INFLUENCE OF CELL POSITION ON PHOTOMORPHOGENIC RESPONSE IN LEAVES OF PEARL MILLET SEEDLINGS

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BY

M. TAMIL SELVI

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD
HYDERABAD-500 046
INDIA

MAY, 1998

ENROLLMENT NO. **91LSPH11**

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD

CERTIFICATE

This is to certify that the thesis entitled **"Influence of cell position on photomorphogenic response in leaves of pearl millet seedlings"** is based on the results of the work done by Ms. **M. Tamil Selvi** for the degree of **Doctor of Philosophy** under my supervision. This work has not been submitted for any degree or diploma of any other University or Institution.

PROF. A. S. RAGHAVENDRA
HEAD,
DEPARTMENT OF PLANT SCIENCES

PROF. R. P. SHARMA,
SUPERVISOR

18/5/98

DEAN,
SCHOOL OF LIFE SCIENCES

DE A N

DEPARTMENT OF PLANT SCIENCES
SCHOOL OF LIFE SCIENCES
UNIVERSITY OF HYDERABAD

DECLARATION

I hereby declare that the work presented in this thesis has been carried out by me under the supervision of PROF. R. P. SHARMA and that this has not been submitted for a degree or diploma in any other University.

Dated: May, 1998
Place: Hyderabad

M. Tamilselvi
M. TAMIL SELVI

PROF. R. P. SHARMA,
SUPERVISOR

To MY PARENTS

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* * *

ABBREVIATIONS

A		absorbance
APS		ammonium per sulphate
B		bundle sheath
Cab		chlorophyll a and chlorophyll b binding protein
Car		carotenoid
Chl		chlorophyll
Chlide		chlorophyllide
d		day
D		dark
DW		distilled water
EDTA		ethylene diamine tetraacetic acid
FW	fresh	weight
h		hour
kD		kilo dalton
L		light
LHCP		light harvesting chlorophyll protein complex
M		mesophyll
min		minute
NF		norflurazon
Pchlide		protochlorophyllide
PAGE		polyacrylamide gel electrophoresis
PEPC		phosphoenolpyruvate carboxylase
PMSF		phenyl methyl sulfonyl fluoride
PVDF		polyvinylidenedifluoride
POR		protochlorophyllide oxidoreductase
RL		red light
Rubisco		ribulose 1,5-bisphosphate carboxylase
s		second
SDS		sodium dodecylsulphate
TEMED		N, N, N',N'-tetramethyl ethylene diamine
TCA		trichloro acetic acid
Tris		(tris[hydroxymethyl] aminomethane)
UV		ultra violet
v/v		volume/volume
w/v		weight/volume
WL		white light
λ_{max}		maximum wavelength

CONTENTS

1. INTRODUCTION

The light dependent development of plant is a complex process. It involves a signal transduction network wherein the environmental signal is perceived by the regulatory genes and in turn causes expression leading to development. The development of the plant has several unique features such as totipotency of cells, plastid development and, continuation of post embryonic development at sites called as meristem. The shoot meristem perpetuates itself and also give rise to new organ particularly leaf primordia. The leaf is an ideal model for studying the importance of cell lineage, local cell interactions and positional information. In grasses such as maize, the developing leaf is established by parallel files of cells originating at the leaf base, which matures in a tip-to-base fashion. Parallel veins running from base to tip, with structural and photosynthetic cells formed around it progressively subdivide the leaf primordia. These two developmental patterns of cell files from a basal meristem and the veins across the leaf appear to provide the specialization of cell types throughout the leaf. It suggests that in order to activate the cell specific expression of photosynthetic genes, bundle sheath (B) and mesophyll (M) cells must interpret positional information distributed locally around each vein. Recent molecular-genetic analysis have defined leaf development specific genes in the organization of leaf primordia, leaf shape, dorsiventral polarity, and the specification of distinct domains within a single leaf (e.g. leaf blade and leaf sheath), (Taylor, 1997).

In higher plants, greening of leaves involves a series of distinct morphological and biochemical processes leading to differentiation of cells and organelles such as chloroplasts. The differential action of light on chloroplast biogenesis in different cells highlights the general fact that tissue specific differentiation, as directed by the developmental program, determines light responsiveness. Chloroplast development requires a complex interaction between nuclear and plastid genomes which are triggered by light and a chloroplast derived signal. In addition, light is also required for photoreduction of protochlorophyllide to chlorophyll (Hoober, 1984).

The differentiation of cell types in plants depends on the continuous

interpretation of positional information. The developing young monocot leaf provides an excellent and highly reproducible tissue for investigating plastid development. Since cell age can be determined as a function of the cell position in the leaf, the appearance of cell constituents can be related directly to cell maturity. As a cell is displaced, it expands and differentiates, and thus the distance between a cell and its origin is a function of both cell age and leaf developmental stage (Schnyder et al., 1990). As cells develop in files, each leaf provides a linear gradient of cell development from base to its tip (Leech 1985). In C_4 plants, development is further accompanied by differentiation of two photosynthetic cell type viz., bundle sheath and mesophyll cells. The photosynthetic reactions are split between these two cell types with each cell type accumulating a specific complement of photosynthetic enzymes (Langdale and Nelson, 1991).

In this study, we used the pearl millet leaf as a model system to examine the effect of light on regulation of cytosolic and chloroplastic enzymes and levels of photosynthetic pigments in relation to the cellular position in developing leaves of pearl millet seedlings. We also used chloroplast developmental mutants of pearl millet to study the nuclear-plastidic interaction on photoresponse in developing pearl millet leaf. The study highlight that expression of photoresponse in pearl millet leaf is determined in hierarchical fashion by cell maturity, chloroplast development and the availability of light.

2. REVIEW OF LITERATURE

After germination, the reserve energy of the seed is mostly used to attain photoautotrophy by acquiring photosynthetic capability in the developing leaf or cotyledon. Leaf development and the achievement of photoautotrophy in angiosperm seedlings are obligatorily dependent on the availability of the light. The influence of light on the process of leaf development can be elegantly studied during seedling development, as young seedlings have enough reserve to sustain development for few days till attainment of photoautotrophy. In the absence of light, dicot leaves or cotyledons do not expand, and in monocots they cannot acquire photosynthetic capability. Plants use signal from the light environment in one or other form to regulate development at all stages of their life cycle. In the absence of light, plants follow a characteristic developmental program called as scotomorphogenesis manifested most prominently during seedling development. It is of interest to learn how light regulates leaf and cotyledon development. The present review summarizes the current information on leaf development.

2.1. Genes regulating leaf development

Leaf is one of the most important organ of angiosperms. The vegetative apical meristems continually develop new cells and form a mass of tissues. At some distance below the apical meristem the leaf primordia originate, and differentiate into leaf. The factors responsible for transition of cells generated by shoot meristem which are undifferentiated, to leaf primordium which are committed to organ formation are not known. The trigger for this transition is possibly provided by positional information by most recently formed organ primordia (Wardlaw, 1968). The analysis of genetic mosaics reveals that leaf development can be distinguished into three distinct phases, recruitment of cells within the meristem, cell division generating the primordium, and post-primordial cell division and differentiation into the mature leaf (Freeling, 1992).

In monocots after the formation of leaf primordium, the subsequent growth of the leaf takes place from continued cell divisions at a basal meristem. Since the monocot leaf elongates by generation of new cells at the basal meristem, the position of cells in the leaf continually changes with reference to the base. In monocot leaves

cell division is followed by cell expansion. In these leaves cells are continually produced at leaf base in meristematic region and expand without division in distal region of elongating zone, while they are pushed forward by younger cells. It takes about 8 h to 4 d for a cell to cross the elongation zone and complete its development. In maize leaves the examination of cell expansion profile in leaf revealed that cell division and expansion occurred in first 20 and 60 mm after ligule respectively (Bernstein et al., 1993; Salah and Tardieu, 1995)

The development of dicot leaf is bidimensional and compared to monocots occurs over a considerably longer duration. The first phase of leaf development is primarily characterized by cell division in the leaf, but little cell expansion takes place. In a recent study it was shown that in sunflower leaves both leaf expansion rates and division rates were similar and constant in all the zones of the leaf (Granier and Tardieu, 1998). In the later phase the cessation of leaf elongation is followed by cessation of cell division leading to formation of mature leaf.

The cyto-differentiation of organelles particularly chloroplasts in leaf cells is also intimately related to leaf development. During leaf development meristematic cells possess only proplastid which differentiates into chloroplast during leaf expansion and elongation in the presence of light. The development of proplastid to chloroplast is regulated by a dual mechanism dependent on differentiation of leaf cells and the availability of light. In the absence of light the proplastid develops into etioplast (Weier and Brown, 1970).

Compared to the floral and inflorescence meristem, information on genes controlling leaf initiation and development is limited. The molecular-genetic analyses have however, identified only a few genes regulating leaf development belonging to the category of homeobox genes. The molecular-genetic analyses have indicated that a number of genes play a role in initiation and maintenance of shoot meristems. These genes can be grouped into three categories based on studies conducted on *Arabidopsis* shoot meristem, *zwille* and *pinhead* are required for shoot meristem initiation, *clv1, clv2, clv3* are required for meristem maintenance and *stm* and *wus* do both functions i.e., are needed for meristem initiation and maintenance (Clark, 1997). The proliferation for cells at shoot meristem is dependent on extensive interaction between these genes.

The genes responsible for leaf initiation are not fully identified, however it is now known that initiation of leaf primordia is preceded by down regulation of the products of the genes expressing in shoot meristem. One of the first gene associated with leaf development was discovered by study of *knotted-] (Kn-1*) mutant locus in maize. Mutations in this locus alter leaf development. The *KN-1* gene codes for a homeobox domain-containing protein, which binds to DNA (Vollbrecht et al., 1991). The gene also has a PEST motif, which acts as a signal for rapid degradation. The *KN-1* gene is similar to many animal homeobox containing gene, which regulates processes determining cell fate. In another mutant of maize *Rs1,* though it is phenotypically similar to the *Kn-1,* the action of mutant loci is confined to the ligular area of the leaf. The molecular analysis of *RS1* gene sequence shows that it also encodes a homeoprotein (Smith and Hake, 1992). The homologues of homeobox genes similar to maize KN1 locus have been found in other systems too. In *Arabidopsis thaliana, KNAT3* is *KN-1* like gene, which express during early development in young leaves. *KNAT3* is expressed in several tissues and at several times during plant development.

The analyses of the *KN1* in leaf development indicate that simple and compound leaves may develop by fundamentally different mechanisms. Misexpression of *KN1* or *KNAT* in leaf primordia of maize (Smith et al., 1992; Schneeberger et al., 1995), *Arabidopsis* (Lincoln et al., 1994; Chuck et al., 1996), and tobacco (Sinha et al., 1993) produces irregularly expanded leaves. By contrast, constitutive expression of *KN1* in transgenic tomato plants has a completely different effect on leaf morphology. Instead of producing reduced leaves, it produces highly ramified compound leaves (Long et al., 1996). Gene fusions of this nature can cause changes in expression patterns that lead to altered morphology and such a phenomena may have played a role in the evolution of the leaf form (Chen et al., 1997).

The information on genes involved in leaf primorida initiation, maintenance of basal meristem in monocots and other processes is still very limited. The preliminary studies indicate that in tomato, expression of *KNOX* gene is detected in floral meristems, emerging leaf primordia and developing leaves. In tomato, *KNOX* genes have different expression pattern in both simple and compound leaves. In tomato, leaf morphology and inflorescence organization are conditioned by the two dominant

mutations *Cu* and *Me,* which resembles the effect of *Knl* like genes in monocot plants (Parnis et al., 1997).

Some information is available on genes involved in regulation of leaf size, cell division orientation, and cell elongatioa In *Antirhinum,* two genes *PHAN* and *GRAM* act in coordination to determine formation of dorsiventral axis of leaf. *Rev~l* mutants of *Arabidopsis* show overgrowth of leaves. These mutants also have defective apical meristem activity resulting in the premature termination of the shoot apex and formation of abnormal or incomplete structures in place of paraclades and flowers. The *REV* gene is required for limiting the growth of the leaf and stem and for the normal growth of apical meristems (Talbert et al., 1995). *MAC* a gene of *Arabidopsis thaliana* plays an important role in regulating the elongation of cells in the longitudinal direction in leaf development (Kim et al., 1997). The *tan1* mutation alters the cell division orientations throughout the maize leaf development without altering the leaf shape (Smith et al., 1996).

A novel *argonaute* mutant (*ago1-1* to *ago1-6*) of *Arabidopsis thaliana* pleotropically affects general plant architecture. The apical shoot meristem generates rosette leaves and a single stem, but axillary meristems rarely develop. Rosette leaves lack a leaf blade but still show adaxial/abaxial differentiation. Instead of cauline leaves, filamentous structures without adaxial/abaxial differentiation develop along the stem and an abnormal inflorescence bearing infertile flowers with filamentous organs is produced (Bohnert et al., 1998). In *Arabidopsis,* mutations that alter palisade development, *rot3 ('rotundifolia')* and *aug ('augustifolia')* fundamentally modify the leaf formation (Tsuge et al., 1996). However in *Arabidopsis* mutations at *pac ('pale cress')* locus which also alter palisade development does not alter the leaf form (Keddie et al., 1996; Chatterjee et al., 1996).

The formation of leaf in *Arabidopsis* appears to be determined by a set of genes whose expression is essential for leaf formation, in absence of these genes leaf transforms to cotyledons (Meinke, 1992). Apparently these genes are involved in the process of leaf determination. Mutations of three genes in *Arabidopsis thaliana-extra cotyledon1* (*xtc1*), *extra cotyledon2 (xtc2),* and *altered meristem programming1* (*amp1*) transform leaves into cotyledons (Conway and Poethig, 1997).

In monocots meristematic cells pass through a series of stages which specify

Table 2.1. Genes that have been identified in regulation of leaf development in different species.

SPECIES	GENE	EXPRESSION PATTERN	REGULATIONS	REFERENCES
Maize	class I *knox* foj *1('knotted')* *rs1(*'rough sheath*')* *stml* ('shoot meristem less'*)*	Shoot apical meristems, axillary meristems and unexpanded leaves	Maintenance of meristem and leaf initiation	Smith et al., (1992); Jackson et al., (1994).
Arabidopsis	*kn1* *Stml*	Shoot apical meristems, axillary meristems and unexpanded leaves	Highly lobed leaves.	Lincoln et al., (1994) Long et al., (1996)
	pac	Immature plastids, leaves have larger intercellular spaces and no palisade		Reiter et al., (1994)
	Cla1	Organ formation	Regulates shoot meristem activity	Clark et al., (1997)
Tomato	*LeT6*	Shoot apical meristems and all leaf primordia	Excessive compound leaves	Chen et al., (1997)
	tkn1	Emerging leaf primordia and developing leaves		Lu et al., (1996)
Tobacco	*lam1*	leaf remains bladeless thick leaf due to the doubled number of mesophyll layer	Controls leaf development	McHale(1993)
	fat		Initiation of leaf blade tissues	

regional differences along the long axis of the leaf. The longitudinal axes of maize leaf is composed of, in proximal to distal order, sheath, ligule, auricle and blade. According to Freeling (1992) model of maize leaf development, sheath is the first stage followed by ligule/auricle and then blade. Mutant alleles in several genes (e.g., *Kn1, Rs1, Lg3, Lg4*) are thought to retard the progression of these stages in leaf development. In maize leaf ligule is the boundary separating leaf blade and leaf sheath, and in ligular mutant this demarcation is diffuse. The gene *liguleless 2* encodes for a leucine zipper (bzip) transcription factor (Walch et al., 1998). In *glossy early flowering* (*gef*) mutant of maize, the juvenile phase of vegetative shoot development is truncated by eliminating the first leaves, which develop on the shoot. Mutation in *gef* affects the identity of the entire leaf, and reduces the number of leaves produced during embryogenesis, but has no effect on the rate of leaf formation after germination (Vega and Poethig, 1996).

In short, the information on genes regulating leaf development is still incomplete. In addition to gene regulation, ectopic expression of apparent structural proteins may control leaf formation. Such an evidence is obtained by application of expansin, a cell wall protein that is needed for cell expansion, and this protein is synthesized by plant cells during the process of cell elongation. The exogenous application of a drop of purified expansin protein on shoot meristem initiated leaf primordia and produced organ like out growth as well as triggered expression of Rubisco (Fleming, 1997; Kahmann and Basse, 1997). The mechanism of expansin action in initiating leaf primordia is however yet to be deciphered.

2.2. Genes regulating chloroplast development

Though the plastid has its own genome, its differentiation into forms such as chloroplasts, chromoplast, amyloplast etc. is determined by its temporal and spatial location, which indicates that there also exists a specific set of nuclear genes which regulates chloroplast development. It is known that though all the plant cells possess plastids, only few tissues of leaf have the capability of developing chloroplasts. Depending on spatial location plastids may also transform to amyloplasts or chromoplasts. An interesting example of spatial differentiation is formation of chloroplast in C_4 plants, where dimorphic chloroplasts are present in bundle sheath

(B) and mesophyll (M) cells. Similarly the guard cell chloroplasts also represent another type of specialized chloroplasts.

Photomorphogenetic differentiation in leaves is accompanied with chloroplast biogenesis and induction of a number of enzymes (Kendrick and Kronenberg, 1994). The emphasis has been laid on identification of components which regulate photomorphogenetic development using *Arabidopsis* as a model system and selecting mutants which possess phenotype of light-grown plants in dark-grown seedlings (Chory et al., 1989). In *Arabidopsis det1* mutant, the loss of DET1 protein lead to ectopic expression of chloroplasts in root cells, indicating that DET1 is needed for correct tissue specific expression of chloroplast in *Arabidopsis* (Chory et al., 1989).

The recent researches have shown that the chloroplast development and morphogenesis of palisade cells in tomato may be intimately linked. In *dcl* mutant of tomato and *dag* mutant of *Antirhinum* which have albino and green sectors on the leaf, palisade cells are normal in green sector but in albino region they do not expand fully. Since *DCL* gene and *PAC* gene encode for a chloroplast destined polypeptide it was felt that this protein is required for both chloroplast development and palisade cell morphogenesis in leaves (Keddie et al., 1996; Chatterjee et al., 1996). There are also mutations which effect plastid division and expansion e.g. *arc* mutant of *Arabidopsis*, possess only two chloroplasts in M cells which are about 20 times the size of WT (wild type) chloroplasts (Pyke et al., 1994). One of the genes that were identified in regulating both leaf and chloroplast development is *pac* gene. The expression of *pac* gene in *Arabidopsis* is regulated by light and is not affected by blocking chloroplast development by NF (Reiter et al., 1994). The *pac* gene encodes an acidic predominantly α-helical protein. The phenotype of *albino* mutant of *Arabidopsis*, which has yellow or white cotyledon and leaves, is due to defect in formation of a chloroplast envelope proteins with homology to proteins present in bacterial membrane and yeast mitochondria (Sunderberg et al., 1997).

Light alone is not a sufficient factor for chloroplast development in leaf. The chloroplast development is also regulated by intrinsic developmental signals that control leaf differentiation. For example, in leaf of C_4 plant B cells from different chloroplasts that are metabolically and structurally distinct from M cell chloroplasts and both of these are different from guard cell chloroplasts. At the same time in

epidermal tissue only guard cells have chloroplasts and the neighboring epidermal cells do not develop any chloroplasts.

Such tissue specific development of chloroplast is apparent in maize *Bsd2-ml {bundle sheath defective 2)* mutant where mutation disrupts B cells resulting in reduction in the photosynthetic enzymes and an aberrant chloroplast development. The mutation also disrupts the pattern of *rbcL* transcript accumulation. It appears that *bs2* gene is necessary for post-transcriptional control and *rbcL* transcript accumulation in B and M cells (Roth et al., 1996). Now it is evident that light stimulus has to act in a concert with M and B cell specific signals to co-ordinate the regulation of nuclear, chloroplastic and mitochondrial genomes in each cell type, which is necessary for establishment of C_4 photosynthesis (Nelson and Langdale, 1992).

The studies on mutations causing aberrant chloroplast development have also highlighted the fact that the mutations at loci other than the genes directly involved in chloroplast development can also cause defect in chloroplast development. In the present study we used *yellow stripe* and *zebra* mutants of pearl millet which are phenotypically similar to maize mutants. In a maternally inherited *yellow stripe* mutant of maize, it was reported that this defect is caused by a defective mitochondrial cytochrome oxidase subunit 2 gene (Newton et al., 1990). By contrast nuclear inherited *yellow stripe* mutants of maize are defective in uptake of iron from media due to defect in phytosiderophore uptake (von Wiren et al., 1994). In an earlier study on pearl millet *yellow stripe* mutant, the plastids were found in yellow regions and their aberrant plastids was defective in chloroplast development and lacked ribosomal RNAs. Plastid specific 23S and 16S rRNAs were absent in yellow regions. There were no detectable differences in M and B plastids in yellow tissues (Reddy et al., 1988). Similarly no differences were observed in the DNA content in both green and yellow regions of the pearl millet *yellow stripe* leaves.

In a recent publication Neuffer et al., (1997) listed at least 7 *zebra* like mutations in maize for a few of which location on chromosomes is known e.g., *zb4* and *zb7* are on short and long arm of chromosome respectively, *zb6* on chromosome 4L, *zb8* on chromosome 9s and *zbi* on chromosome 8L. On comparison of the phenotypes described earlier, the pearl millet *zebra* mutant is somewhat similar to

maize *zb6* and *zb4*, where similar to pearl millet yellow cross band on leaves are promoted by cooler temperature. While several other mutant loci from maize have been cloned (Neuffer et al., 1997) the molecular nature *of zebra* mutation is not known.

In maize *zebra* stripe mutants, (*zb1, zb2, zb3, zb4, and zb7*), affect chloroplast development by reducing thylakoid membrane formation and the number of grana. Mutant plants typically express transverse yellow band on the leaves. Adult double mutants were stunted compared to wild type and single mutant siblings. Western analysis of plastidic proteins of double mutants using antibodies against four chloroplast photosystem complexes protein (Sub 4, psaD, psbA, and ATPase) were conducted to assess the nature of the defect. The analysis of wild type, *zb3, zb7* and the *zb3-zb7* double mutants with specific antibodies using juvenile leaves of the double mutants revealed that these leaves lack psaD. The analysis of adult *zb3-zb7* double mutant leaves revealed a small amount of the psaD protein, suggesting that though the juvenile leaves lack PSI completely, the mutant recovers small amounts of PSI in the adult leaves (Surdam and Garcia, 1997).

