmer, J.D. 1990. Contrasting modes and tempos of genome evolution in land plant organelles. Trends Genet. 6: 115-120.

Potrykus, I. 1970. Mutation und Ruckmutation extrachromosomal vererbter plastidenmerkmale von *Petunia.* Z. Pflanzenzucht. 3: 24-40.

Rajasekhar, V.K. 1991. Regulation of nuclear gene expression plastid biogenesis as affected by development stage of plastid. Biochem. Physiol Pflanzen. 187: 257-271.

Rao, P.N. 1975. Plastid inheritance in Job's tears, *Coix lacrymajobi* L. Theor. Appl. Genet. 45: 363-367.

Rapp, J. and Mullet, J. 1991. Chloroplast transcription is required to express the nuclear genes *rbcS* and *cab.* Plastid DNA copy number is regulated independently. Plant Mol. Biol. 17: 713-823.

Rapp, J.C., Baumgartner, B.J. and Mullet, J. 1992. Quantitative analysis of transcription and RNA levels of 15 barley chloroplast genes: transcription rates and mRNA levels vary over 300-fold; predicted mRNA stabilities vary 30-fold, J. Biol. Chem. 267: 21404-21411.

Ratnaswamy, M.C. 1960. Cytoplasmic inheritance of variegation in bajra the pearl millet *(Pennisetum typhoides,* stapf and Hubbard). The Madras Agri. J. 47: 109-117.

Reddy, M.K. 1986. Nuclear gene controlled alteration and reversion of plastids in *Pennisetum americanum* (L., Leeke). Ph.D. Thesis, University of Hyderabad.

Reddy, M.K., Subrahmanyam, N.C., Appa Rao, S. and Mengesha, M.H. 1988. Ultrastructural and molecular characterization of altered plastids in nuclear gene controlled yellow stripe mutant of *Pennisetum americanum.* Hereditas 109: 253-260.

Reddy, M.K. and Subrahmanyam, N.C. 1988a. Inheritance and transmission of plastid alterations in a green-white stripe mutant of pearl millet *(Pennisetum americanum)*. Can. J. Bot. 66: 179-182.

Reddy, M.K. and Subrahmanyam, N.C. 1988b. Nuclear gene induced plastid alterations in *Pennisetum americanum.* Genome 30: 147-151.

Redei, G.P. 1973. Extra-chromosomal mutability determined by a nuclear gene locus in *Arabidopsis,* Mut. Res. 18: 149-162.

Redei, G.P. and Plurad, S.B. 1973. Hereditary structural alterations of plastids induced by a nuclear mutator gene in *Arabidopsis.* Protoplasma, 77: 361-380.

Rhoades, M.M. 1943. Genic induction of an inherited cytoplasmic difference. Proc. Natl. Acad. Sci. (U.S.A.) 29: 327-329.

Rhoades, M.M. 1946. Plastid mutations. Cold Spring Harbor Symp. Quant. Biol. 11: 202-207.

Robbelen, G. (1966). Chloroplasten differenzierung nach geninduzierter plastomon mutation bei *Arabidopsis thaliana,* Z. Pflanzen Physiol. 55: 387-403.

Rochaix, J.D. and Erickson, 1988. Function and assembly of photosystem II: genetic and molecular analysis. Trends Biochem. Sci. 13: 56-59.

Roth, L. 1927. Untersuchungen uber die periklinal bunten Rassen von *Pelargonium zonale.* Z. Vererbungslehre. 45: 125-259.

Rüdiger, W., Benz, J. and Guthoff, C. 1980. Detection and partial characterization of activity of chlorophyll synthetase in etioplast membranes. Eur. J. Biochem. 109: 193-200.

Rüdiger, W. and Schoch, S. 1988. Chlorophylls. In Chemistry and Biochemistry of Plant Pigments. T.W. Goodwin (ed.) pp. 1-59, Academic Press New York.

Sager, R. 1955. Inheritance in the green alga *Chlamydomonas reinhardii.* Genetics 40: 476-489.

Sambrook, J., Fritsch, E.F., Maniatis, R. 1989. Molecular cloning: A laboratory manual, 2nd ed. Cold Spring Harbor, Laboratory Press. Cold Spring Harbor, New York.