2.3. Nucleus plastid **interaction** on gene expression

Plastid gene expression during plant growth and development requires several interdependent processes. Although the plastid contains all the basic components for gene expression, it must rely on the import of nuclear-encoded proteins to carry out plastid biogenesis and photosynthesis. Developmental and light-dependent regulation of plastid gene expression has been studied in plants and algae. The assembly of photosynthetic machinery accompanies the developmental changes in plastid morphology. This involves an enhanced and co-ordinate expression of plastid and nuclear genes. Though nuclear genes regulate chloroplast development, now it is evident that chloroplasts also send a signal back to nucleus and control expression of proteins that are targeted to the chloroplasts.

Plastid biogenesis, stimulates the expression of nuclear genes for plastid proteins (Oelmüller, 1989; Taylor, 1989; Susak et al., 1993). The light activated signal, cannot operate in the absence of functional plastids (Oelmüller et al., 1986) indicating an intracellular connection between the cytoplasmic signaling, control of

plastid biogenesis and expression of nuclear genes is required for synthesis of plastid proteins. The photocontrolled accumulation of nuclear-encoded thylakoid polypeptides in tobacco seems to be regulated at both the post-transcriptional and post-translational levels (Herrmann et al., 1992; Palomares et al., 1993).

Plastid proteins are encoded in two genomes, one in the nucleus and the other in the organelle. The expression of genes in these two compartments is co-ordinated during development and in response to environmental parameters such as light. Two converging approaches reveal features of this coordination: the biochemical analysis of proteins involved in gene expression, and the genetic analysis of mutants affected in plastid function or development. Because the majority of proteins implicated in plastid gene expression are encoded in the nucleus, regulatory processes in the nucleus and in the cytoplasm control plastid gene expression, in particular during development. Many nucleus-encoded factors involved in transcriptional and post-transcriptional steps of plastid gene expression have been characterized (Goldschmidt-Clermont, 1998).

In recent years emphasis has been laid on to deciphering the hypothetical 'plastid factor' which is sent from plastid to nucleus to regulate gene expression. Chory's group (1989) isolated specific *Arabidopsis* mutants, which uncouples nuclear gene expression from chloroplast development, to find the above factor. These mutants are called gene 'uncoupled' *{gun)* mutants and are characterized by uncoupling of chlorophyll a/b binding proteins of PSII and small subunit of Rubisco gene expression from the functional state of chloroplast. *gun* mutants were selected for their ability to express *CAB3* genes under conditions in which chloroplast did not develop. The *gun* alleles are recessive and consist of six complementation groups, *gun1-6*. Susek et al., (1993) suggest that non-functional plastids send a signal to repress the nuclear photosynthetic gene expression (Susek et al., 1993). *gun]* mutant in *Arabidopsis* seedlings develop normally in dark, but the development of plastids were slow compared to wild type when transferred to light, it slow down the accumulation of chlorophyll. *gunl* mutant seedlings also lack the prolamellar body and do not accumulate POR A protein. The results suggest that *gunl* mutant seedlings play an important physiological role in the signaling pathway between nucleus and

chloroplast (Mochizuki et al., 1996).

The possibility that some pigment precursors may be putative plastid factor was highlighted by studies of *Chlamydomonas* where plastid-derived chlorophyll precursors replaces light in the induction of two nuclear heat-shock genes *(HSP70A* and *HSP70B)* and thus qualify as plastidic signal. These chlorophyll precursors specifically activate the light signaling pathway. This finding suggests a specific interaction of defined chlorophyll precursors with factor(s) that regulates nuclear gene expression (Kropat et al., 1997).

2.4. Positional regulation in leaf development

The monocot leaves are polarized in both cell division and expansion. In monocots such as maize, a basal meristem generates files of cells that remain aligned from the base to the tip of the leaf (Poethig, 1984). In monocot leaves, cells at the tip are most photosynthetically developed whereas at basal location, leaves contain cells at progressively younger stages of differentiation (Nelson and Langdale, 1992). The above developmental gradient in monocot leaves has been utilized to examine various aspects of leaf development in barley (Viro and Kloppstech, 1980), wheat (Dean and Leech, 1982), oat (Taylor and Mackender, 1977) and maize (Baker and Leech, 1977; Williams and Kennedy, 1978). The expression of genes in the developing leaves occur after integrating the information about cell types, position and light level. There have been many studies to understand how this information is generated and expressed at the molecular/genetic level. The present information is far from complete. The available information related to parameter studied in this thesis is summarized.

2.4.1. Regulation of plastidic proteins/pigment

The gradient of cell age upwards from the base of the graminaceous leaf has been mostly exploited to study plastid development and the role of the chloroplast and nuclear genomes in the assembly of the photosynthetic apparatus (Viro and Kloppstech, 1980; Dean and Leech, 1982; Martineau and Taylor, 1985). One of the most apparent effect of cell age on plastids formation, is the distribution of the plant pigment chlorophyll, which is also visually apparent. The examination of chlorophyll

and carotenoid distribution from the base to the tip in *Lolium tubulentum* leaf revealed that the levels of chlorophyll and carotenoid increased with increasing distance from the base, reaching their maxima between segment 4 to 6.5 cm from leaf base. Appreciable amounts of chlorophyll was present within 1.5 cm from basal meristem and at least 2 cm below the point at which the leaf emerges into full light from the sheath formed by the bases of older leaves (Davies et al., 1989). Robertson and Laetsch (1974) examined the chlorophyll synthesis in five regions of etiolated barley leaf using both excised and intact leaf segments. The older leaf segments synthesized chlorophyll at faster rate than younger leaf section. Also there was a marked delay in onset of chlorophyll formation in younger section compared to older section.

The pattern of chlorophyll distribution in monocot leaves is also correlated with the state of plastid development. Dark-grown barley leaves possess proplastids at the leaf base and well differentiated etioplasts at the leaf tip (Robertson and Laetsch, 1974) while the light-grown leaves possess proplastids at the leaf base followed by amyloplasts, protochloroplasts present little above the leaf base and chloroplasts in the middle and tip region (Wellburn et al., 1986). In barley the illumination stimulates plastid differentiation in all segments of leaf within 48 h (Robertson and Laetsch, 1974).

The distribution of precursor for chlorophyll biosynthesis, Pchlide has not been examined, however the information is available for distribution of phytochrome POR both in etiolated and light-grown leaves. The photoconversion of protochlorophyllide to chlorophyllide is brought about by the action of plastidic enzyme POR. In angiosperms it is now established that POR consists of two distinct isozymes of POR A and POR B encoded by two different genes, which are identified as two distinct isozymes of POR A and POR B (Holtorf et al., 1995). The POR A is predominantly present in etiolated seedlings and upon exposure of seedlings to light POR A level is down regulated. The greening leaves of barley possesses POR B which has properties distinct from the POR A present in etiolated leaves. However differentiation of etioplasts in dark-grown leaves is regulated by the expression of POR A (Sperling et al., 1998). In fourth leaf of *lolium*, 2 bands of POR were identified with MW of 39 and 41kD respectively. 39 kD band was present in the leaf up to 4 cm from base, and 41 kD band was at a constant level throughout the leaf

length. Since these leaves were light-grown it may be assumed that it reflected distribution of POR B in the leaf. Similarly in 7-d old light-and dark-grown maize leaves the level of POR increased from leaf base to leaf tip (Reddy, 1995) and the distribution pattern of POR was similar in both light-and dark-grown leaves. Similarly in light-grown barley leaves the distribution of POR protein followed a similar gradient to *lolium* and maize (Schunmann and Ougham, 1996), but POR mRNA was present at a maximal level at the leaf base in leaf elongation zone.

The influence of cell position on both protein and transcript levels have been examined for several nuclear encoded plastidic proteins and in most cases the accumulation of protein is maximal at the leaf tip which has the oldest cells. The examination of several plastidic proteins in maize such as Cyt/(Davies et al., 1989), LHCP, rbcL, rbcS (Mayfield and Taylor, 1984; Martineau and Taylor, 1985), showed that the level of the plastidic proteins increased from leaf base to leaf tip. In the expression of the transcript for the plastidic proteins in maize such as such as LHCP, rbcL, rbcS (Nelson et al., 1984; Martineau and Taylor, 1985), plastocyanin, the coupling factor (Mayfield and Taylor, 1984) is similar to protein distribution, but protein and mRNA level was not exactly parallel (Martineau and Taylor, 1985). In contrast in barley accumulation of LHCP and RbcS protein and mRNA is highly co-ordinated from leaf base to leaf tip (Viro and Klopstech, 1980) in wheat leaves. In barley leaves, the expression of LSU of Rubisco and Rubisco activase transcripts follows a gradient with increasing level towards the tip, but *cab* transcript level peaked at 2 cm from the leaf base and its level declined towards the tip of the leaf (Zidlinski et al., 1989). The expression of photosynthetic genes in C_4 plants is also subjected to positional regulation due to differentiation of Kranz anatomy. The accumulation of these proteins were examined in the light-grown leaves, but in the etiolated leaves of maize many of the plastidic proteins did not appear, for example, in LHCP as their synthesis is closely linked to development of functional chloroplasts.

The examination of the different stages of wheat leaf development shows an increasing number of plastids per cell and also the amount of chloroplast (cp) DNA per cell increased with cell age but cpDNA per plastid remains constant. Plastids per cell increased continuously but cpDNA per plastid decreases (Boffey and Leech,

1982). Increasing gradient of Rubisco and chlorophyll was observed along the length of the wheat leaf M cells. Chlorophyll development takes place 2 cm above the leaf base, and completes its development within 36 h, whereas total DNA content varied reflecting the changes in nuclear and chloroplast DNA synthesis during different phases of cellular and chloroplast division (Dean and Leech, 1982a). Dean and Leech (1982b) reported that Rubisco protein is increased along the length of three species of wheat leaves. The increasing content of Rubisco is constant for all three species. Increase in Rubisco per M cells to nuclear DNA per M cells was observed. Increased nuclear DNA leads to the increasing synthesis of Rubisco. Protein and RNA content also increased along the length of the leaf.

The changes in photosynthetic capacity that characterize leaf ontogeny appear to be part of developmental program that specifies alterations in the expression of nuclear and chloroplasts genes for photosynthetic protein (Huffaker, 1986; Gepstein, 1988). Jiang and Rodermel (1995) showed that expression pattern of photosynthetic proteins altered along the length of fully expanded tobacco leaves, such as Rubisco activase, Cyt/and CAB. But the alterations were similar throughout the development of WT and antisense mutant of tobacco leaves. Alterations in the Rubisco content during WT leaf development were primarily a function of co-ordinate changes in *RbcS* and *rbcL* transcript abundance. Similar observations were also made in soybean (Jiang et al., 1993), wheat and *Phaseolus* (Brady, 1988; Bate et al., 1991). The signals that integrate *RbcS* transcription in the nuclear cytoplasmic compartment with *rbcL* transcription in the plastids involve both endogenous (hormones) and exogenous environmental (light) factors (Mullet, 1988; Taylor, 1989; Susek and Chory, 1992).

In maize, the pattern of leaf development is more complicated and being C_4 plant it also has chloroplastic and cellular dimorphism. Maize leaves possesses two major photosynthetic cell types; B cells containing agranal plastids, and M cells with chloroplast exhibiting extensive thylakoid membrane stacking (Martineau and Taylor 1985). The cell developmental gradients in maize have been exploited to characterize the appearance of C_4 activities (Perchorowitz and Gibbs, 1980), proteins (Martineau and Taylor, 1985) and mRNAs (Martineau and Taylor, 1985). Above studies reveal that while the levels of C_4 proteins increase towards the tip of leaf, the levels of the corresponding mRNAs peak near the base of the blade and decrease towards the tip.

Immunolocalization methods have shown that C_4 protein accumulation is always associated with a mature vein and bundle sheath anatomy even in relatively young (basal) regions of a developing maize leaves (Nelson and Langdale, 1992)

C_4 photosynthesis requires cooperative interactions between M and B cells and selective cell type specific expression of genes encoding the C_4 enzymes. In maize, it has been found that the cell type-specific expression of C_4 pathway genes is controlled by cell position within a leaf and by light (Sheen and Bogorad, 1986, 1987; Langdale et al., 1987, Nelson and Langdale, 1989). The control of C_4 expression in both M and B cells appears to takesplace primarily at the transcriptional level (Langdale and Nelson, 1991: Langdale et al, 1991). In C_4 plants like maize, gene expression depends on positional signals. Both light and cell position relative to vein influence C_4 photosynthetic gene expression. Light induces a switch from C_3 type to C_4 type of gene expression pattern in etiolated tissue. However, cell position is more important than light induction in the regulation of C_4 photosynthesis. Cells that are distant from veins do not accumulate C_4 enzymes even with light induction. On the other hand, cells that are close to a vein are able to overcome the effect of low light, which would, by default, result in C_3 type of gene expression patterns in cells more distant from a vein.

In C_4 plants PEP-carboxylase, malate dehydrogenase, and pyruvate phosphate dikinase act in the M cells, Rubisco and maleic enzyme act in the B cells. Thus the expression of the above genes is light dependent and cell specific (Langdale et al., 1987). However, on illumination of maize leaves, there is a suppression of Rubisco in M cells and expression in B cells leading to appearance of the observed distribution gradient of the plastidic enzymes in B cells alone (Nelson and Langdale, 1992). Moreover, after onset of illumination, Rubisco in M cells is degraded at a faster rate than that in B cells. It is therefore evident that in addition to cell position, the differentiation of veins also plays an important role in determining accumulation of several plastidic proteins in M and B cells (Nelson and Langdale, 1992);

Another protein, which shows a distinct gradient of accumulation in monocot leaves is LHCP (Nelson et al., 1984; Nelson and Langdale, 1992). The LHCP of PS-II make up less than 50% of Chlorophyll containing polypeptides of thylakoid membranes isolated from chloroplasts. It is encoded by a multi-gene family in barley

and wheat, and light regulates the LHCP gene expression in plant leaves. In maize leaves six different LHCP gene have been identified, and 95% of total LHCP mRNAs is present in B and M cells. Three genes are preferentially expressed in M cells and their mRNAs constitute about 54% of the total LHCP transcripts of illuminated dark-grown maize leaves. Most interestingly the RNA of one gene that contributes about 8% of the total LHCP transcripts in leaves greening for 24 h is present in much higher level in B cells than M cells. Immunolocalization studies, using an LHCP monoclonal antibody, demonstrate the presence of LHCP polypeptides in chloroplasts of both cell types (Vainstein et al., 1989).

In pearl millet leaves light induces the appearance of a chloroplastic specific α-amylase, which is localized both in M and B cells (Vally and Sharma, 1995). The analysis of distribution of a-amylase in these leaves revealed a distribution profile in conformity with other plastidic proteins. The level of plastidic a-amylase increased in pearl millet leaves from leaf base to leaf tip, in fact in first 2-3 cm of leaf its activity was below the limit of detectability (Vally, 1994).

By contrast another plastidic enzyme in pearl millet phosphorylase was exclusively localized only in B cells and no activity could be detected in M cells chloroplast in green leaves. In etiolated leaves it was present in all segments of leaf with maximal activity near the leaf tip (Vally et al., 1995). The exposure to light suppressed the appearance of plastidic phosphorylase in the basal segment of leaf, and its level increased only after 2-3 cm from leaf base with maximal level in leaf tip.

2.4.2. Regulation in extra plastidic compartments

Relatively few studies have examined effect of cell position on expression of photoresponses in non-plastidic compartments and many of the examined responses were related to plastid development. The monocot leaf is polarized in terms of cell age, and it reflects in turn accumulation of three essential components of cellular machinery viz. DNA, RNA and protein.

The examination of cell division and cell expansion pattern in 5-d old etiolated barley leaves revealed that cell division was restricted to the leaf base and segment distance grew by cell elongation (Robertson and Laetsch, 1974). There is relatively little information on pattern of cellular DNA replication in monocot leaves.

It is known that cell division at the leaf base is followed by a rapid elongation. However it is also known that most M cells are polyploid. While the cells may not be actually dividing in the zone of leaf extension they may undergo DNA replication, reflecting increasing ploidy level. In wheat leaf the distribution of cell cycle control proteins $p34^{cdc2}$ show a maximal expression near the leaf base and its level declines to low level and remains constant after 4 cm (John et al., 1990). The responsibility of cells to re-differentiate or resume cells division also depends on cell age gradients.

In 7-d old wheat leaf on per cell basis, the leaf base had maximal amount of DNA, followed by a low level near the leaf base between 0.5-3 cm, where cells were undergoing rapid cell expansion and then DNA level stabilized to a constant value, indicating a complete cessation of cell division (Topping and Leever, 1990). By contrast the distribution of total RNA and protein in the same leaf showed a distinct gradient with increasing level towards the leaf tip (Topping and Leever, 1990). It may be argued that the protein gradient in these leaves basically reflects a large scale build up of Rubisco in chloroplasts. However in Norflurazon (NF) treated pearl millet leaf which do not develop chloroplast, the maximal amount of protein was also observed in the leaf tip (Vally, unpublished results) indicating that protein gradient in these leaves is related to cell maturity.

In wheat leaf a selective response of cells has been observed with respect to hormone promoted cell division, where the cells which are distant from the basal meristem are unable to resume cell division, even after the administration of auxins, although all cells of the leaf possess equal rates of uptake and metabolism of auxins (Wernicke and Milkovits, 1987a,b).

The profile of distribution of few major proteins along the length of *Lolium* leaf was examined (Davies et al., 1989) by using SDS-PAGE. As expected the level of plastidic proteins such as rbcL and rbcS showed a higher level towards the leaf tip. However several proteins, whose location was not identified, and could also be presumably cytosolic protein having a MW 21 kD and 30 kD respectively, were maximal at the leaf base and their level declined towards the leaf tip (Davies et al., 1989).

7-d old *Lolium temulentum* L leaves has highest proportion of cell division. In *Lolium* leaves the majority of the cell divisions are confined to the basal region

between 0.8-1.6 cm. Electrophoretic separation of soluble proteins on starch or native page polyacrylamide gels followed by activity staining revealed many enzymes increase from the leaf base to tip. The number of different bands was observed to decrease with increasing distance from the leaf base. Most of the metabolically important isozymes exhibited a gradient of activity with respect to length of leaf. Acid phosphatase had more activity at leaf base and gradually declined towards the leaf tip. Glutamate oxaloacetate transaminase also showed a trend similar to that of acid phosphatase activity. However the activity of phosphoglucoisomerase was uniform throughout the length of the leaf. In case of isocitrate dehydrogenase the activity was very low at the base and increased towards the leaf tip (Oughum et al., 1987a).

Contrasting gradients of two developmentally regulated polypeptides, a-tubulin and chlorophyll a/b harvesting protein complexes were observed in the developing leaf of *Lolium temulentum* (Oughum et al., 1987b)

A positional effect on the expression of enzyme pattern in monocot leaves was obtained for the regulation of phosphorylase in pearl millet. Pearl millet leaves has two isozymes of phosphorylase, one localized in the cytosol, and the other in chloroplast. The plastidic isozymes are present in the B cells, whereas, the cytosolic isozymes are present in M cells. In etiolated leaves, where both the isozymes are present in all segments of the leaf, the distribution pattern was not altered. In light-grown leaves, the distribution pattern was altered. While light stimulated the activity of the cytosolic phosphorylase in the region close to the leaf base, it stimulated the activity of the chloroplastic isozyme towards the tip of the leaf (Vally et al., 1995). This indicates that cell position in the monocot leaves play an important role in determining the expression pattern of light-regulated enzymes. In light-grown pearl millet leaves the level of the cytosolic isoform of phosphorylase is maximum at the base of the leaf, declining towards the leaf tip. In contrast, the level of the chloroplastic isoform of phosphorylase increases from a very low amount at the base and reach maximal level at the leaf tip (Vally et al., 1995). In this case, light regulated the level of two phosphorylase enzyme in diametrically opposite way, and light stimulated cytosolic enzyme at the leaf base, but suppressed the plastic enzyme at the base. In the upper half of the leaf it suppressed the activity of cytosolic phosphorylase

and had no effect on activity of plastidic phosphorylase (Vally et al., 1995).

The above studies indicate that the action of light on regulation of cytosolic enzyme in monocot leaves follow different profile than that observed for plastidic proteins. Interestingly the effect of light on P-amylase activity in maize (Datta, 1996) and pearl millet (Vally, 1994) also showed similar profile. Though β-amylase is the starch hydrolyzing enzymes in pea and mustard the enzyme is cytosolic in localization (Levi and Preiss 1978; Manga and Sharma 1990). The cytosolic localization of (3-amylase has also been observed in wheat, barley and *Chenopodium rubrum* (Lin et al., 1988).

In etiolated pearl millet leaves though the p-amylase activity increased from leaf base to leaf tip, however exposure to light altered the pattern of distribution. In pearl millet leaves exposure to light stimulated p-amylase activity in leaf, independently of chloroplast development and was localized near the leaf base (Vally and Sharma, 1991). In addition to light in monocot leaves, p-amylase activity is also upregulated by stress. The water stress induced P-amylase in barley leaves was also predominantly present in basal region of leaf (Jacobsen et al., 1986). Similarly in green barley leaves high levels of mRNA of light stress-inducible proteins such as HL-2 and ELIP (early light inducible protein) are observed in basal region of leaf (Pötter et al., 1996). In NF-treated and albostrians mutant barley leaves, three isozymes of P-amylase were found. The white tissue possessed 3-5 fold higher activity of all 3 p-amylase isozymes compared to green tissue. The NF-treatment also induced P-amylase to a maximal level in light-grown plants, which were completely photobleached. The increase in p-amylase activity is interpreted as a stress response to high light intensity. The p-amylase activity also increases by other stresses like elevated temperature, low water potential resulting from sorbitol or salt treatment. The increased stress response to high light intensity in green tissue may be due to deficiency in carotenoid and chlorophyll (Dreier et al., 1995).

The examination of mitochondrial gene expression revealed that at least in developing wheat leaf, mitochondrial gene expression follows an inverse gradient compared to chloroplast. The maximal amount of mitochondrial gene expression was found near the base of the leaf and it declined towards the tip of the leaf (Topping and Leever, 1990). Interestingly the examination of mitochondrial DNA level along the

length of the leaf reveals that maximal amount of mitochondrial DNA, which may also reflect the number of mitochondria per cell, was near the leaf base within basal one cm. In, more distal part of the leaf the level of mitochondrial DNA, the transcripts for mitochondrial encoded genes such as *coxII*, *Cob*, *alpA* were at a very high level near the leaf base and gradually declined to a low level by 2.5 cm from leaf base. These data indicate that complete mitochondrial development in wheat leaf takes place near the leaf base, which is inverse to that of observed pattern of gene expression and chloroplast development in wheat leaves. It would have been ideal if Topping and Leever (1990) could have also examined the level of transcript of those nuclear encoded protein which are destined to mitochondria to see if the expression pattern of these transcripts also show a similar pattern as that of mitochondrial transcripts.

The distribution of many cytosolic proteins is determined by chloroplasts, where the accumulations of these cytosolic proteins are in parallel with the development of chloroplasts. One of the most prominent of these proteins is PEP-carboxylase, which act in concert with chloroplast enzymes to fix CO_2 in C_4 plants. Studies were conducted on expression of PEP-carboxylase, which though is a cytosolic enzyme in C_4 plants is intimately linked to chloroplast function. PEP-carboxylase is a ubiquitous enzyme occurring in cytosol of photosynthetic and nonphotosynthetic tissues of higher plants, algae, bacteria, and legume root nodules (Andreo et al., 1987; Deroche and Carrayol, 1988; Vance and Gantt, 1992). The enzyme is responsible for primary fixation of CO_2 in C_4 and CAM plants. Interestingly, the cell specific expression of PEP-carboxylase is related to its methylation at a specific site at its gene sequence at 5'end (Langdale et al., 1991). The appearance of PEP-carboxylase mRNA and its polypeptides along the length of leaf follows a distinct gradient, e.g., light stimulates PEP-carboxylase mRNA from the base of the leaf and increases level of transcript towards the leaf tip (Nelson et al., 1984 ; Mayfield and Taylor, 1984; Martineau and Taylor, 1985).

Similar to PEP-carboxylase the level of another cytosolic enzyme pyruvate orthophosphate dikinase also increases from leaf base to leaf tip (Mayfield and Taylor, 1984). Similarly in pearl millet leaf the cytosolic α-amylase distribution show a clear correlation to distribution of plastidic proteins (Vally, 1994).

The information about distribution of peroxisomal enzymes in monocot leaves is very limiting. One of the marker enzymes of peroxisome is catalase, which is involved in removal of hydrogen peroxide (H_2O_2) in plants. Catalases converts H_2O_2 to H_2O and O_2 ($2\ H_2O_2 \rightarrow 2\ H_2O+O_2$). Thus catalase prevents oxidative damage to the cell by eliminating H_2O_2 accumulation and preventing H_2O_2 from reacting with O_2 to generate the more potent oxidant OH. The presence of multiple isoforms of CAT has been reported for a number of plant species. Phytochrome mediates the appearance of leaf type peroxisomes and of specific catalase isozymes. Catalase is the primary H_2O_2 antioxidant enzyme present in the peroxisomes and mitochondria, at least in maize (Scandalios et al., 1980; Prasad et al., 1994). In maize catalase is present as four different isozymes (Scandalios et al., 1984) only two of which are present in dark-grown seedlings. In dark-grown leaves of maize CAT-2 isozyme is absent, in continuous white light-grown leaves CAT 2 protein level increases. Cat2 mRNA was equally distributed both in light-and dark-grown maize leaves. In maize out of three *cat* genes expression, *call* is light dependent while expression of *cat2* and *cat3* is light responsive.

In many cases the light action on developmental process is brought about by the perception of three photoreceptors, namely UV-B-A/Blue, UV-B, and phytochrome. In etiolated maize leaf the examination of distribution of phytochrome reveals that Phy A level was more in the basal half of the leaf, and was less on the middle region and then increases towards the leaf tip (Reddy and Sharma, 1998). A similar profile of distribution was observed for distribution of Phy B in the maize leaf cells. In light-grown maize leaf the distribution of Phy A was similar to etiolated leaf and was not altered by light response, whereas in light-grown plants Phy A level was below detectable level due to the down regulation. Interestingly, on transfer of light-grown seedlings to darkness the Phy A reappeared in leaf within 12-18 h with a profile similar to that observed for dark-grown plants, indicating that cellular position primarily determines phytochrome distribution.

To summarize, though the information on influence of cell position on quantitative and qualitative development of photoresponse are limited, it is evident that the final level of a given protein pigment in a cell is subjected to multiple control, of which cell age, positional location, intracellular localization, organelle

development and availability of light all participate in a co-ordinate fashion to bring out the response.

3. MATERIALS AND METHODS

3.1. Plant material

Pearl millet evolved in the dry and semi-arid regions of Africa. In Sub-Saharan West Africa, pearl millet land races have been grown since the beginning of agriculture. So far, the introduction of improved pearl millet cultivars is limited. Farmers rely on land races with good production stability that are rarely out-yielded by improved cultivars. Farmers save seed from their own crop, often selecting for spike length, spike compactness, and kernel size. The artificial selections coupled with natural survival in the local environment have led to establishment of ecologically well adopted land races. The cultivars are the improved variety that evolved from the cultivated plant. In this study we have used a few of the mutants collected by 1CRISAT (International Crop Research Institute for Semi Arid Tropics) for our experiments.

Pearl millet seeds (*Pennisetum americanum* WGC-75) were obtained from Andhra Pradesh State Seed Corporation, Hyderabad, India. In addition, we obtained several mutants of pearl millets from ICRISAT. Table 3.1 shows the description of the pearl millet mutants that were obtained from, ICRISAT, Hyderabad, India.

The multiplication of mutant seeds was carried out by growing the plants in pots at 25°C in green house. We also obtained the seeds by growing plants in field during July-February.

3.2. Plant growth condition

Control (WGC-75) and other mutant seedlings were grown either under continuous white light or under photoperiod consisting of 16 h white light and 8 h dark photoperiod. Light sources used are described below. The seeds were first soaked in distilled water at room temperature (25 °C) for 2 h and then sown on 4 layers of moist germination papers (Jayashree traders, New Delhi) in plastic trays (40 cm *l*x 30 cm *b* x7 cm *h*). The trays were covered with glass plates for first 4 days. The seedlings were watered daily with distilled water.

Table 3.1. The characteristics of different mutants of pearl millet obtained from ICRISAT.

MUTANT	ORIGIN	DESCRIPTION	LIKELY DEFECT
glossy IP 8282	Sudan	upto 25 days	
early flowering IP 4021	Gujarat	33 days to flower, continuous flowering	May be deficient in phytochrome.
yellow stripe and white stripe on a leaf *IP 8292* *SW-1*	Nigeria ICRISAT	Yellow stripe White stripe	Defect in chloroplast development.
zebra *IP 8283* *IP 8284*	Ghana Tanzania	Transverse yellow bands Transverse yellow bands below 10°C	Altered meristem activity at low temperature leading to defect in chloroplast development

3.3. Multiplication of *zebra* mutant and its growth condition

The seeds of pearl millet mutant *zebra* were first sterilized with 0.4% Na-hypochlorite solution, and were washed thoroughly with water. Thereafter the seeds were sown in moist soilrite mixture. Soilrite mixture contains sphagnum peat moss, perlite, nutrients and wetting agent. *zebra* seedlings grown in a soilrite mixture were watered once in day with Hoagland's solution. *zebra* mutant was subjected to the following cold treatment. Four-day-old seedlings were transferred to below 10°C in night for 8 h and then transferred to growth room for 16 h at 25°C. After emergence of the seedlings the pearl millet mutant plants were grown under field conditions in pots. After 60 days, the pearl millet spikes were harvested, dried and the seeds were stored for experiments. The morphology of *zebra* seedlings grown in field as well as in laboratory conditions are shown in Figs. 3.1 and 3.2.

3.4. Light sources

All light and dark treatments were performed in an air-conditioned area at 25°C. Seedlings were grown under normal sun light (992 umol m^2 s'), continuous white light (100 umol m^2 s') (WL) which was obtained by using four tube lights, three of which were white (Phillips no. 6500 K) and one was brown (Phillips no. 2700 K) or in complete darkness at 25°C. Red light (λ max 650 nm, 0.30 μmol m^2 s^1) was obtained by filtering the light from two cool white fluorescent tube lights (40 W) through two red plexiglass sheets (λ max 650 nm). The green safe light (<0.01 μmol m^2 s^1) was obtained from a cool white fluorescent tube light wrapped in 6 layers of green cellophane paper (X max 530 nm). The intensity of the light sources was measured with a Skye unit SKP-215, SKP-110 (Skye Instruments, Powys, UK).

3.5. Norflurazon treatment

For Norflurazon (Sandoz) treatment, the seeds were soaked in 0.4 mM NF solution for 2 h and then sowed on germination papers presoaked with NF solution. The NF solution was prepared by dissolving NF in minimum amount of methanol, and making up the volume with the requisite amount of distilled water. The seedlings were grown in red light and in darkness, irrigated with NF.

Figure 3.1. Phenotype of pearl millet *zebra IP 8284* mutant. The leaves show alternate green and yellow cross band on leaves.

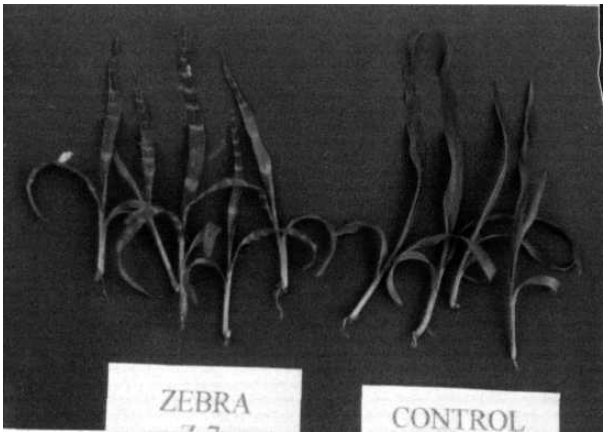

Figure 3.2. Seedlings of pearl millet mutant *zebra* IP 8284 showing alternate green and yellow bands on leaves. The seedlings were grown under photoperiodic cycle consisting of 16 h light period at 25°C and 8 h dark at about 8 h 10°C.

3.6. Protein estimation

Protein was estimated according to the procedure of Lowry et al., (1951). The protein in the crude extracts was estimated after precipitation with an equal volume of 10% (w/v) trichloro acetic acid. The mixture was incubated for 30 min at -20°C and then centrifuged at 10,000*g* for 10 min. The precipitate was dissolved in 0.5 ml of 1 N NaOH. A standard curve for the protein estimation was prepared by using bovine serum albumin fraction V (Fig. 3.3). The absorbance of the protein sample was measured at 500 nm after 30 min incubation with the reagent mixture.

3.7. Chlorophyll estimation

Chlorophyll was extracted from leaf tissue by homogenizing in 5 ml of 80% acetone at room temperature and centrifuged at 8,000*g* for 10 min. The chlorophyll content of the supernatant was estimated according to Vernon (1960) as:

Chl a = 11.63 A_{665} - 2.39 A_{649} = µg Chl a /ml

Chl b = 20.11 A_{649} - 5.18 A_{665} = ug Chl b /ml

Total Chl = 6.45 A_{665} + 17.72 A_{649} = ug Chl /ml

Carotenoid were estimated according to Liaaen-Jensen and Jensen (1971) as:

A_{473}× dilution of sample x 4= ug carotenoid/ml

3.8. Absorption spectroscopy

In vivo absorption spectra were recorded from 80% extract of pearl millet leaves by using Hitachi 557 spectrophotometer or 922 Uvikon spectrophotometer. The absorption spectra of the samples were recorded at room temperature between the ultraviolet and the visible region i.e. 300-800 nm using 80% acetone as a reference solvent.

3.9. Fluorescence spectroscopy

Fluorescence emission spectra was recorded using a Jasco FP-777 spectroflurometer at room temperature with 440 nm as excitation wavelength and the emission was recorded between 600-700 nm. The spectra was recorded using emission scan mode with a scan speed of 200 nm/min. Eighty percent acetone was used as a reference solvent.

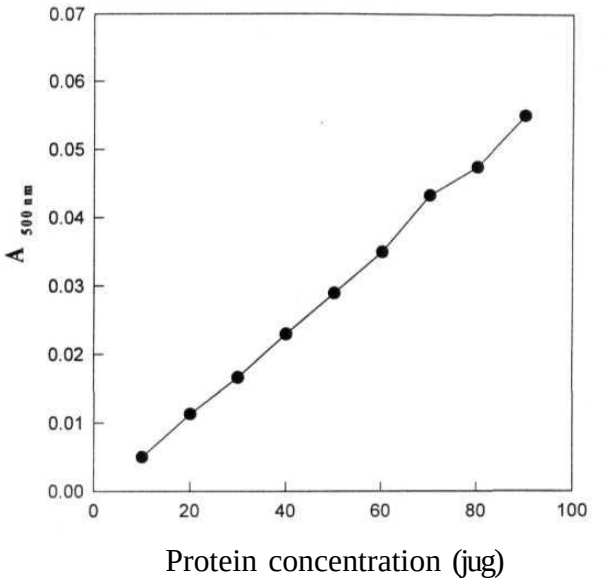

Figure 3.3. Standard curve for the estimation of protein by Lowry method (1951)

3.10. Peroxidase assay

Leaves were homogenized (1:3 ratio, w/v) in ice cold pestle and mortar by using Tris-HCl buffer pH 7.2 followed by centrifugation for 30 min at 8,000*g* at 4°C. Aliquots of supernatant 100 µl were immediately added to the assay mixture, which contained 0.32 ml of 5% (w/v) pyrogallol, 0.16 ml 0.147 M H_2O_2, 0.32 ml of 0.50 M phosphate buffer pH 7.2 and 2.0 ml distilled water. Increase in absorbance at 440 nm was monitored at every 15 s for 1.5 min in spectrophotometer. The unit of peroxidase activity was expressed as µmoles/min.

$$\frac{\text{Mean value of absorbance x total volume of assay mixture}}{\text{total number of seedlings}} \quad \text{Total activity of peroxidase}$$

3.11. Catalase assay

Leaves were homogenized in a ice cold pestle and mortar (1:3 ratio, w/v) in 50 mM Tris-HCl buffer pH 7.2 and centrifuged for 30 min at 7,500g at 4°C. 100 µl of the supernatant was mixed with assay mixture contains 1:1.5 proportion of 50 mM potassium phosphate buffer pH 7.0. The enzyme assay was initiated by adding 10 µl of substrate, 20 mM of H_2O_2. Enzyme activity was estimated by measuring absorbance at 240 nm at every 5 sec interval for 30 s. The difference in absorbance at 240 nm per unit time is a measure of activity.

$$\frac{\text{Mean value of absorbance x total volume of assay mixture}}{\text{total number of seedlings}} \quad \text{Total activity of catalase}$$

3.12. β-Amylase extraction

To extract β-amylase protein, leaves were homogenized, in a pre-cooled mortar and pestle on ice, with 50 mM Tris-HCl buffer (w/v) 1:3 pH 7.2 containing 10 mM $CaCl_2$, 5 mM β-mercaptoethanol and 1 mM phenymethylsulfonylfluoride (PMSF). The homogenate was clarified by centrifugation at 7,500*g* for 30 min at 4°C.

3.13. Protochlorophyllide oxidoreductase extraction

The harvested leaf samples were frozen in liquid nitrogen and ground (w/v) 1:2 in a pre-chilled mortar and pestle at 4°C in an extraction buffer consisting of 50% ethyleneglycol (EG), 100 mM Tris- HC1, pH 8.3, 140 mM ammonium sulfate, 10 mM

Na_4 EDTA, 20 mM sodium bisulfite. The homogenate was centrifuged at 10,000*g* for 30 min at 4°C. Proteins were separated on 10% SDS-PAGE gels using the buffer system of Laemmli (1970) to polyvinylenedifluoride (PVDF) membrane. Western blotting was done according to Towbin et al., (1979).

3.14. Light harvesting chlorophyll complex (LHCP) extraction

The procedure used for the extraction of LHCP protein was similar to that of protochlorophyllide oxidoreductase. The harvested leaf samples were frozen in liquid nitrogen and ground in a pre-chilled mortar and pestle at 4°C in an extraction buffer (1:2) (w/v) consisting of 50% ethyleneglycol (EG), 100 mM Tris- HC1, pH 8.3, 140 mM ammonium sulfate, and 10 mM Na_4 EDTA, 20 mM sodium bisulfite. The homogenate was centrifuged at 10,000*g* for 30 min at 4°C.

3.15. Extraction of PEPC

Leaves were extracted with 100 mM HEPES-KOH pH 7.3, w/v (1:4) containing 10 mM $MgCl_2$, 2 mM K_2HPO_4, 1 mM EDTA, 20% (v/v) glycerol, 2 mM PMSF and 10 mM 2-mercaptoethanol. The extract was filtered through 4 layers of cheese cloth and rapidly centrifuged at 15,000*g* for 5 min.

3.16. α-Amylase extraction

To extract α-amylase protein, leaves were homogenized in a pre-cooled mortar and pestle on ice along with (w/v) 1:3, 50 mM Tris-HCl buffer pH 7.2 containing 10 mM $CaCl_2$, 5 mM β-mercaptoethanol and 1mM phenymethylsulfonylfluoride (PMSF). The homogenate was clarified by centrifugation at 7,500*g* for 30 min at 4°C.

3.17. SDS-PAGE

The electrophoresis of denatured proteins in polyacrylamide gel in the presence of an ionic detergent sodium dodecyl sulfate (SDS) was carried out according to Laemmli (1970). 1 mm thick separating gels of 10% (w/v) were polymerized in 0.375 M Tris-HCl buffer (pH 8.8) containing 0.1% (w/v) SDS, 0.1% (w/v) ammonium persulphate, and 0.01% (v/v) TEMED. Stacking gel 5% (w/v) was made in 0.125 M Tris-HCl (pH 6.8), containing 0.1% (w/v) SDS, 0.1% (w/v)

ammonium persulphate, and 0.012% (w/v) TEMED.

Samples were prepared in buffer containing 50 mM Tris-HCl (pH 6.8), 2% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromophenol blue and 5% (v/v) p-mercaptoethanol. The samples were boiled for 4 min for uniform coating of detergent. After cooling, the samples were centrifuged and aliquot was used for electrophoresis.

Electrophoresis was carried out at room temperature at a current of 10 mA stacking gel and 20 mA for separating gels respectively. The gel running buffer was made of 25 mM Tris (pH 8.3), 250 mM glycine and 0.1% (w/v) SDS.

3.18. Electroblotting

Proteins were electroblotted onto nitrocellulose membrane using semi-dry blotting method. The membrane was cut into the size of the resolving gel and floated on the surface of distilled water for 5-10 min for nitrocellulose membrane and dipped in water followed by transfer buffer containing 48 mM Tris base, 39 mM glycine, 20% (v/v) methanol and 0.037% (w/v) SDS. In the case of PVDF membrane, the membrane was first wetted with methanol for 5-10 sec, washed with distilled water and soaked in transfer buffer. Whatman No.3 chromatographic papers were cut to the size of the membrane and soaked in the transfer buffer.

After SDS-PAGE, the stacking gel portion was excised from the resolving gel and the bottom left corner of the gel was marked with a small cut. The gel was washed twice with distilled water followed by transfer buffer.

The semi-dry blotting was carried out in a custom made apparatus. Both anode and cathode graphic plates were washed thoroughly with distilled water. On the anode plate, three sheets of Whatman No.3 papers were layered carefully, avoiding trapping of air bubbles between the sheets, the membrane was layered over it and a small cut was made in the bottom left corner of the membrane which coincides with the gel. The gel was carefully layered on the top of the membrane on which three more sheets of Whatman papers were layered. Finally, the cathode plate was placed over the Whatman papers and the whole sandwich consisting of the plates, papers, membrane and gel was tightened by clamps. The blotting unit was connected to a power supply which was set to a constant current supply of 0.8 mA/sq.cm area for 1.5 h. After the run, the efficiency of the transfer of proteins onto the membrane was checked by

staining the proteins with Ponceau S dye.

3.19. Antibodies used

The POR and LHCP polyclonal antibody was raised against barley protochlorophyllide oxidoreductase and LHCP (Prof. K Apel, Zurich). The a-amylase and β-amylase polyclonal antibody was raised against maize seed a-amylase (Subbarao, 1992). PEP-carboxylase polyclonal antibody was raised against *Amaranthus* species (Prof. A. S. Raghavendra, Hyderabad).

3.20. Western blotting

Immunoblotting was done following the procedure of Towbin et al., (1979) at room temperature. After electroblotting, the PVDF membranes were blocked for three min with blocking buffer containing 2% (v/v) polyoxyethylene sorbital monolaurate (Tween 20), 20 mM Tris-Cl (pH 7.5), and 500 mM NaCl. Immediately after blocking, the membranes were washed with a buffer containing 20 mM Tris-Cl (pH 7.5), 500 mM NaCl and 0.05% (v/v) Tween 20. The membranes were further washed three times with 20 ml of washing buffer (TBST) containing 20 mM Tris-Cl, 150 mM NaCl and 0.05% (v/v) Tween 20 for 10 min each. Next, the blots were incubated with the primary antibody (1:500 dilution) for 1 h in incubation buffer (TBS) containing 20 mM Tris-Cl (pH 7.5), 150 mM NaCl and 1% (w/v) low fat skim milk powder. Thereafter, the blots were washed three times with TBST as above for 10 min each. Membranes were again incubated in TBS for 1 h containing anti-rabbit secondary antibodies (1:30,000 dilution) conjugated with alkaline phosphatase. Thereafter, the blots were washed three times as above with TBST for 10 min each. The blots were stained by adding alkaline phosphatase substrate; 66 ul of nitroblue tetrazolium (50 mg/ml) and 33 ul 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml) in 10 ml alkaline phosphatase buffer containing 100 mM Tris-Cl (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$. After the bands become clearly visible staining was stopped by washing the blot with distilled water.