Schoch, S. 1978. The esterification of chlorophyllide a in greening bean leaves. Z. Nature Forsch. 33C: 712-714.

Scholtz, F. 1954. Uber Plastiden Konkurrenz bei penothera. Planta (Berlin). 43: 182-240.

Schrubar, H., Wanner, G. and Westhoff, P. 1990. Transcriptional control of plastid gene expression in greening *Sorghum* seedlings. Planta 183: 101-111.

Senge, **M.O.** 1993. Recent advances in the biosynthesis and chemistry of the chlorophylls. **Photochem.** Photobiol. 57: 189-206.

Sharp, P.J., Kries, **M.** , Shewry, P.R., Gale, **M.D.** 1988. Location of **β-amylase** sequences in wheat and its relatives. Theor. **Appl.** Genet. 75: 286-290.

Sheel, H. 1956. Untersuchungen uber die variegation bei *Barbarea vulgaris* R. Br. var. *Variegata* hort. Z. **Pflanzenzuchtg.** 54: 225-246.

Shinozaki, K., **Ohme,** M., Tanaka, **M.** , Wakasugi, T., Hayshida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., **Yamaguchi** Shinozaki, K., Ohto, C., Torazawa, K., **Meng,** B.Y., Sugita, **M.** , Deno, H., **Kamogashira., Yamada,** K., Kusudo, J., Takaiwa, F., **Kata,** A., Todoh, **N.,** Shimada, H. and Sugiura, M. 1986. The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression, EMBO J. 5: 2043-2049.

Siemenroth, A., Borner, T and **Metzger,** U. 1980. Biochemical studies on the *iojap* mutant of maize. Plant Physiol 65: 1108-1110.

Siemenroth, A., Wollgiehn, R., Neumann, D. and Borner, T. 1981. Syntheses of **ribosomal** RNA in **ribosome-deficient** plastids of the mutant **"albostrains"** of ***Hordeum*** *vulgare* L. Planta. 153: 547-555.

Simpson, J., Van Montagu, M. and L. Herrera-Estrella, 1986. Photosynthesis-associated gene families: differences in response to **tissue-specific** and environmental factors. Science 233: 34-38.

So, M. 1921. On the inheritance of variegation of barley. Japan. J. Genet. 1: 21-36.

Sodmergen, T.S., Kawano, S., **Nakamura,** S., Kurowa, I. 1992. Behaviour of organelle nuclei (nucleoids) in generative and vegetative cells during maturation of pollen in ***Lilium longiflorum*** and *Pelargonium zonale.* **Protoplasma.** 168: 73-81.

Sodmergen, Y.Y.L., Kuroiwa, T. and Hu, S. Y. 1994. **Cytoplasmic** DNA aportionment and plastid **differentiation** during male gametophyte development in *Pelargonium zonale.* Sexual Plant Repro 7:51-56.

Spano, A.J. and **Timko,** M.P. 1991. Isolation, characterization and partial **amino** acid sequence of a chloroplast-localized porphobilinogen deaminase from pea (*Pisum sativum* L.). Biochim. Biophys. Acta 1076: 29-36.

Stockhaus, J., Schell, J., and **Willmitzer,** L. 1989. Correlation of the expression of the nuclear photosynthetic gene **ST-LSI** with the presence of chloroplasts, EMBO J. 8: 2445-2451.

Stroup, D. 1970. **Genic** induction and maternal transmission of variegation in *Zea mays. J.* hered. 61: 139-141.

Subrahmanyam, N.C., Satyaprasad, M., Appa Rao, S. and Mengesha, M.H. 1986. Pattern dependent maternal plastid inheritance in ***Pennisetum americanum.*** Can. J. Boi. 64: 1081-1085.

Sugiura, M. 1992. The chloroplast genome. Plant **Mol. Biol.** 19:

149-168.

Sugita, M., Kato, A., Shimada, H. and Sugiura, M. 1984. Sequence analysis of the junctions between a large inverted repeat and single-copy regions in tobacco chloroplast DNA. Mol. Gen. Genet. 194: 200-205.