3.21. Non-dissociating PAGE

Non-denaturing polyacrylamide gel electrophoresis (PAGE) was conducted in slab gels to separate enzymes in their native state according to Davis (1964). 10%

resolving gel mixture containing 10% (w/v) acrylamide, 0.375 M Tris-HCl (pH 8.8), 0.075% (w/v) 0.05%(v/v) N,N,N',N'- Tetramethylethylenediamine (TEMED) was polymerized in a 1 mm thick gel assembly. The gel was overlaid carefully with distilled water. Stacking gel containing 4% (w/v) acrylamide, 0.125 M Tris-HCl (pH 6.8), 0.075% (w/v) ammonium persulphate, 0.075% (v/v) TEMED was polymerized on top of the resolving gel.

6 nkat of enzyme activity in 20% (v/v) glycerol, 0.005% (w/v) bromophenol blue was loaded and the gel was run at constant voltage of 100 volts for 2.5 h at 4°C. The gel running buffer was made of 0.025 M Tris, 0.190 M glycine, pH 8.3.

3.22. Activity staining of native gels

a. Peroxidase

After completion of the electrophoresis the gel was stained first by submerging in a benzidine and acetic acid mixture containing 2.4 mM benzidine and 7 mM acetic acid for 10 min and then 3 mM H_2O_2 for 2 min. Intense blue bands which turned brown on storage.

b. Catalase

After completion of electrophoresis, the gels were first washed three times with distilled water for 15 min each and then incubated in 0.003% (v/v) H_2O_2 with constant shaking for 10 min. Thereafter gel was washed with distilled water for 10 min and transferred to in a freshly prepared solution of 10% (w/v) ferric chloride and 10% potassium ferricyanide III. Yellow colored catalase bands were visible and stable for several hours and was stored in dark.

3.23. CO_2 dependent photosynthetic oxygen evolution

Oxygen evolution was monitored by using a Clark type oxygen electrode (Model DW2, Hansatech Ltd, King's Lynn, U.K.). The sample chamber of the monitor was filled with equal parts of CO_2 buffer containing 7 parts of 0.1 M $NaHCO_3$ 3 parts of 0.1 M Na_2CO_3 and Avron's medium containing 15 mM Tris-HCl pH 7.8, 2 mM NaCl, 4 mM $MgCl_2$ and 4 mM K-phosphate (Sharma et al., 1979). The pearl millet leaves were excised into pieces, which were finely excised about to 2 mm in size. A constant temperature of 30°C was maintained during the course of

experiment by circulating water through the outer jacket of the reaction chamber from a constant temperature refrigerated circulating water-bath. The electrode was calibrated with air-saturated distilled water. The samples were first incubated in the dark to establish the respiration rate, and then the oxygen evolution was monitored by exposing the samples to light (1,200 $\mu E\ m^{-2}\ s^{-1}$). A constant rate of oxygen uptake due to respiration was established as the basal rate before irradiation from which the rate of oxygen evolution was calculated. The chlorophyll level of segments was determined independently to calculate the O_2 evolution rate, which was expressed as nmoles O_2/mg Chl/h.

3.24. Quantification of western blots /PAGE bands

The relative amount of protein in the immunoblots was measured by scanning the bands using a gel documentation (Molecular Dynamics) system. Alternatively bands were scanned on a HP4C Scanjet scanner and the images were quantified by Image tool software (UTHSCA). In order to minimize the error in the scanning at a single point within a band, the bands in different lanes were inserted in separate windows and the total volume of the bands was measured. The background value was deducted from the total value of the band. The obtained values were used for plotting the graphs.

Since the intensity of the band on blots and gels represents the relative pattern of distribution, the intensity of the most bands on blot was used as a standard to calculate the relative amount of the band. The densitometric values of most intense band on gel/blot was taken as 100% and the relative amount of weaker band was calculated from that intense band.

4. RESULTS

The monocot leaf offers an ideal model to examine interrelationship between cell maturity and various developmental and growth responses. In comparison to dicot leaves, studying these processes in monocot leaves has an advantage; for example, the dicot leaf initiates as a leaf primordia, thereafter its expansion is restrained, and its further expansion and development is dependent on availability of light. By contrast, the process of leaf expansion in monocot leaf is not obligatorily dependent on light, and leaf expansion continues, even in darkness without acquisition of photosynthesis. In addition, the monocot leaf develops from a meristem located at the leaf base. By virtue of this, the developing monocot leaves possess cells of different maturity along the length of the leaf. The existence of cell maturity gradient in the monocot leaf has been exploited to investigate the effect of light on gene expression in these leaves, particularly those related to development of photosynthesis. On exposing dark-grown monocot leaves to light, the initiation and magnitude of light-induced responses can be examined along the length of the leaf cells having different maturity.

Light-regulated responses in monocot leaves most prominently include the acquisition of photosynthesis. Therefore, in the present study an extensive characterization of this process was done by using key markers of this process, such as increase in the level of photosynthetic pigments and associated proteins. Since the influence of light on leaf development is not necessarily confined to chloroplast alone, we also examined the level of few cytosolic enzymes such as β-amylase, peroxidase and catalase.

In view of paucity of studies on pearl millet as a physiological system, in the first phase of the present study we made an extensive characterization of photosynthetic pigment formation in leaves to correlate with the observation made in other plant systems. The representative markers for chloroplast development are chlorophyll, carotenoid and protochlorophyllides. Of these carotenoid and protochlorophyllide is synthesized in darkness and are present in etioplast. The exposure of light accelerates the biosynthesis of these pigments. Exposure of dark-grown leaves to light also converts protochlorophyllide to chlorophyllide, and this

conversion in turn acts as a signal to stimulate chlorophyll biosynthesis. The light also stimulates carotenoid biosynthesis, which acts as a photosynthetic accessory pigments. Since the carotenoid and chlorophyll associate together to form an active photosynthetic antenna complex, these pigments are required to be synthesized in predetermined proportions. Therefore their biosynthesis is highly coordinated to maintain a strict stoichiometry between chlorophyll and carotenoid amounts. We also examined, the effect of light on levels of plastidic protein protochlorophyllide oxidoreductase (POR), which accumulates to high level in dark-grown seedlings and on exposure to light, the level of POR protein rapidly declines, marking the onset of chlorophyll biosynthesis and acquisition of photosynthesis. The effect of light on cytosolic compartment was investigated using β-amylase and peroxidase as a marker enzyme. The influence of light on other subcellular compartments such as peroxisome was analyzed using catalase as a marker enzyme.

4.1. Morphological features of the pearl millet seedlings

Pearl millet seeds are ovoid and about 2 mm in length. On imbibition radicle and shoot tip protrude from seed coat in about 24 h after sowing. During initial phase of seedling development, mesocotyl along with shoot tip expands and the leaf remained enclosed in coleoptile for 3-4 d from sowing. After that, the expanding leaf ruptures the coleoptile and continues its expansion. The light-and dark-grown seedlings manifest significant morphological differences in their developmental patterns, characteristic of photomorphogenic and scotomorphogenic development pathways. The light-grown seedlings have green expanded leaves and a short mesocotyl, because exposure to light suppresses elongation of mesocotyl. The mature pearl millet leaves after few days of expansion can be distinguished as consisting of two distinct portions, upper portion as leaf blade, and a lower portion as leaf sheath. In leaf blade portion, the leaf is wider, whereas leaf sheath at the base is narrow and tubular. Similarly the leaf blade portion is dark-green while the basal leaf sheath is pale green. In comparison to light-grown seedlings the dark-grown seedlings possess a long mesocotyl, but the first leaf is smaller in size than the light-grown leaves. The dark-grown leaf is yellow in color as it lacks chlorophyll and therefore accumulation of yellow colored cartoenoid is visualized.

There was no apparent influence of light on germination as the germination percentage was similar in both light-and dark-grown seedlings. In this study, pearl millet seedlings were raised on only distilled water, therefore after 12-d in light and after 9-d in dark the leaf begin to senescence. Hence, experiments on first leaf of pearl millet seedlings were carried out only upto 9-d from sowing. The primary leaf was about 11 cm long in 10-d old light-grown seedlings, whereas in dark-grown seedlings, the leaf expansion ceased completely after 8-d, and the leaf size remained about 7 cm. In the present study effect of light on leaf development was studied by comparing continuous red light and white light-grown seedlings with seedlings grown in darkness.

4.2. Chlorophyll and Carotenoid

The marker of acquisition of photosynthesis in a developing leaf is initiation of chlorophyll accumulation. In order to characterize it, the profile of chlorophyll accumulation was analyzed in pearl millet leaf, from the day the first leaf begins expanding i.e. 3-d from sowing till 9th d. Since the leaves of dark-grown seedlings possess no chloroplasts, the accumulation of chlorophyll was monitored in the first leaf harvested from WL-and RL-grown seedlings. In initial period of leaf growth, leaves are shielded by coleoptiles up to 4-d from sowing, and then first leaf emerges out of the coleoptile and is directly exposed to light. Pearl millet leaf consists of two parts, the upper region as leaf blade and the lower region as leaf sheath, however for estimation of the pigment accumulation, chlorophyll and carotenoid content of the entire leaf was measured.

Fig. 4.1 shows the profile of chlorophyll accumulation in the first leaf excised from WL-and RL-grown seedlings from 3-9-d. It is evident that in both WL and RL light-grown leaves the amount of chlorophyll continually increases with increase in the age. However, the amount of chlorophyll was more in WL-grown leaves compared to RL-grown leaves. It is evident that among the two lights, exposure of seedlings to WL induced greater rate of chlorophyll accumulation, compared to seedlings exposed to the RL. The higher level of chlorophyll in leaf by ninth day signifies the fact that the chlorophyll level in the leaf increases along with the increase in the length of the leaf.

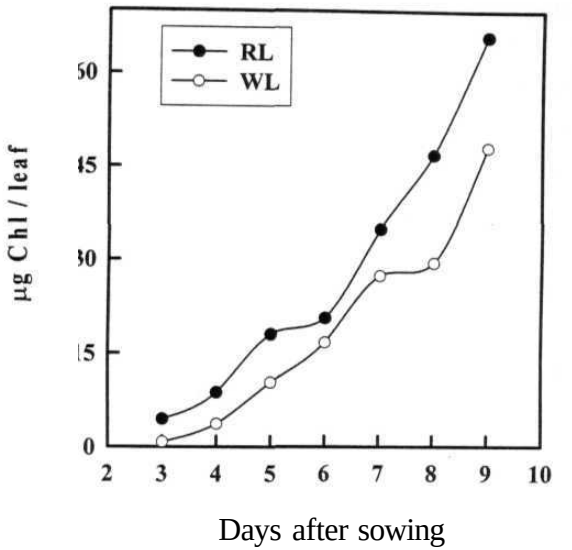

Figure 4. 1. Time course of chlorophyll accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown under WL (○) and RL (●) from sowing. At the time point (days) indicated, the first leaf was excised at the mesocotyl junction, and the chlorophyll amount was estimated.

Since in higher plants chlorophyll consists of two species viz. chlorophyll a and chlorophyll b. We examined the profile of accumulation of chlorophyll a and chlorophyll b during the period of leaf expansion. Fig. 4.2 shows that both RL-and WL-grown leaves accumulate more chlorophyll a than chlorophyll b. It is evident that under WL higher level of chlorophyll b is accumulated than under RL. Basically the accumulation profile of both chlorophyll a and chlorophyll b follow a pattern similar to that observed for total chlorophyll.

We also examined whether the formation of chlorophyll a and chlorophyll b in pearl millet leaf maintains a co-ordinated balance in their accumulation, by examining Chl a/b ratio. Fig. 4.3 shows that Chl a/b ratio in RL-grown leaf varies between 1.63-3.77, with a highest value on 5th day from sowing. Thereafter the Chl a/b ratio declined and is stabilized at 2.45. Similarly Chl a/b ratio in WL-grown leaf also increased from a low level of 1.95 to highest level of 3.22 on 6th day and its value by 1 Oth day was similar to that observed from RL-grown leaf.

The accumulation of carotenoid in pearl millet leaf followed a profile similar to the accumulation of chlorophyll in developing leaf (Fig. 4.4). In dark-grown leaves the amount of carotenoid was quite low, however, its level increased slowly with the age of the seedlings. The exposure of seedlings to WL or RL enhanced the accumulation of carotenoid in the first leaf. Among the two light treatments used, WL stimulated carotenoid accumulation more significantly than RL. However, carotenoid accumulation follows a similar profile under both RL and WL.

Since carotenoid also acts as photosynthetic accessory pigments, a strict stoichiometry is required to be maintained between the levels of carotenoid and chlorophylls. Moreover in absence of carotenoids, chlorophyll molecules are photooxidized by light, so the absence of carotenoids leads to photooxidation of chlorophylls and loss of the functional chloroplasts. The profile of Chl/Car ratio is stabilized at nearing 5.0 in both continuous WL-and RL-grown seedlings showed in Fig. 4.5.

The influence of light on the level of chlorophyll was also examined by transferring dark-grown seedlings to light, to elicit the possible duration of lag between exposure to light and initiation of chlorophyll biosynthesis. The results presented in Fig. 4.6 shows that on exposure to WL or RL, there is a rapid formation

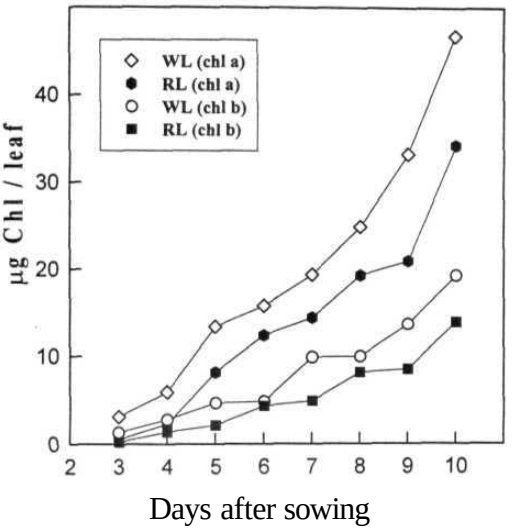

Figure 4. 2. Time course of chlorophyll a and chlorophyll b accumulation in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (◇, ○) or RL (•, •) from sowing. At the time points indicated (days), the first leaf was excised at the mesocotyl junction, and the amount of chlorophyll a (◇, •) and chlorophyll b (o, •) was estimated.

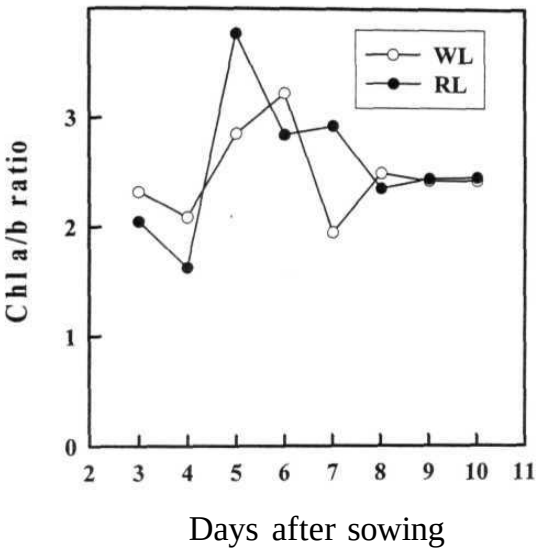

Figure 4. 3. Chl a/b ratio in the first leaf of pearl millet (WGC-75), during the period of leaf expansion. Seedlings were grown under WL (o) and RL (•) from sowing. At the time point (days) indicated, the first leaf was excised at the base and the Chl a/b ratio was estimated.

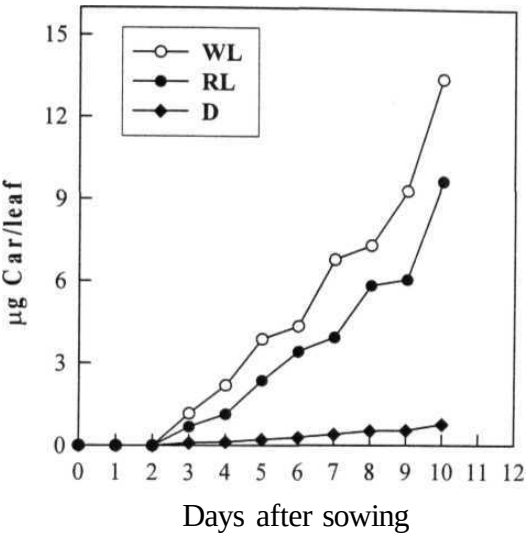

Figure 4.4 Time course of carotenoid accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown under WL (○), RL (•) and D (◆) from sowing. At the time point (days) indicated the first leaf was excised at the mesocotyl junction, and the carotenoid amont was estimated.

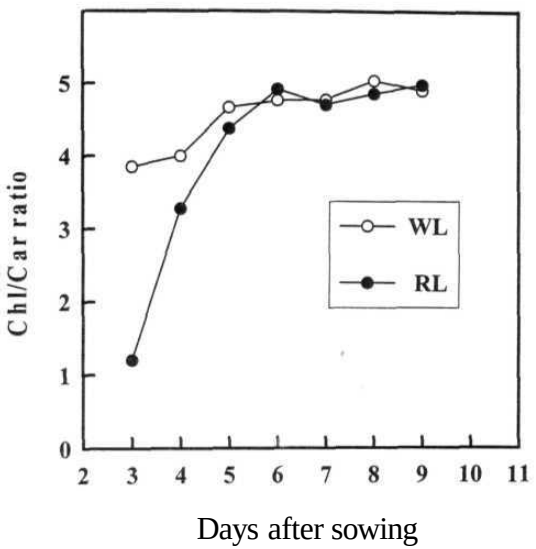

Figure 4.5. Chlorophyll/carotenoid ratio in first leaf of pearl millet (WGC-75), during the period of leaf expansion. Seedlings were grown under continuous WL (○) and RL (•) from sowing. At the time point indicated, (days) the first leaf was excised at the base and the Chl/Car ratio was estimated.

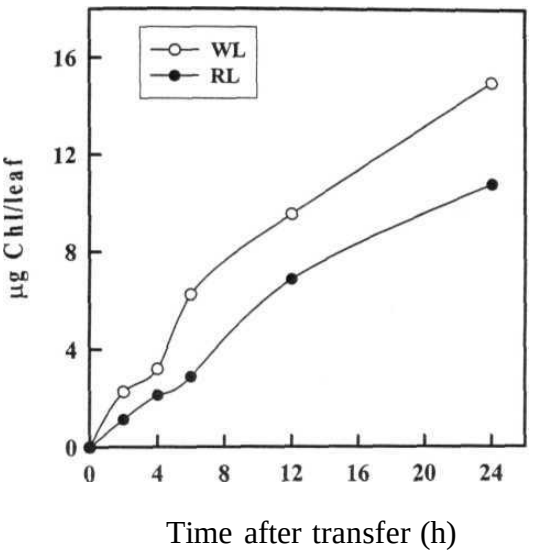

Figure 4.6. Time course of chlorophyll accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown in continuous darkness upto 6-d from sowing and then transferred to either continuous WL (○)or (•)RL. At the time points indicated (h), the first leaf was excised at the mesocotyl junction, and the chlorophyll amount was estimated.

of chlorophyll. Both in WL and RL the chlorophyll accumulation showed nearly equivalent levels at 2 h after transfer to light. Thereafter the chlorophyll accumulation was more rapid in WL-than in RL-grown seedlings. On comparing levels of chlorophyll after 24 h exposure of RL-and WL-grown seedlings (Fig. 4.6), with that of seedlings grown under continuous WL and RL (Fig. 4.1) it is evident that even after 24 h of light exposure the level of chlorophyll in seedlings transferred to light is only about half of that grown under continuous light.

The influence of light on synthesis of individual Chl species was examined by monitoring levels of chlorophyll a and chlorophyll b in first leaf after transfer of dark-grown seedlings to light. Fig. 4.7 shows that after 2 h of transfer from darkness to light the chlorophyll a and chlorophyll b amounts were nearly equal. However, on prolonged light exposure, chlorophyll a was predominantly synthesized than chlorophyll b. The profile of chlorophyll a and chlorophyll b accumulation were similar both in WL-and RL-grown seedlings.

The analysis of Chl a/b ratio in seedlings transferred to light shows that at 2 h (Fig. 4.8) after transfer to light, the Chl a/b ratio is close to 1.4. Thereafter, the leaves form more chlorophyll a than chlorophyll b. By 12 h of transfer a balance between Chl a and Chl b formation is achieved. (Fig. 4.8). Thereafter in WL Chl a/b ratio value stabilized at 2.0 whereas in RL stabilized at 2.50.

The transfer of etiolated seedlings to light also stimulated the carotenoid formation (Fig. 4.9). The dark-grown leaves possessed a basal amount of carotenoids, which however remains at a low level (Fig. 4.4) throughout the period of leaf expansion. The exposure to light stimulated the formation of carotenoid both in WL- and RL-grown seedlings, with WL exposure being more effective for stimulating carotenoid formation than the RL (Fig. 4.9).

The stoichiometry between carotenoid and chlorophyll formation was also analyzed (Fig. 4.10). In dark-grown seedlings at the time of transfer to light, during the initial phase of transfer Chl/Car ratio was low. Thereafter it increased rapidly due to accumulation of chlorophyll in the leaf upto 12 h. Thereafter Chl/Car ratio reached about 3.78, 3.58 for both WL and RL respectively.

While we examined the chlorophyll and carotenoid level in whole first leaf, a visual inspection of leaf clearly shows that leaf has more chlorophyll in the upper leaf

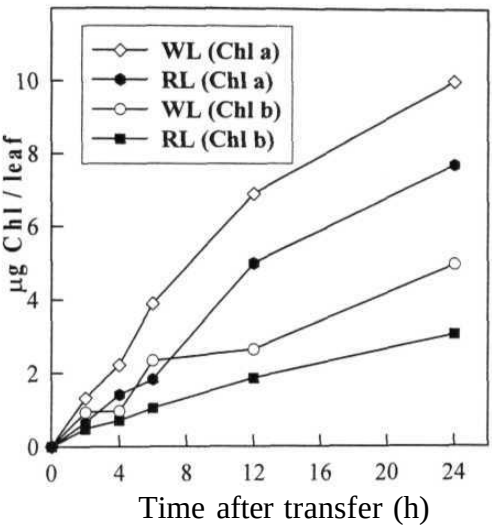

Figure 4. 7. Time course of chlorophyll a and chlorophyll b accumulation in the first leaf of pearl millet (WGC-75). Seedlings were grown in continuous darkness upto 6-d from sowing and then transferred to either continuous WL (◇, o) and or RL (■, ●).At the time points (h) indicated the first leaf was excised at the mesocotyl junction, and the Chl a (◇, •) and Chl b (■, ○) was estimated.

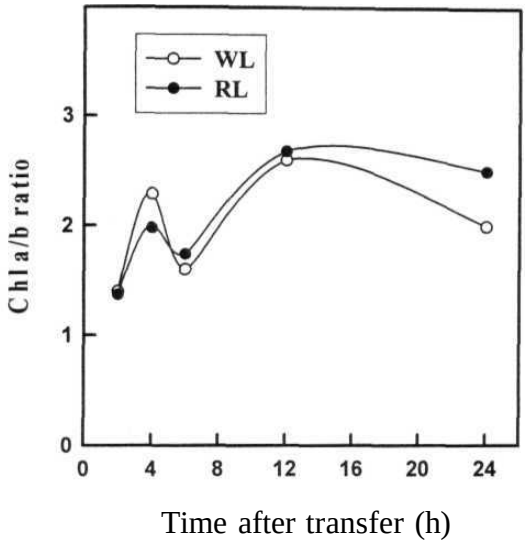

Figure 4. 8. Chl a/b ratio in first leaf of pearl millet (WGC-75) after transfer to light. Seedlings were grown in continuous darkness upto 6-d from sowing and then transferred to either continuous WL (o) or RL (•). At the time points indicated (h), the first leaf of pearl millet was excised at the mesocotyl junction, and the Chl a/b ratio was estimated.

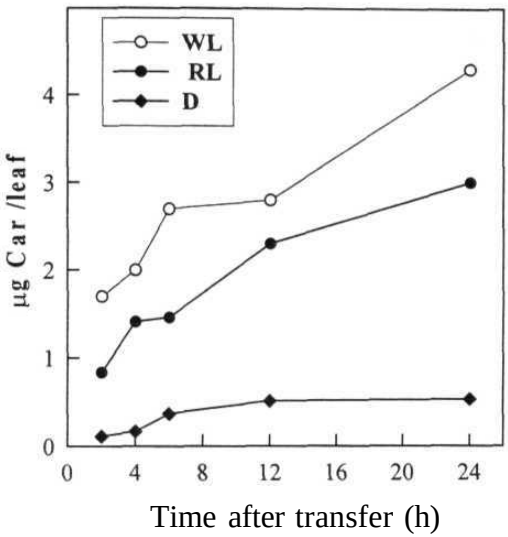

Figure 4.9. Time course of cartotenoid accumulation in first leaf of pearl millet (WGC-75) leaves. Seedlings were grown in continuous darkness (D) (•) upto 6-d from sowing, and then transferred to either continuous WL (•)or RL (o)for different period of time. At the time points (h) indicated the first leaf was excised at the mesocotyl junction, and the carotenoid amount was estimated.

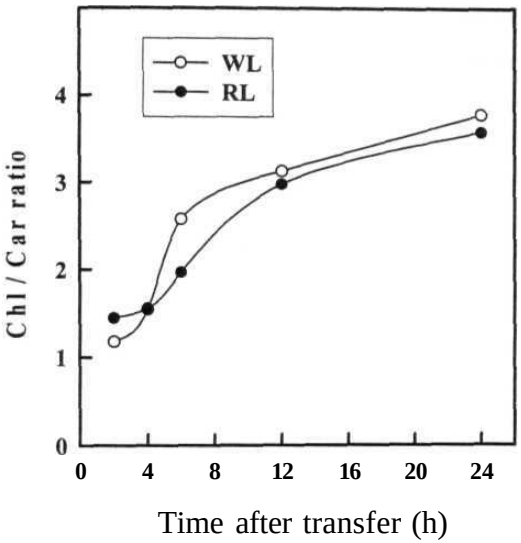

Figure 4. 10. Chl/Car ratio in first leaf of pearl millet (WGC-75) during the period of light exposure. Seedlings were grown in continuous darkness upto 6-d from sowing and then transferred to either continuous WL (○) or RL (●). At the time points (h) indicated, the first leaf was excised at the mesocotyl junction, and the Chl/Car ratio was estimated.

blade region than the lower sheath region. Since pearl millet leaf is continually expanding due to the activity of basal meristem. We examined the influence of cell position on accumulation of both chlorophyll and carotenoid in the leaf. To achieve this after harvesting the first leaf was segmented into 1 cm long segments numbered from base to leaf tip and pigment amount was estimated.

Fig. 4.11 shows that there is a distinct gradient of chlorophyll accumulation in first leaf harvested from seedlings grown under continuous WL and RL. The maximal amount of chlorophlyll was observed near the leaf tip and a minimal amount was observed at the leaf base. The profile of chlorophyll accumulation was similar both in RL-and WL-grown leaves, but the amount of chlorophyll was lower in RL-grown leaves.

The profile of chlorophyll a and chlorophyll b accumulation in leaf segment was followed along the length of leaf (Fig. 4.12). Similar to total chlorophyll the amount of both Chl a and Chl b increased from leaf base to leaf tip with more Chl being present in WL-grown leaves. In case of RL-grown leaves, Chl a level follows a profile similar to that of Chl a under WL, but the profile of Chl b distribution was less in RL-grown seedlings.

The fact that leaves of 7-d old RL-grown seedlings make less Chl b than WL-grown seedlings is evident by the comparison of Chl a/b ratio in different segments along the length of the leaf. In case of WL-grown seedlings showed a Chl a/b ratio between 2.13-2.5, while in RL-grown leaves showed a low Chl a/b ratio in basal segment (1.90), its level increased along the length of leaf and was stabilized at 3.17. It was evident that among the two light treatments, red light-grown leaf shows less formation of Chl b thereby leading to high Chl a/b ratio in RL-grown seedlings (Fig. 4.13).

The analysis of acetone extract of individual segments by absorption spectroscopy also correlated with the earlier results. The peak at both red and blue absorption regions, signifies the absorption of chlorophyll and carotenoid respectively, it is evident that the amount of both chlorophyll and carotenoid increases towards the tip of the leaf (Fig. 4.14).

Analogous to chlorophyll distribution, the distribution of carotenoid also showed increase in level towards the tip of the leaf. However comparison between

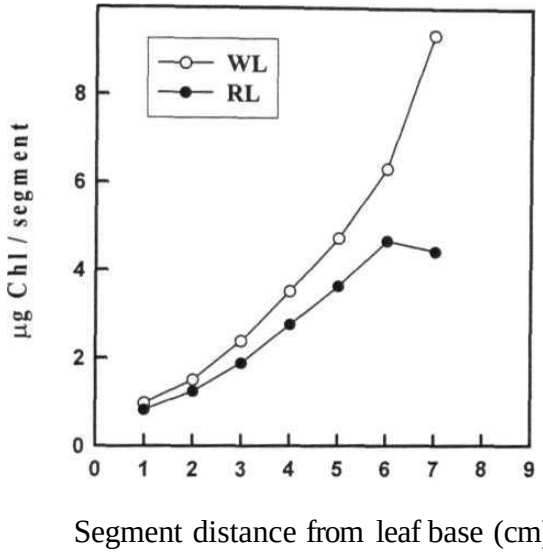

Figure 4. 11. Distribution of chlorophyll along the length of first leaf of pearl millet (WGC-75) were grown under continuous WL (○) or RL (•) from sowing. The first leaves of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of chlorophyll. Segments are numbered from base to the leaf tip.

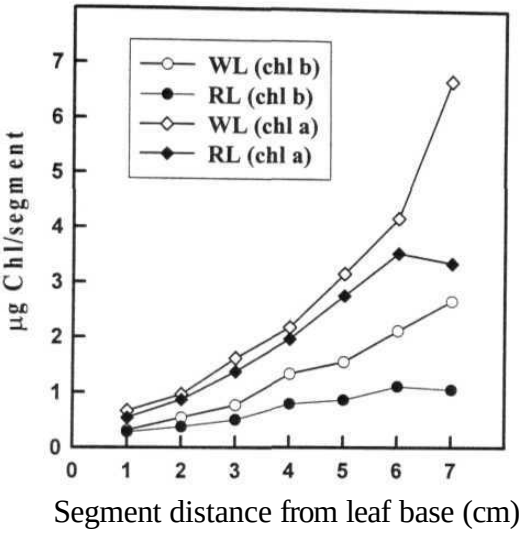

Figure 4. 12. Distribution of chlorophyll a and chlorophyll b along the length of first leaves of pearl millet (WGC-75). Seedlings were grown under continuous WL (o, ◇) or RL (•, •) from sowing. The first leaves of 7-d old seedlings were excised at the mesocotyl junction. The leaves were then cut into 1 cm long segments from the leaf base to tip, and used for estimation of chlorophyll a (o, •) and chlorophyll b (◇, •) The segments were numbered from base to the leaf tip.

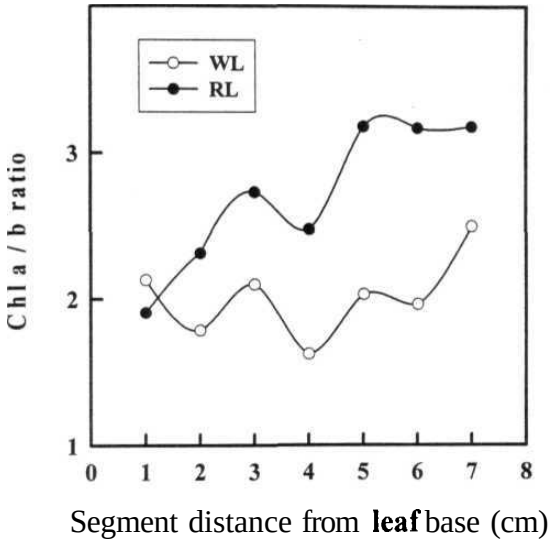

Figure 4. 13. Profile of chl a/b ratio in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (o) or RL (•). The first leaf of 7-d- old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of Chl a/b ratio. Segments were numbered from base to the leaf tip.

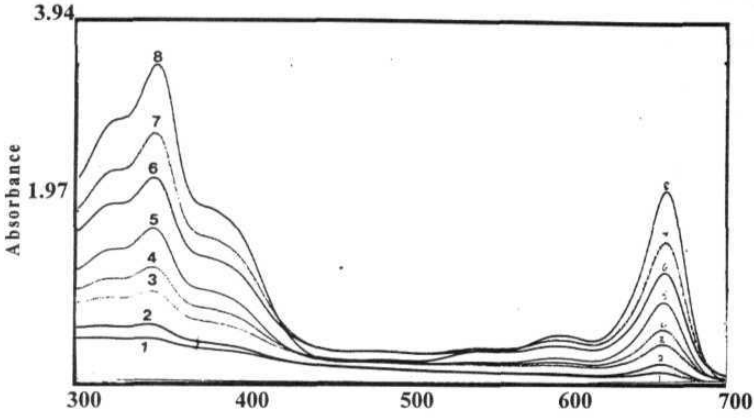

Figure 4.14. Absorption spectra of 80% acetone extracts from first leaf of 7-d-old pearl millet (WGC-75). Seedlings were grown under continuous WL from sowing. The first leaf was harvested and excised to 1 cm segments from leaf base to tip, and used for pigment extraction for spectral analysis. Segments are numbered from base to the leaf tip. The number in spectra indicates the segment number.

dark-and light-grown leaves clearly show that transfer to light significantly effect the level of carotenoid accumulation in the leaf. Exposure to light stimulates carotenoid accumulation in a distribution profile, which is parallel to cell maturity gradient. It is evident that the older leaf segment shows a high accumulation of carotenoid level (Fig. 4.15).

Apparently the profile of carotenoid (Fig. 4.16) accumulation is imprinted and regulated by leaf development. The comparison of distribution of carotenoid in leaf of different age from 3-d to 9-d old shows a similar profile. In all cases the amount of carotenoid level at leaf tip is more than the leaf base. Fig. 4.16 clearly shows that carotenoid accumulation gradient are correlated to cell maturity in pearl millet leaf. The absorption spectra of acetone extracts of etiolated leaf clearly shows that leaf tip has higher amount of carotenoid than the leaf base (Fig. 4.17).

The Chl/Car ratio in pearl millet leaf showed that in WL-grown leaf Chl/Car amount was in the range of 1.17-0.94, in RL-grown leaves the Chl/Car ratio was 1.03 at leaf base and declines to 0.68 at the tip. It is evident that red light is not as effective as WL in stimulating Chl biosynthesis in basal segments of the leaf (Fig. 4.18).

4.3. Chlorophyll and carotenoids in pearl millet mutant

In *yellow stripe* mutant leaves, yellow and green tissues were dissected carefully, and its absorption was examined. Yellow portion contains very little amount of chlorophyll but show a considerable level of carotenoid (Data not shown) showing absorption spectra similar to wild type. In case of *white stripe* mutant, white portion of leaf contains insignificant amount of carotenoids and chlorophyll. The similar analyses were also done for *zebra* mutant that has transverse yellow zebra bands. The yellow stripe, which appears on low temperature treatment, has only carotenoids, and chlorophyll level could not be detected. The above studies highlighted that in *zebra* mutant on lowering temperature below 10°C shuts off chlorophyll biosynthesis but allowed normal carotenoid synthesis to proceed. Fig. 4.19 shows the quantitative analysis of chlorophyll and carotenoid pigments in green and yellow segments of *stripe* and *zebra* mutant. The result shows that light promotes the synthesis of chlorophyll and carotenoid levels in green tissues whereas in yellow tissues the chlorophyll development is arrested at the early stages of plastid

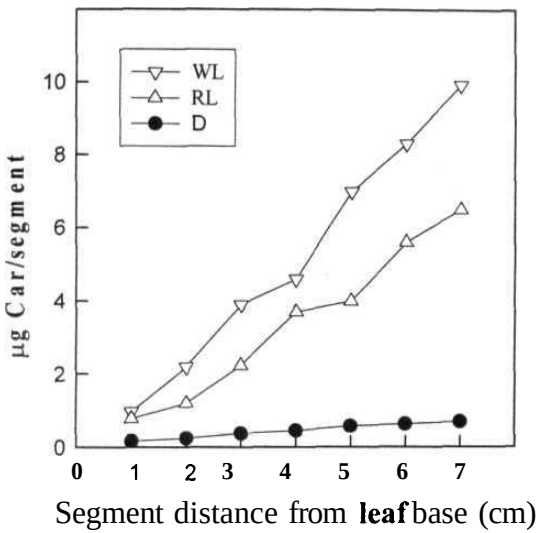

Figure 4. 15. Distribution of carotenoid along the length of first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (v) or RL (△) or D (•) from sowing. The first leaves of 7-d-old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of carotenoids. Segments were numbered from base to the leaf **tip.**

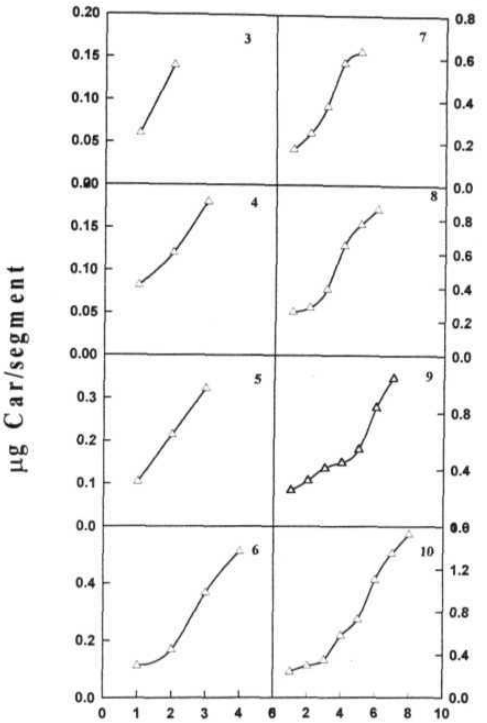

Figure 4. 16. Time course of carotenoid accumulation in first leaf of pearl millet (WGC-75) seedlings. Seedlings were grown under continuous darkness from sowing. At the time point indicated (days), the first leaves of seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of carotenoids. Segments were numbered from base to the leaf tip.

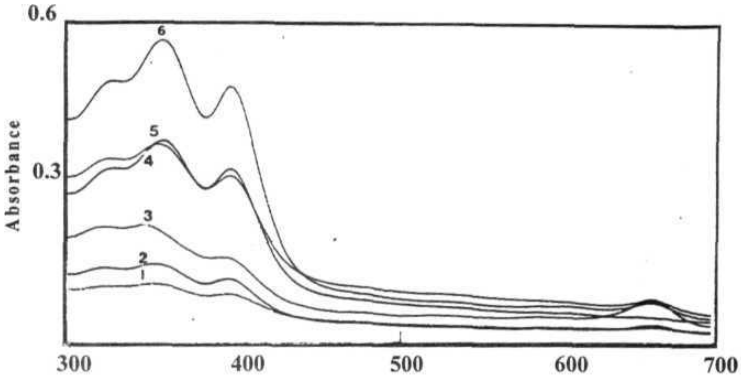

Figure 4.17. Absorption spectra of 80% acetone extracts from first leaf of 7-d-old pearl millet (WGC-75). Seedlings were grown under continuous WL from sowing. The first leaf was harvested and excised to 1 cm segments from leaf base to tip, and used for pigment extraction for spectral analysis. Segments are numbered from base to the leaf tip. The number in spectra indicates the segment number.

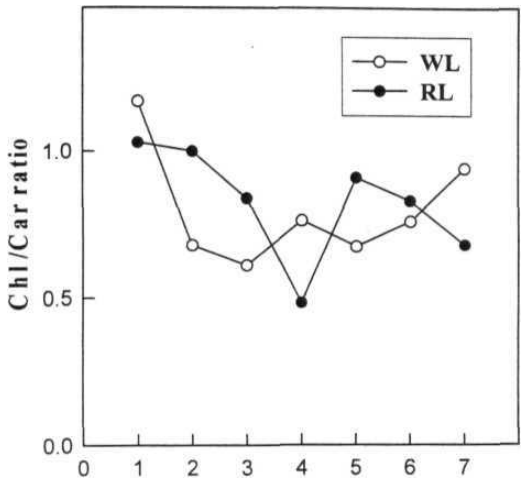

Figure 4. 18. Profile of Chl /Car ratio in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous WL (o) and RL (•) from sowing. The first leaves of 7-d- old seedlings were excised at the mesocotyl junction. The leaves were then excised to 1 cm long segments from leaf base to tip, and used for estimation of Chl/Car ratio. Segments were numbered from base to the leaf tip.

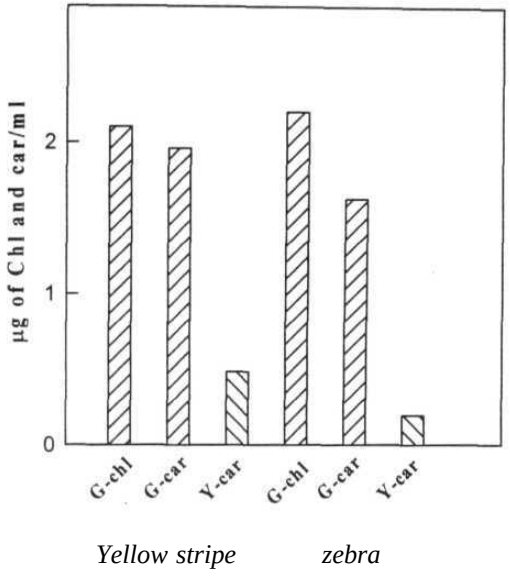

Figure 4. 19. Distribution of chlorophyll and carotenoid in yellow (Y) and green (G) sector of *yellow stripe* (IP 8284) and *zebra* (IP 8283) mutant seedlings were grown under a photoperiod consisting of continuous white light for 16 h and complete darkness for 8 h at 25°C. In *zebra* four days under same conditions, thereafter seedlings were transferred to 8 h darkness at 10^0C and 16 h continuous WL at 25°C. The green and yellow band leaf tissues were separated 1 cm below the tip of the leaves and estimated for chlorophyll and carotenoids.

development when insignificant amount of chlorophyll is present.

4.4. Protochlorophyll(ide) Pchl(ide)

In angiosperms, biosynthesis of chlorophyll is obligatorily dependent on the light. When seedlings are grown in continuous darkness, the proplastids of the developing leaves differentiate into etioplast instead of chloroplasts. These etioplasts accumulate the precursor of chlorophyll biosynthesis Pchlide. The reduction of Pchlide to Chlide is a light dependent step in angiosperms. Chlide act as the intermediate form between the Pchlide and chlorophylls. When dark-grown seedlings are exposed to light the accumulation of chlorophyll is initiated. The phototransformation of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) also involves structural changes in the pigment protein complexes of etioplasts, which occurs during the conversion of Pchlide to Chlide and spectroscopically detected as Shibata shift (Shibata, 1957).