Sujatha, M. and Subrahmanyam, N.C. 1991. Characterisation of nuclear gene controlled yellow stripe mutant of *Pennisetum glaucum* (L.) R. Br. Plant Sci. 73: 55-64.

Susek, R.E. and Chory, J. 1992. A tale of two genomes: role of a chloroplast signal in coordinating nuclear and plastid genome expression. Austr. J. Plant Physiol. 19: 387-399.

Suzuki, T., Kawano, S., Sakai, A., Fujie, M., Kuroiwa, H., Nakamura, H and Kurowa, T. 1992. Preferential mitochondrial and plastid DNA synthesis before multiple cell divisions in *Nicotiana tabacum*. J. Cell Sci. 103: 831-837.

Taylor, W.C., Barkan, A. and Martienssen, R.A. 1987. Use of nuclear mutants in the analysis of chloroplast development. Dev. Genet. 8: 305-320.

Taylor, W.C. 1989. Regulatory interactions between nuclear and plastid genomes. Ann. Rev. Plant Physiol. Plant Mol. Biol. 40: 211-233.

Tewari, K.K. and Goel, A. 1983. Solubilization and partial purification of RNA polymerase from pea chloroplasts. Biochemistry 22: 2142-2148.

Thompson, D., Walbot, V. and Coe, E.H. Jr. 1983. Plastid development in *iojap* and chloroplast mutator affected maize plants. Ame. J. Bot. 70: 940-50.

Tilney-Bassett, R.A.E. 1963. Genetics and plastid physiology in *Pelargonium.* Heredity. 18: 485-504.

Tilney-Bassett. R.A.E. 1965. Genetics and plastid physiology in *Pelargonium* II. Heredity. 20: 451-466.

Tilney-Bassett, R.A.E. 1970a. Genetics and plastid physiology in *Pelargonium* III. Effect of cultivar and plastids on fertilization and embryo survival. Heredity. 25: 89-103.

Tilney-Bassett, R.A.E. 1970b. The control of plastid inheritance in *Pelargonium.* Genet. Res. 16: 49-61.

Tilney-Bassett, R.A.E. 1978. The inheritance and genetic autonomy of plastids. - In: The Plastids (eds. J.T.O. Kirk and R.A.E. Tilney-Bassett), Elsevier/North Holland Biomedical Press, Amsterdam, P. 251-254.

Towbin, H., Staehelm, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications.Proc. Natl. Acad. Sci. U.S.A. 61: 4350-4354.

Vainstein, A., Ferreira, P., Peterson, C.C., Verbeke, J.A. and Thornber, J.P. 1989. Expression of the major light harvesting chlorophyll a/b protein and its import into thylakoids of mesophyll and bundle sheath chloroplasts of maize. Plant Physiol. 89: 602-609.

Vinchon, R. 1949. Etude Sur la genetique des Pennisetum cultives (study of the genetics of the cultivated *Pennisetum* varieties). — Agron. Trop. 4: 451-485 (in French).

Von Wettstein, D., Hennigsen, K.W., Boynton, J.E., Kannangara, G.C. and Nielsen, O.F. 1971. The genic control of chloroplast

development in barley. pp. 205-223. In "Autonomy and Biogenesis of Mitochordria and Chloroplasts". Amsterdam: Boardman, N.K., Linane, A.W., Smillie, R.M. (eds.): North Holland.

Walbot, V. and Coe, E.H. Jr. 1979. Nuclear gene *iojap* conditions a programmed change to ribosome - less plastids in *zea mays.* Proc. Natl. Acad. Sci. (U.S.A.) 76: 2760-2764.

Witty, M., Wallace-Cook, A.D.M., Albrecht, H., Spano, A.J., Michel, H. , Shabanowitz, J., Hunt, D.F., Timko, M.P. and Smith, A.G. 1993. Structure and expression of chloroplast-localized porphobilinogen deaminase from pea (*Pisum sativum* L.) isolated by redundant polymerase chain reaction. Plant Physiol. 103: 139-147.

Zhu, T. , Mogensen, H.L., Smith, S.E. 1992. Heritable paternal cytoplasmic organelles in alfalfa sperm cells - ultra structural reconstruction and quantitative cytology. Eur J. Cell Biol. 59: 211 - 218.