The accumulation of Pchlide in pearl millet leaves was examined by, dissecting 1 cm long segments of first leaves from base to tip, harvested from dark-grown seedlings. The accumulation of Pchlide in pearl millet leaves was found to be strongly influenced by cellular position in leaf. In dark-grown pearl millet leaves the maximal amount of Pchlide was observed in the leaf region near to the leaf tip (Table. 4.1), while the same leaf possessed least amount of Pchlide at the base. In essence, the gradient of Pchlide accumulation in dark-grown leaves was similar to Chl accumulation gradient in light-grown leaves (Table. 4.1)

Similar examination of fluorescence spectra of leaves grown in continuous WL-and RL showed that the level of Pchlide was below the level of detectability, and the chlorophylls was predominantly present in Chlide form (Table. 4.2). Moreover these results also indicate that maximal amount of Chlide was present in leaf tip and least amount is present in the leaf base. To study the complete conversion of Pchlide to Chlide, 7-d-old dark-grown pearl millet control seedlings were transferred to continuous RL for 30 minutes. 30 min of RL is sufficient to complete the conversion of Pchlide to Chlide. It is evident that though maximal amount of chlorophyll in leaves belong to Chlide, a little amount of Pchl is also presents (Table. 4.3).

Table 4.1. Distribution of Pchlide and Chlide along the length of dark-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous darkness upto 7-day from the time of sowing. Thereafter first leaves of 7-day old seedlings were excised at the mesocotyl junction. The leaves were excised into 1 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to the leaf tip.

Segment distance from leaf base (cm)	Chlide amount (μg/segment)	Pchlide amount (μg/segment)
1.	0.0013	0.573
2.	0.0017	0.717
3.	0.0013	0.544
4.	0.0023	0.946
5.	0.0021	0.889
6.	0.0025	1.061

Table 4.2. Distribution of Pchlide and Chlide along the length of WL or RL-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous WL or RL upto 7-day from the time of sowing. Thereafter first leaves of 7-day old seedlings were excised into 1 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to leaf tip.

Segment	White light		Red light	
	Chlide (peak nm)	Chlide (jig/segment)	Chlide (peak nm)	Chlide (μg/segment)
1	669.0	0.043	668.0	0.070
2	670.0	0.139	669.0	0.206
3	671.0	0.438	674.0	0.620
4	672.0	1.11	670.0	1.70
5	674.0	2.53	670.0	2.54
6	676.2	3.93	673.4	4.32
7	676.4	4.73	673.3	3.45

Table 4.3. Distribution of Pchlide and Chlide along the length of dark-grown pearl millet (WGC-75) leaves. Seedlings were grown under continuous darkness upto 6-day from the time of sowing. Thereafter seedlings were transferred to continuous RL for 30 min and the first leaf was excised at the mesocotyl junction. The leaves were then cut into 2 cm long segments from leaf base to tip, and used for estimation of Pchlide and Chlide. The segments were numbered from base to the leaf tip.

Segment	Chlide (μg/segment)	Pchlide (μg/segment)	Chlide peak position (nm)
Base	0.020	0.001	674.0
Middle	0.043	0.005	673.2
Tip	0.051	0.007	673.0

4.5. Protochlorophyllide in pearl millet mutant

Since accumulation of Pchlide in tissue signifies a block in chloroplast biosynthesis, we analyzed the *zebra* and *yellow stripe* **mutants to examine this** possibility. These yellow mutants which have yellow stripe parallel to green stripe were grown under WL and green and yellow leaf tissues were harvested and analyzed for Chlide amount (Tables. 4.4 and 4.5). This indicates that white segments of the leaf have no functional chloroplast, and Chl. It is therefore evident that defect in Chl synthesis in white segment of the leaf lies down stream to Pchlide formation step, therefore, Pchlide cannot be converted to Chlide due to the absence of the functional chloroplast.

4.6. Protochlorophyllide oxidoreductase (POR)

In etiolated leaves of monocot seedlings, proplastid develops into etioplast, possessing a central prolamellar body, consisting of several proteins. One of the important proteins in etioplast is POR, which accumulates at high level. POR also acts as one of the marker enzyme for initiation of light mediated transition of etioplast to chloroplast. The POR is primarily responsible for catalyzing reduction of Pchlide to Chlide on exposure to light. In etioplast, POR forms a ternary complex with NADH and Pchlide, which on illumination instantaneously reduces Pchlide to Chlide. Recent researches have shown that angiosperms have two POR proteins; namely POR A, and POR B. Of these POR A is present in etioplasts and exposure of leaves to light initiates a rapid decline in its level and it participates in chlorophyll biosynthesis only for a limited duration. POR B is present in chloroplasts and is responsible for the formation of chlorophyll in chloroplasts. In present study, we used POR as a marker enzyme to study the transition of etioplast to chloroplast. In addition we also compared the amount of POR in wild type chloroplasts versus mutant chloroplasts. The polyclonal antibodies used in this study were raised against a mixture of POR A and POR B of barley, which also cross-reacted with pearl millet POR.

The POR protein levels were compared in WL-and dark-grown leaves of pearl millet by western blotting. Fig. 4.20 shows that in dark-grown leaves (WGC-75) POR A protein predominates, whereas in light-grown leaves POR B is a dominant protein. Since polyclonal antibodies were used in this study to recognize both POR A and

Table 4.4. Distribution of Chlide in green and white stripes of leaves of 7-d old pearl millet (SW-1) mutant. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25°C from the time of sowing. The green and white tissues of the leaves were excised and used for estimation of Chlide.

Segment	Chlide (μg/segment)	Chlide peak position (nm)
Green	3.070	674.0
White	1.029	673.2

Table 4.5. Distribution of Chlide in green and yellow stripes of leaves of pearl millet (IP 8292) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25°C for 4 days. Thereafter the seedlings were transferred to 16 h light at 25°C and 8°C for 8 h upto 7day from the time of sowing. The yellow and green tissues of the leaves were excised and used for estimation of Chlide.

Segment	Chlide peak position (nm)	Chlide (μg/segment)
Yellow	678.0	0.178
Green	675.0	1.35
Green	676.0	1.04

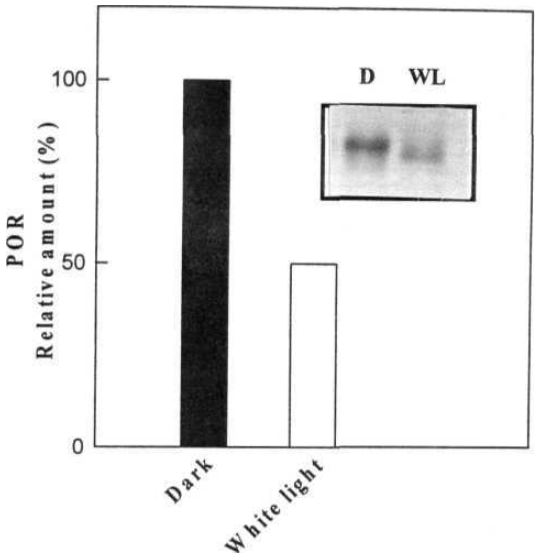

Figure 4.20. Quantification of POR protein in pearl millet (WGC-75) leaves. Seedlings were either grown in continuous white light (WL) or darkness (D) upto 7-d from the time of sowing. Thereafter first leaf was harvested, the POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%. Note the difference in mobility of POR in WL- grown leaf, which is light stable POR B.

POR B, the distinction between POR A and POR B was found to be primarily based on the respective MW. It is known that POR B of light-grown leaves has a MW which is about 2 kD less than that of POR A present in etioplast, whose molecular weight is about 36 kD. It is evident from results presented in Fig. 4.20 that POR of dark-grown leaves has higher MW than that of light-grown leaves indicating that these are POR A and POR B protein respectively.

The profile of distribution of POR protein along the length of dark-grown seedlings was analyzed by western blotting. The leaf was segmented in 1 cm long pieces numbered from base to the leaf tip, and the amount of POR was analyzed in each segment. Fig. 4.21 show that in dark-grown leaves the level of POR A protein increases from base to the leaf tip. In contrast the light-grown leaves, which has only POR B protein shows a massive decline in level of POR B protein (Fig. 4.22). Particularly in the segments near the leaf base the level of POR B protein is very low. However, inspite of decline in the level of POR B protein, its average pattern of distribution was not altered. Most interestingly the tip of the light-grown leaf showed a high content of POR B protein.

The analysis of POR protein in *yellow stripe* mutant showed that in dark-grown leaves POR A predominantly present. In contrast, in the green segment excised from leaf only POR B is present. Similarly in yellow segments excised from leaf only POR B appears to be present. On a comparative scale, though green and yellow sector of leaf are adjacent to each other, the yellow segment appears to have higher level of POR B protein than in green portion (Fig. 4.23). On a similar analysis of a *white stripe* mutant, the white portion of leaf showed no POR A but faint band of little POR B protein (Fig. 4.24). Evidently the white segment of leaves was not only devoid of chlorophylls, but also devoid of carotenoid and POR protein and apparently did not posses either etioplasts or chloroplasts.

4.7. LHCP

The LHCP is a marker protein for the development of thylakoid membranes in the chloroplasts. Its presence in the chloroplast marks the operation of an active photosynthetic system. The etiolated seedlings are devoid of LHCP and exposure of light to etiolated seedlings initiates LHCP formation. We used the LHCP in leaves as

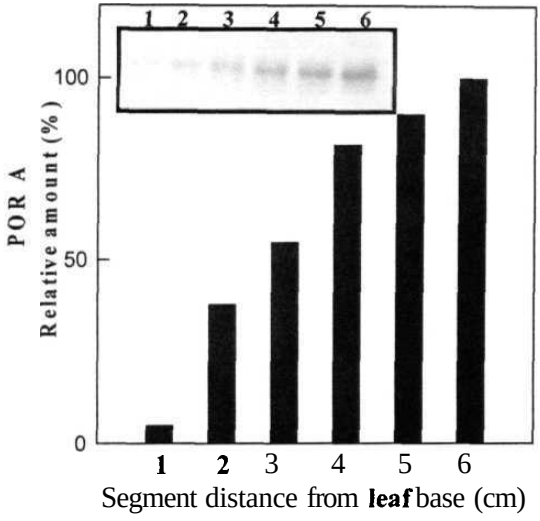

Figure 4. 21. Distribution of POR A protein in pearl millet leaves (WGC-75). Seedlings were grown under continuous darkness (D) for 7-d from the time of sowing. Thereafter, the first leaf was harvested and the leaf was dissected in to 1 cm long segments and numbered from base to the tip. POR A level was determined by western blotting (Inset). The relative amount of POR A was calculated using the intensity of POR A band present in leaf tip as 100%.

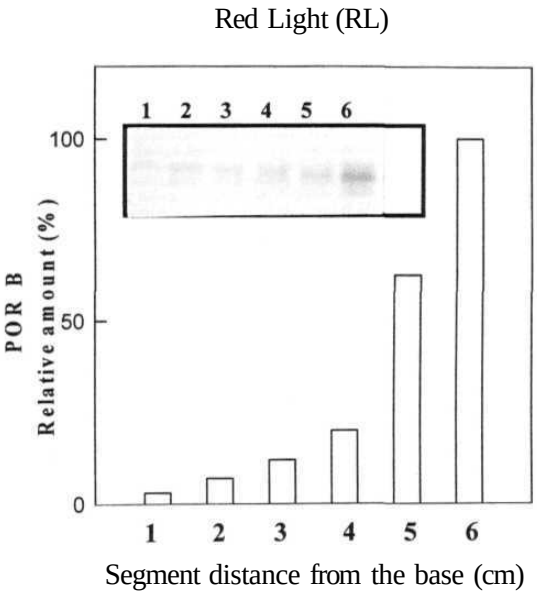

Figure 4.22. Distribution of POR B protein in pearl millet (WGC-75) leaves. Seedlings were grown under continuous RL for 7-d from the time of sowing. Thereafter the first leaf was harvested and the leaf was dissected in to 1 cm long segments and numbered from base to the tip. POR B level was determined by western blotting (Inset). The relative amount of POR B was calculated using the intensity of POR B band present in leaf tip as 100%.

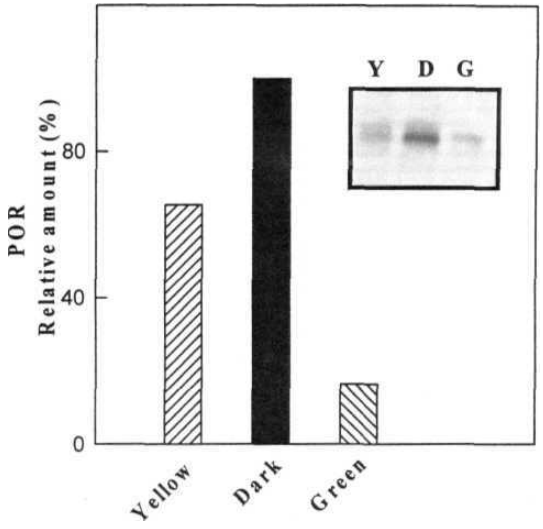

Figure 4. 23. Quantification of POR protein in *yellow stripe* (IP 8292) leaves. Seedlings were either grown in continuous white light or darkness (D) upto 7-d from the time of sowing. There after the first leaf was excised, yellow (Y) and green (G) band of the leaves were separated. The POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%.

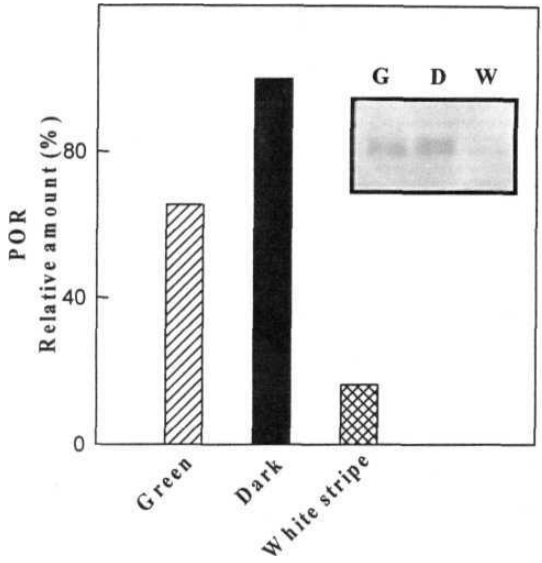

Figure 4.24. Quantification of POR protein level in *white stripe* (SW-1) leaves. Seedlings were grown in continuous white light (W) or darkness (D) upto 7-d from sowing. Thereafter the first leaf was harvested, white (W) and green (G) bands of the leaves were separated. The POR level was determined by western blotting (Inset). The relative amount of POR was calculated using the intensity of POR band present in dark-grown leaf as 100%.

a marker to decipher the photosynthetic efficiency of green and yellow segments of mutant leaves. The influence of light on LHCP level in pearl millet leaves was examined by immunoblotting of extracts obtained from leaf of WCG-75 variety which was used as a control (Figs. 4.25 and 4.26). It is evident from that the formation of LHCP in pearl millet leaf is obligatorily dependent on exposure to light (Fig. 4.25). In dark-grown leaves only an insignificant amount of LHCP protein was detected. By contrast the light-grown leaves had a high level of LHCP protein indicating that LHCP protein is formed after exposure to light. The fact that light is essential for LHCP formation is evident since it was observed that the transfer of etiolated seedlings to light induces the formation of LHCP protein in leaves within few hours of exposure.

Fig. 4.26 shows that in light-grown leaves the level of LHCP increases from leaf base to leaf tip. In the segments near the leaf base level of LHCP is low, and it gradually increase towards the leaf tip. The content of LHCP was also examined in leaves of pearl millet mutant such as *zebra,* which possesses alternate yellow and green stripes of leaf. Immunoblotting of extracts of yellow and green sectors of leaf excised from *zebra* mutant showed that yellow sector of leaf contains little amount of LHCP as compared to green sector (Fig. 4.27). Similarly in *yellow stripe* mutant the LHCP level was low in yellow region and high in green region (Fig. 4.28). These results clearly indicate that yellow region of leaves of *zebra* and *yellow stripe* mutant is deficient in LHCP a marker for functional chloroplast and performs photosynthesis at a reduced rate.

4.8. Phosphoenol pyruvate (PEP) carboxylase

Since pearl millet is a C_4 plant, the primary fixation of CO_2 in leaves is carried out by the activity of enzyme PEP-carboxylase. In C_4 plants PEP-carboxylase activity is localized in M cells, and this enzyme can be considered as a marker for cytosolic enzyme in leaves. The relative level of PEP-carboxylase was examined in dark-and light-grown leaves of pearl millet, using antibody raised against *Amaranthus* species. The observations indicated that *Amaranthus* antibody was able to detect pearl millet PEP-carboxylase. It is well known that in C_4 plants like *Amaranthus* and maize, the formation of PEP-carboxylase is induced by light. In dark-grown leaves PEP-

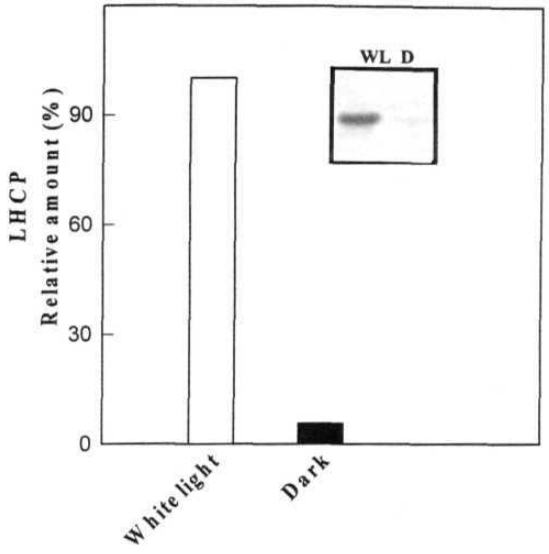

Figure 4. 25. Quantification of LHCP level in pearl millet(WGC-75) leaves. Seedlings were either grown in continuous WL or darkness upto 7-days from the time of sowing. The first leaves were harvested and the level of LHCP was determined by western blotting (Inset). The relative amount of LHCP was calculated using the intensity of LHCP band present in the WL-grown leaf tissue as 100%.

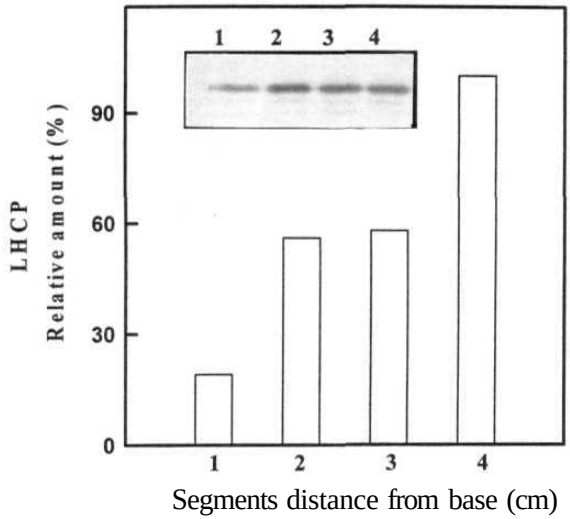

Figure 4.26. Distribution of LHCP protein in pearl millet (WGC-75) leaves. Seedlings were grown in continuous WL-for 9-days from the time of sowing. The first leaves were harvested and the leaf was segmented into two cm long segments from base to the tip. The segments were numbered from base to the leaf tip. The LHCP level in segments was determined by western blotting (Inset). The relative amount of LHCP level was calculated using the intensity of LHCP band present in the leaf tip (segment 4) as 100%.

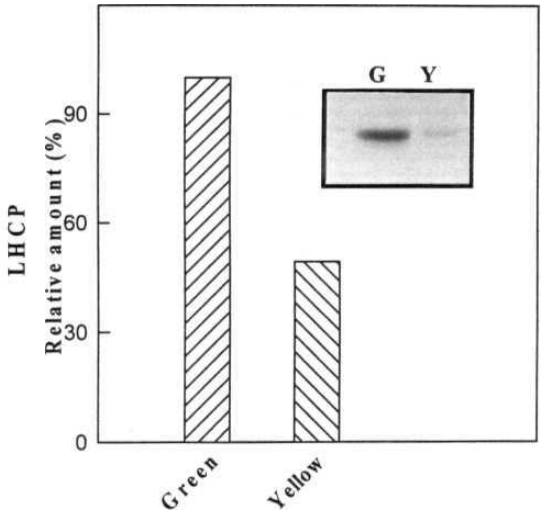

Figure 4.27. Quantification of LHCP level in *zebra* (IP 8283) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 °C for 4 days thereafter seedlings were transferred to photoperiod consisting of 16 h light at 25 °C and 8 h dark at 10°C. The yellow (Y) and green portion (G) of the leaves were dissected carefully and the LHCP level was determined by Western blotting (Inset). The relative amount of LHCP was calculated using the intensity of LHCP band is present in green leaf tissue as 100%.

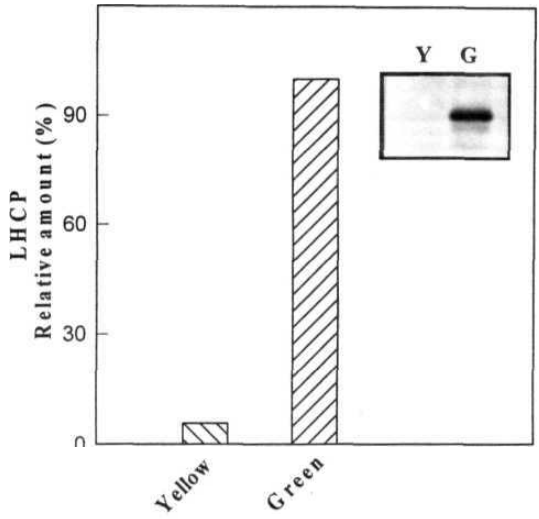

Figure 4.28. Quantification of LHCP level in of *yellow stripe* (IP 8292) mutant leaves. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 °C for 4 days thereafter seedlings were transferred to a photoperiod consisting of 16 h light at 25 °C and 8 h dark at 10°C. The first leaf was harvested, yellow (Y) and green portion (G) of the leaves were dissected carefully. The LHCP level was determined by western blotting of protein (Inset). The relative amount of LHCP was calculated by using the intensity of LHCP band in the green leaf tissue as 100%.

carboxylase level was below the level of detectability. In comparison, light-grown pearl millet seedlings showed a distinct band of PEP-carboxylase in leaf (Fig. 4.29).

In light-grown pearl millet leaves, distribution of PEP-carboxylase was similar to that observed for LHCP. As a representative example, Fig. 4.30, shows PEP-carboxylase distribution in *glossy* mutant leaves where its level increases from leaf base to leaf tip.

4.9. Oxygen evolution

In order to determine the photosynthetic capacity of the pearl millet leaf, light mediated O_2 evolution was examined from leaf harvested from etiolated-and WL-grown seedlings. The leaf segments from etiolated seedlings displayed no light-dependent oxygen evolution (data not shown). However light-grown leaf segments on exposure to light showed a light-dependent O_2 evolution. Using photosynthetic O_2 evolution as a marker for operation of functional chloroplasts, we compared the photosynthetic O_2 evolution of leaf strips excised from green and yellow leaf region of *zebra* mutant. Fig. 4.31 shows that the yellow segments, has low ratio of photosynthetic O_2 evolution compared to green segments. Interestingly the yellow region of the leaf also showed sluggish respiration rate, which was nearly half of the green segment (data not shown).

4.10. α-Amylase

In pearl millet leaves α-amylase is present both in extra-plastidic fractions and in chloroplasts (Vally and Sharma, 1995). The distribution profile of total a-amylase activity in pearl millet leaves show increasing activity towards the leaf tip. Pearl millet leaf a-amylase is immunologically similar to maize seed a-amylase, which is evident by the fact that the antibodies raised against maize seed a-amylase also recognizes pearl millet leaf α-amylases (Vally and Sharma, 1995). Our earlier studies showed that in pearl millet leaves light stimulates the a-amylase synthesis, inducing the appearance of a new isoform of a-amylase, which is exclusively localized in chloroplast. In this study the correlation of light with existence of functional chloroplast and of a-amylase protein was examined by using western blotting.

In dark-grown seedlings the level of a-amylase protein in pearl millet leaf is

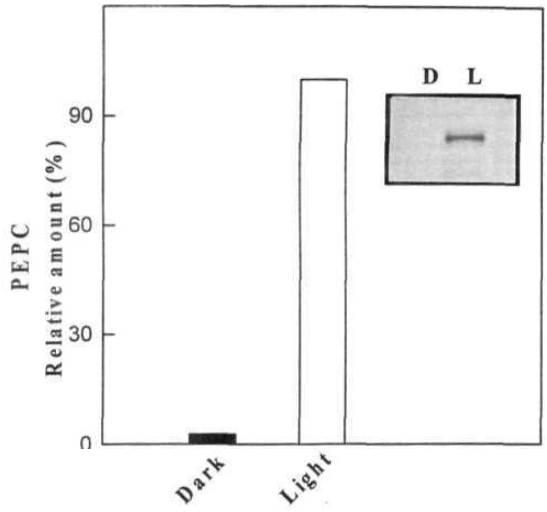

Figure 4. 29. Quantification of PEP carboxylase level in pearl millet (WGC-75) leaves Seedlings were grown either in continuous (W) or darkness (D) for 7-days. The first leaves were harvested and the PEP- carboxylase level was determined by western blotting (Inset). The relative amount of PEP- carboxylase was calculated using the intensity of PEP- carboxylase band present in light grown leaf as 100%.

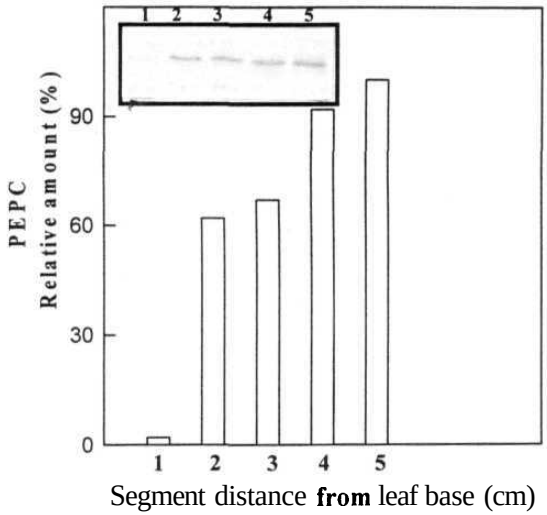

Figure 4.30. Quantification of PEP- carboxylase level in glossy mutant (IP 8282) leaves. Seedlings were grown in continuous white light (WL) for 9-days from the time of sowing. The first leaves were harvested and the segments were dissected into two cm long segments and were numbered from base to the leaf tip. The PEP-carboxylase level was determined by western blotting (Inset). The relative amount of PEP- carboxylase was calculated using the intensity of PEP- carboxylase band present in leaf (segment 5) tip as 100%.

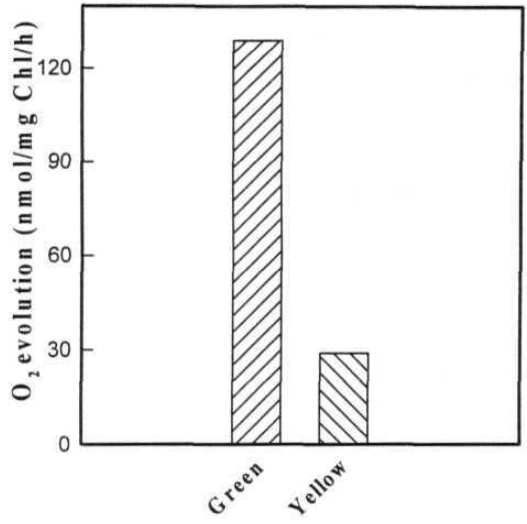

Figure 4. 31. Light dependent oxygen evolution in leaves of *zebra* (IP 8284) mutant. Seedlings were grown under a photoperiod consisting of 16 h light and 8 h dark at 25 ºC for 4 days after it transferred to 16 h light at 25 ºC and 8 h dark at less than 10ºC. The yellow (Y) and green portion (G) of the leaves were excised into 1-3 mm long stripes and O_2 evolution was measured using a clark type O_2 electrode.

quiet low, but in light-grown seedlings the α-amylase protein level is about two folds difference (Fig. 4.32). This pattern is also observed in mutant seedlings of pearl millet when the α-amylase protein level is compared between light-and dark-grown seedlings. The examination of a-amylase in *early flowering*, mutant showed that it was similar to wild type a low level of a-amylase protein in dark-grown seedlings (Fig. 4.33).

The possibility that the presence of chloroplast is necessary for a-amylase induction was ascertained by estimating the a-amylase protein in yellow and green sector of the *yellow stripe* mutant of pearl millet. The yellow and green sectors were carefully dissected and the a-amylase protein was determined by western blotting. Fig. 4.34 show that in the same leaf, the green portion has high level of a-amylase protein compared to yellow portion of the leaf. Quantitatively the level of a-amylase protein in green portion was about twice than the yellow portion.

4.11. β-Amylase

An analysis of intracellular distribution of p-amylase in pearl millet leaves indicated that p-amylase is predominantly localized in M cells and is absent in B cells. Additionally in pearl millet leaves p-amylase is localized outside the plastid, most likely in the vacuole. Our earlier studies showed that p-amylase level in dark-grown leaves increases from base to tip, whereas in light-grown leaves it is predominantly localized at the leaf base (Vally, 1995).

The quantitation of P-amylase protein in pearl millet leaves was carried out with polyclonal antibodies raised against maize seed β-amylases and these antibodies have been shown to specifically cross react with pearl millet leaf P-amylase in the double immunodiffusion reaction and also by immunoblotting. It is evident that in NF-treated light-grown leaves, the decline in p-amylase activity is arrested in all segments (Fig. 4.35). The analysis of p-amylase distribution along the length of leaf by western blotting revealed that the level of p-amylase protein in dark-grown pearl millet seedlings increases from base to the tip of the leaf (Fig. 4.36). In light-grown leaves P-amylase protein was predominantly localized near the base of the leaf. The level of p-amylase protein was maximal in the first segment and declined in more mature segments (Fig. 4.37).

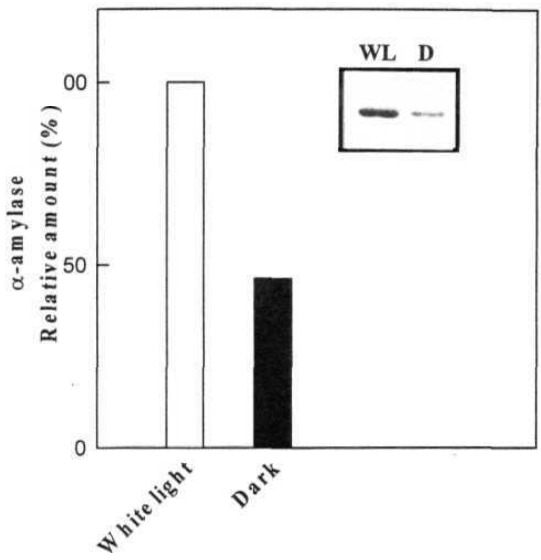

Figure 4.32. Quantification of a-amylase level in pearl millet (WGC-75) leaves. Seedlings were grown in continuous white light (WL) or darkness (D) upto 7-d from the time of sowing. Thereafter first leaf was harvested, the level of a-amylase was determined by Western blotting (Inset). The relative amount of a-amylase level was calculated using the intensity of a-amylase band present in WL- grown leaves as 100%.

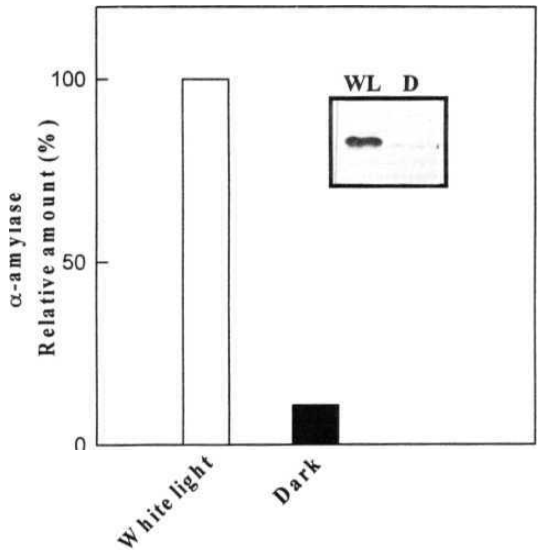

Figure 4.33. Quantification of a-amylase level in *early flowering* (IP 4021) mutant leaves. The mutant seedlings were grown in 16 h white light (WL) and 8 h darkness (D) at 25°C or continuous darkness (D) upto 9-d from the time of sowing. Thereafter first leaf was harvested, the level of a-amylase was determined by Western blotting (Inset). The relative amount of a-amylase was calculated using the intensity of a-amylase band present in WL- grown leaves as 100%.

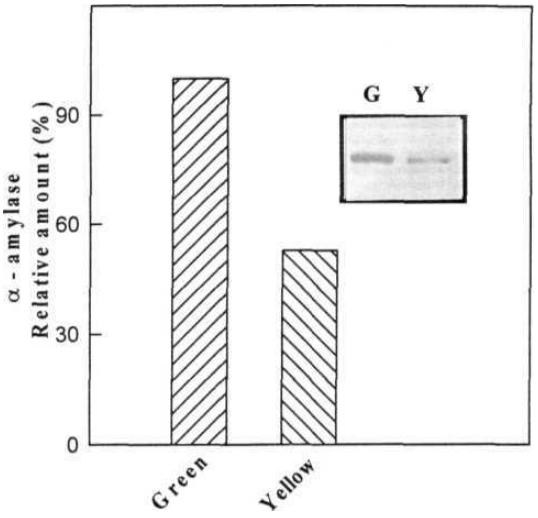

Figure 4. 34. Quantification of a-amylase level in *yellow stripe* (IP 8292) mutant leaves. The mutant seedlings were grown under a photoperido consisting of 16 h light and 8 h dark at 25°C upto 9-d from the time of sowing. Thereafter first leaf was harvested, yellow (Y) and green (G) leaf tissues were separated carefully, the level of a-amylase was determined by Western blotting (Inset). The relative amount of a-amylase level was calculated by using the intensity of a-amylase band present in green leaf as 100%.

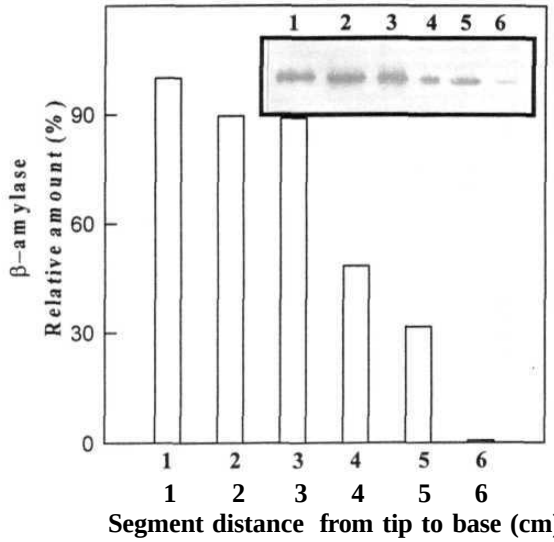

Figure 4.35. Distribution of P-amylase in 0.4 mM NF treated pearl millet (WGC-75) leaves. Seedlings were grown under continuous red light (RL) for 7-d from the time of sowing. Thereafter the first leaf was excised and the leaf was dissected in to 1 cm long segments from base to the tip. The level of P-amylase was determined by western blotting (Inset). The relative amount of p-amylase was calculated using the intensity of P-amylase band present in leaf tip (segment 6) as 100%.

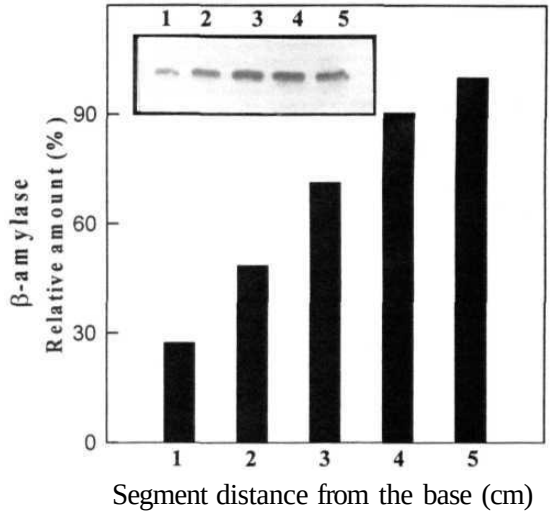

Figure 4.36. Distribution of P-amylase in dark-grown pearl millet (WGC-75) leaves. Seedlings were grown in continuous darkness upto 7-d from the time of sowing. The first leaf was harvested and the leaf was dissected in to 1 cm long segments were numbered from leaf base to the leaf tip. Thereafter the level of P-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of p-amylase band present in leaf tip (segment 5) as 100%.

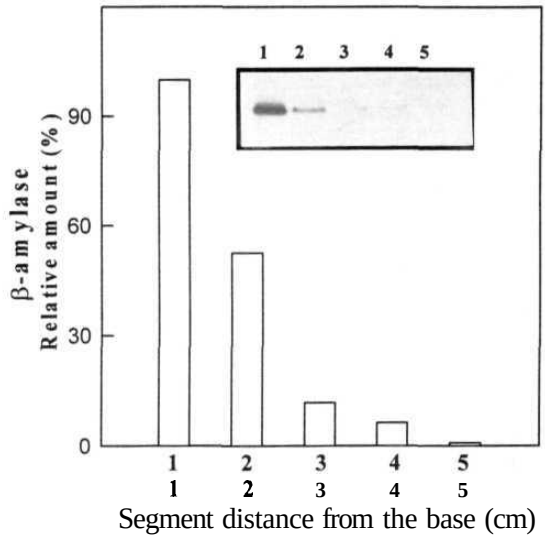

Figure 4. 37. Distribution of β-amylase level in pearl millet (WGC-75) leaves. Seedlings were grown in continuous red light (RL) for 7-d from the time of sowing. The first leaf was harvested and the leaf was dissected in to 1 cm long segments and numbered from base to the tip. Thereafter the level of p-amylase was determined by western blotting (Inset). The relative amount of P-amylase was calculated using the intensity of P-amylase band present at leaf base (segment 1) as 100%.

The studies on regulation of P-amylase in pearl millet leaves revealed that in light-grown seedlings NF-treatment, which generate albino seedlings due to photooxidation of chloroplasts, stimulates P-amylase activity in pearl millet seedlings by 7 fold, when compared to light-grown seedlings grown in distilled water. Since NF-treatment significantly stimulates P-amylase level, the likely effect of loss of chloroplast on P-amylase distribution was examined by quantifying along the length of the leaf.

The stimulation of p-amylase level in NF based light-grown leaves indicated an inverse relationship between the presence of functional chloroplasts and level of P-amylase protein. The fact that NF-treatment stimulates P-amylase only in light-grown seedlings, which had no functional chloroplasts, reinforced this view. The possibility of p-amylase induction level by loss of chloroplasts was examined by using mutant of pearl millet, which have lost chloroplasts in certain sector either due to mutation or it has reduced chloroplast functions due to reduction of photosynthetic protein. The analysis of pigment protein i.e. LHCP and O_2 evolution as presented in earlier sections indicated that yellow sector of *zebra* and *yellow stripe* leaf are deficient in photosynthetic functions. We examined the P-amylase level in yellow and green sector. of mutant leaves by western blotting. The above analysis revealed that the yellow leaf sector had a significant higher level of p-amylase protein than green sector. The western blotting analysis revealed that, yellow sector has about 2 fold difference in p-amylase protein than green sector (Fig. 4.38).

The analysis of P-amylase level in *zebra* mutant, which makes alternate yellow and green sector, was also done. The yellow and green bands were excised and P-amylase level was examined. The profile of p-amylase protein for *zebra* mutant is similar to that observed with yellow and green sector isolated from *yellow stripe* mutant leaf. In this mutant too, yellow band has higher level of p-amylase protein than green band indicating that loss of functional chloroplasts stimulates p-amylase level in yellow band (Fig. 4.39).

4.12. Peroxidase activity

Peroxidase is an enzyme, which has multifarious function in plants, such as biosynthesis of lignin, polymerization of extensin, defense against attack by

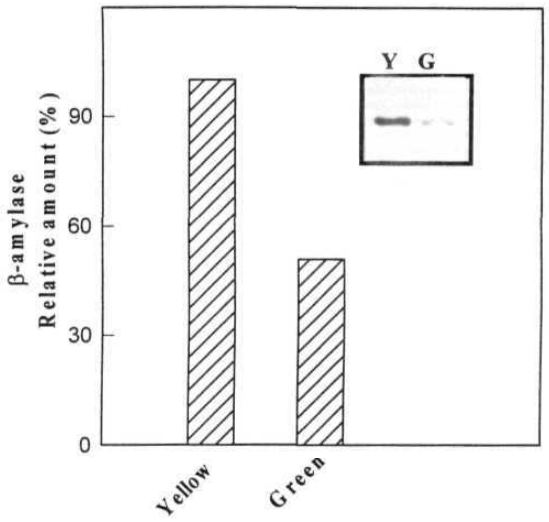

Figure 4.38. Quantification of β-amylase level in *zebra* (IP 8284) leaves.The mutant seedlings were grown under a photoperiod consisting of 16 h white light (L) and 8 h darkness (D) at 25°C for 4 days. Thereafter the mutant seedlings were transferred to a photoperiod consisting of 16 h light at 25°C and 8 h darkness at 10°C upto 9-d. Thereafter the first leaf was excised and the yellow (Y) and green (G) tissues separated carefully. The level of P-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of p-amylase band present in yellow leaf tissue as 100%.

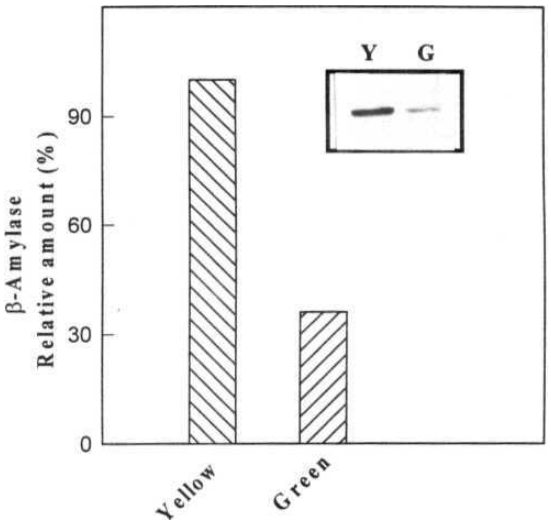

Figure 4.39. Quantification of p-amylase level in *yellow stripe* (IP 8292) mutant leaves. The mutant seedlings were grown under a photoperiod consisting of 16h light and 8 h darkness at 25°C upto 9-d from the time of sowing. Thereafter the first leaf was excised and the yellow (Y) and green (G) tissues were separated carefully. The level of β-amylase was determined by western blotting (Inset). The relative amount of P-amylase level was calculated using the intensity of P-amylase band present in yellow leaf tissue as 100%.

pathogens, in the healing response to wounding and the oxidative metabolism of auxin. In plants most peroxidase activity is localized in cytosol, therefore we used it as a marker enzyme to examine effect of cell maturity on the level of a typical cytosolic enzyme. Analysis of peroxidase activity in first leaf of pearl millet seedlings during the course of leaf elongation revealed that in young leaf the level of peroxidase is significantly higher in light-grown leaves compared to dark-grown leaves. With the increase in the leaf length, peroxidase activity declined in the light-grown leaves. In contrast the dark-grown leaves had low level of peroxidase activity and the peroxidase activity increased with the age. Interestingly 9-d old light-grown leaf had peroxidase activity equivalent to 3-d old dark-grown leaves (Fig. 4.40).

Since the young dark-grown leaves possessed a low level of peroxidase activity compared to light-grown leaves, we examined the effect of NF treatment on peroxidase activity of pearl millet seedlings. The NF mediated loss of carotenoids in pearl millet seedlings significantly stimulated peroxidase activity in dark-grown seedlings (Fig. 4.41), However it had no drastic effect on the level of peroxidase activity in RL-grown leaves. In both cases the peroxidase activity declined to a low level with increase in the age of the seedlings.

The 7-d-old light-grown leaves of pearl millet posses nearly similar activity of peroxidase in dark-grown leaves, therefore pearl millet leaves were dissected to examine the distribution of peroxidase activity in leaf. Fig. 4.42 shows that though the peroxidase activity in 7-d old RL-and dark-grown seedlings are nearly equal (Fig. 4.40), their distribution in leaf is opposite to each other. In dark-grown leaf peroxidase activity increases from leaf base to tip, and in contrast RL-grown leaf peroxidase activity declines from leaf base to tip.

The isozyme profile of peroxidase in different segment of leaf was analyzed using the native PAGE. The analysis of peroxidase activity by PAGE along the leaf axis showed that in dark-grown leaves the basal segment possess low activity, and it increases towards tip of the leaf (Fig. 4.43). On the contrary, in the light-grown leaves the peroxidase distribution was different from the dark-grown leaf. In light-grown leaf maximal peroxidase activity was observed in the base of the leaf and then it gradually declined towards the leaf tip (Fig. 4.44). The above isozyme profiles on PAGE also clearly correlated with peroxidase activity distribution along the length of pearl millet

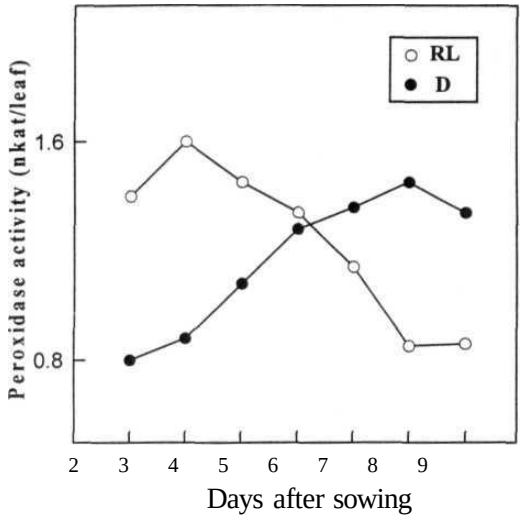

Figure 4. 40. Time course of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (•) or darkness (○) from the time of sowing. At the time point indicated (days) first leaf was excised at the base and the peroxidase activity was determined.

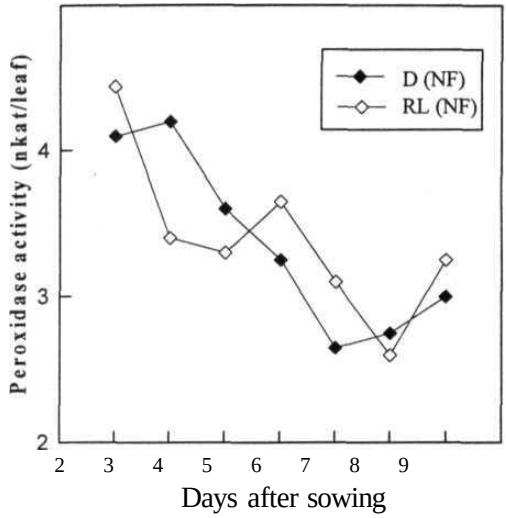

Figure 4. 41. Time course of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (◇) or darkness (◆) with 0.4 mM NF solution from the time of sowing. At the time point indicated (days) first leaf was excised at the base and the peroxidase activity was determined.

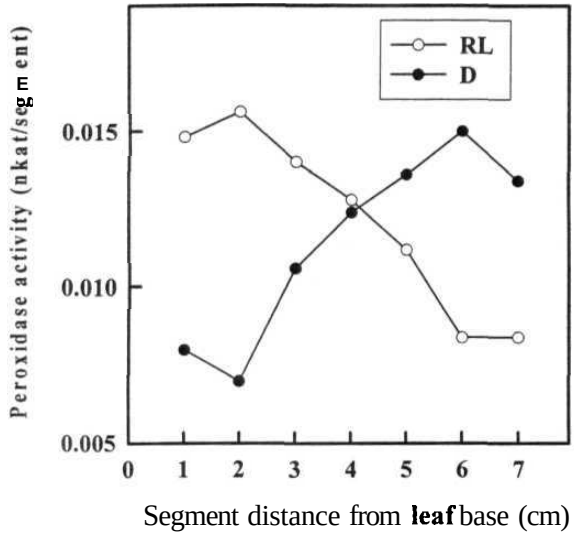

Figure 4. 42. Distribution of peroxidase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (O) or continuous darkness (•) upto 7-d from the time of sowing. At the time point indicated the first leaf was excised at the base and segmented into 1 cm long segments from leaf base to tip. The segments were numbered from leaf base to tip and the peroxidase activity was determined in segments.

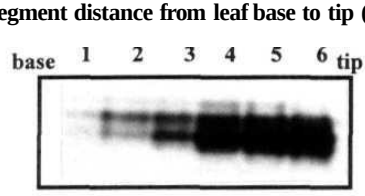

Figure 4. 43. Distribution of peroxidase isozymes along the length of pearl millet (WGC-75) leaves. The leaf was excised from 7-d old dark-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for peroxidase activity.

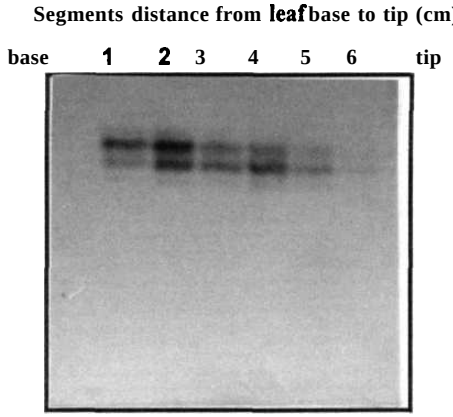

Figure 4.44. Distribution of peroxidase isozymes along the length of pearl millet (WGC-75) leaves. The leaf was excised from 7-d old RL-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for peroxidase activity.

leaf (Fig. 4.42).

4.13. Catalase Activity

The catalase enzyme is predominantly present in peroxisomes in leaves and act as a scavenger of H_2O_2 generated in plant metabolism. It is also believed that it may act as a salicylic acid binding protein *in vivo* and plays a role in defense mechanism. In the present study, we examined that the light induced expression of catalase activity in the pearl millet leaves. Fig. 4.45 shows the time course of catalase activity during the period of leaf elongation in pearl millet seedlings. The exposure of light had no effect on the level of catalase activity. In both dark-and light-grown leaves catalase activity increased with age. However at 8-d old RL-grown leaf had significantly higher catalase activity than dark-grown leaves.

The distribution of catalase activity as well as its isozymes was studied along the length of the 7-d old leaves. The primary leaf was dissected into one cm long segments, numbered sequentially from base to tip, and the catalase isozyme level was measured by native PAGE. The pearl millet leaf showed two catalase isozyme both in dark-and light-grown leaves. The analysis of catalase isozymes by native PAGE along the length of light-grown leaf showed a distinct gradient of catalase level from leaf base to tip (Fig. 4.46). In contrast dark-grown leaf did not show a distinct gradient of catalase accumulation. It had nearly the same catalase activity on PAGE except in the leaf tip region (Fig. 4.47).

Since the effect of light on catalase activity was noticed only after 6-d, the effect of light on catalase activity was examined after transferring dark-grown seedlings to RL for 12 h. In seedlings exposed to RL the level of catalase activity increased along the length of the leaf compared to dark-grown leaves and showed a pattern similar to light-grown leaves. This indicated that the level of catalase activity was positively regulated by light (Fig. 4.48) during the later phase of leaf elongation.

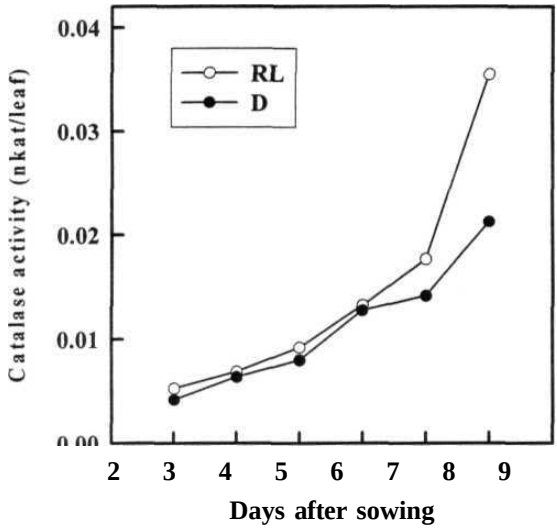

Figure 4. 45. Time course of catalase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous RL (O) or complete darkness (•) from the time of sowing. At the time point indicated (days), the first leaf was excised at the base and used for estimating the catalase activity.

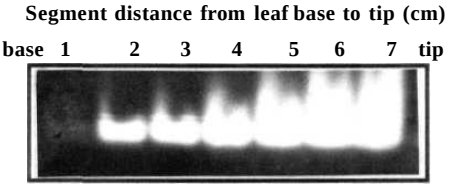

Figure 4. 46. Distribution of catalase isozymes along the length of pearl millet (WGC-75) leaf. The leaf was excised from 7-d old RL-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for catalase activity.

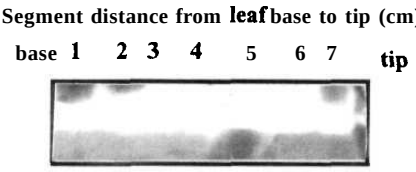

Figure 4.47. Distribution of catalase isozymes along the length of pearl millet (WGC-75) leaf. The leaf was excised from 7-d old dark-grown pearl millet seedlings. The leaf was excised at the base and cut into 1 cm long segments and numbered from base to the leaf tip. The segments were homogenized and centrifuged. The amount of proteins were calculated in supernatants and equal amounts of proteins were loaded onto each lane and after completion of native PAGE, stained for catalase activity.

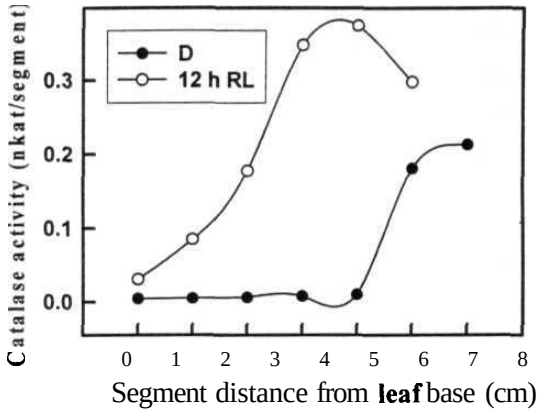

Figure **4.48.** Distribution of catalase activity in first leaf of pearl millet (WGC-75). Seedlings were grown under continuous darkness (•) upto 7-d from the time of sowing and then transferred to continuous RL (○) for 12 h. The first leaf was excised at the base and segmented into 1 cm long segments from leaf base to tip. The segments were numbered from leaf base to tip and the catalase activity was determined in segments.

5. DISCUSSION

Light controls the plant growth and development in a pleiotropic manner, and dictates a pattern of gene expression that leads to photomorphogenesis (Kendrick and Kronenberg, 1994; Chory, 1993; Deng, 1994; Reed and Chory, 1994; von Arnim and Deng, 1996). In dicots the post-primordial development of the leaf, particularly the leaf expansion is strongly dependent on the availability of light. In monocots, leaf development follows a different strategy, by virtue of continuation of leaf development at basal meristem and indeterminate nature of leaf length. Consequently, the monocot leaf possesses along its length a cell maturity gradient with oldest cells at the tip. In the present study, using this gradient we examined the interaction between cellular position and light mediated induction/inhibition of photoresponses located in plastidic, cytosolic, peroxisomal compartments of pearl millet leaf.

5.1 Pigments

One of the prominent response in the leaf development is greening of leaf, and therefore we first examined interaction between cellular position and light-mediated chlorophyll formation. The information on factors regulating biosynthesis of chlorophyll and its assembly into functional photosynthetic active complexes is not complete. Though, it is a well established fact that light stimulates chlorophyll biosynthesis in angiosperms, the mechanism of control is apparently exercised at several points, including requirement for light for a direct photoreduction of protochlorophyllide to chlorophyllide. However, there also exists the possibility that certain amount of chlorophyll biosynthesis in angiosperms may also take place indirectly via the action of light independent Pchlide reduction (Adamson and Packer, 1984; Tripathy and Rebeiz, 1987), but it is very small and its significance is not known.

It is apparent from time course profile of chlorophyll level in light-grown pearl millet seedlings, that light stimulates the chlorophyll biosynthesis and the effect of light on this process is both temporally and spatially determined (Fig. 4.1). The light-induced formation of chlorophyll in pearl millet leaf could be detected right after the formation of leaf. Even though in 3-4 d old seedlings, leaf was ensheathed in

coleoptile, and therefore receives reduced amount of light, it made a substantial amount of chlorophyll. On a comparative scale WL-exposure induced a higher accumulation of chlorophyll than RL (Fig. 4.6). The relative efficiency of WL compared to RL in stimulating chlorophyll biosynthesis may result from difference in light intensity between two light sources, which were more than 333.3-fold. Alternatively, under WL all the photoreceptors regulating chlorophyll biosynthesis, such as blue/UV-A photoreceptor and phytochrome are active, while under RL only phytochrome would be active.

Among two species of chlorophyll in angiosperm, chlorophyll a is dominant species and present at higher level than chlorophyll b. It is believed that chlorophyll b are synthesized by enzymatically controlled side chain modification of chlorophyll a to chlorophyll b. It is believed that reverse conversion of chlorophyll a to chlorophyll b cannot take place because reduction of formyl group to methyl group is a difficult reaction. The control mechanism maintaining stoichiometry between chlorophyll a and chlorophyll b formation is not known. The work published by several groups particularly of Rebeiz group indicates that formation of chlorophyll b from precursors of chlorophyll biosynthetic pathway possibly can follow at least 10 different pathways indicating heterogeneity in the biosynthetic pathway of chlorophyll b formation.

Interestingly not only chlorophyll b is formed by multiple routes, under certain condition chlorophyll b itself can revert back to chlorophyll a to allow effective formation of photosynthetic complexes (Ohtsuka et al.,1997). The difference in ratio of chlorophyll a/b observed during the greening of leaves (Fig. 4.7) may in part either represent their differential rate of biosynthesis or it may even represent interconversion between chlorophyll a and chlorophyll b.

It is also well known that chlorophyll and its precursor are toxic molecules, which generate active oxygen under illumination, therefore chlorophyll a/b ratio *in vivo* has to be regulated in such a way that it does not cause accumulation of free chlorophylls or its precursors. The interconversion between chlorophyll a and chlorophyll b therefore can reduce the amount of free chlorophyll and protect photosynthetic complexes against possible photodynamic damages. Such a reconversion of chlorophyll a to chlorophyll b has been observed in cucumber

chloroplast (Ito et al., 1996), where chlorophyll b associated with its binding protein converted to chlorophyll a via 7-hydroxymethyl chlorophyll via lipid bilayer and then chlorophyll b was used for formation of core complexes of photosystems (Beale and Weinstein, 1990).

In the present study, dark-grown pearl millet leaves has very low carotenoid content (Fig. 4.4), however exposure to light strongly stimulates carotenoid biosynthesis (Fig. 4.9). The role of light in regulating carotenoid biosynthesis is clearly apparent from the fact that similar to chlorophyll, seedlings grown in continuous light accumulate high level of carotenoids (Fig. 4.4). Though it is known that carotenoid synthesis is stimulated by light, the potential sites regulating action of light are still not fully known. In mustard it has been shown that phytochrome mediated carotenoid accumulation takes place during conversion of etioplast to chloroplasts (Frosh and Mohr, 1980). The dilemma of site where light regulates carotenoid biosynthesis is now partly resolved. It has been shown by examining transcript levels of three carotenoid biosynthetic genes such as geranylgeranyl pyrophosphate synthase, phytoene synthase, and phytoene desaturase in mustard and Arabidopsis. Among these three enzymes involved in carotenoid synthesis, the light strongly regulates the level of phytoene synthase (von Lintig et al., 1997), indicating that phytochrome action on carotenoid biosynthesis is exercised at the level of phytoene synthase.

The profile of chlorophyll accumulation under continuous illumination (Fig. 4.1) represents a situation where irrespective of cell maturity each cell is exposed to light continually and once it becomes competent to respond to a light signal for a particular developmental response, the response begin instantaneously. In comparison to this, in seedlings grown in darkness, the leaves are full etiolated and though they are competent to develop chloroplast and chlorophyll, they need the triggering stimulus of light. The transfer of such a leaf to light allows one to examine how the same light signal, is perceived in different cells to initiate pigment and protein synthesis. It also allows one to monitor the interdependence of accumulation of these pigments in different cells of leaves.

On transforming 6-d old etiolated pearl millet seedlings to light, chlorophyll synthesis is initiated with a lag period of about 2 h, with white light being more

effective than RL (Fig. 4.6). Similarly a rapid induction of formation of carotenoid is also initiated in these leaves (Fig. 4.9). The examination of chlorophyll/carotenoid distribution along the length of the leaf (Figs. 4.11 and 4.15) highlights the fact that the younger cells of leaf do not synthesize chlorophyll/carotenoid at the same rate as the older cells near the leaf tip. A distinct gradient of both chlorophyll and carotenoid formation is apparent from profile of distribution of both chlorophyll and carotenoids, which is a minima at the leaf base and a maxima at the leaf tip (Figs. 4.1, 4.4, 4.6, 4.9, 4.11 and 4.15).

The results highlight the fact that though the signal to induce chlorophyll and carotenoid synthesis (i.e. light) was equally available to all cells of leaf, however the age of leaf cells is primarily responsible for quantitative difference in induction level of these pigments at different portion of leaf. On light exposure the younger cells of leaf forms relatively less amount of chlorophyll and carotenoid, in comparison with older cells near the leaf tip. Evidently, the light mediated quantitative formation of pigments in pearl millet leaf is determined by a preformed cell maturity gradient in leaf. It is of interest to note that the above gradient which determines the quantitative formation of pigments also determines the level of several other plastidic proteins, or other photoresponses which are metabolically linked to chloroplast development, and have been described below in more detail.

The mechanism maintaining stoichiometry between chlorophyll/carotenoid is not known, however, a co-ordinated balance between two is needed for effective assembly of photosystems. Carotenoid such as luetin is also required for specific and accurate assembly of light-harvesting complex (Plumley and Schmidt, 1987). It is often difficult to separate process of chlorophyll from carotenoid biosynthesis as on exposure to light both chlorophyll and carotenoid biosynthesis are triggered simultaneously. Moreover, any block in carotenoid biosynthesis either by mutation or herbicide treatment leads to a complete photooxidation of accumulated chlorophyll molecules leading to albino tissues. In general *albino* or *white stripe* mutants represent operation of a process where either both chlorophyll and carotenoid fail to accumulate due to a chloroplast development specific mutations, or block in the carotenoid biosynthesis causing albino phenotype due to

photooxidation of chlorophyll. By contrast, carotenoid biosynthesis can continue even in the absence of chlorophyll biosynthesis, as carotenoids do not undergo photooxidation, which is apparent from observation of most chlorophyll biosynthetic mutants, where accumulation of carotenoids can be visualized as yellow leaf phenotype. However, it is observed that the accumulation of carotenoid is also closely linked to the level of chlorophyll in tissue, at least in photosynthetic tissues of leaf (Frosch and Mohr, 1980; van Lintig et al., 1997).

The dependence of carotenoid biosynthesis on chlorophyll biosynthesis have been indicated in several earlier studies (Frosch and Mohr, 1980; Malhotra et al., 1982). The recent studies using mustard and *Arabidopsis* mutants have shown that though light enhances the level of carotenoid biosynthesizing enzyme phytoene synthase, the level of carotenoid *per se* does not increase in the seedlings in similar proportion, unless it is also accompanied by chloroplast synthesis. In fact, the increase in carotenoid level is intimately associated with corresponding increase in chlorophyll levels. Therefore, under far-red light though the level of phytoene synthase is enhanced, the carotenoid level increases only under those light condition, that also favors chlorophyll synthesis such as red light, blue light or white light (von Lintig et al., 1997).

Relatively few studies have examined the mechanism controlling balance between chlorophyll and carotenoid biosynthesis. A model developed by Mohr's group (Frosh and Mohr, 1980; Malhotra et al., 1982) on light-regulated carotogenesis in higher plants envisage a 'push and pull' mechanism, where light acting through phytochrome push the biosynthesis of carotenoid and chlorophyll acting as a complementary pigment is needed to pull the synthesis of carotenoid (Frosh and Mohr, 1980; Malhotra et al., 1982). This model of light-induced carotogenesis in higher plants has been confirmed using a monocot-sorghum and dicot-mustard system. The data obtained in pearl millet leaf with respect to chlorophyll/carotenoid distribution along the length of the leaf (Figs. 4.11 and 4.15) supports the Mohr's model and further highlights that in addition to push and pull mechanism, the cell position also determines the quantitative accumulation of both chlorophyll and carotenoid in monocot leaves.

In etiolated leaves the biosynthesis of chlorophyll is blocked at the level of the

formation of Pchlide, as subsequent conversion of it to Chlide needs light dependent enzymic action (Griffiths, 1991). *In vivo,* Pchlide exits as a complex with holoprotein in the prolamellar body of the etioplasts. In this study we did not examine the distribution of Pchl-holoprotein complexes by examining *in vivo* spectra of the different segments of leaf, but relied on direct quantification of Pchlide in acetone extracts. The distribution profile of Pchlide highlighted that dark-grown leaf accumulates Pchlide in increasing amount from base to leaf tip with highest amount of Pchl near the leaf tip (Table. 4.1). The exposure to light induces the conversion of Pchlide to Chlide, whose distribution also follows an identical pattern (Table. 4.3). The examination of Pchilde distribution in pea epicotyls (Boddi et al., 1994) and bean hyopcotyls (Mc Ewen et al., 1994), also revealed distribution profiles where maximal amout of Pchilde was present in the upper region of the organ near the developing leaves or shoot apical meristem. However, unlike the pearl millet leaf where more Pchlide is present in older leaf cells, in these two dicot seedlings viz pea and bean, more Pchlide is present in younger cells near the meristems (Boddi et al., 1994; Mc Ewen et al., 1994).

5.2. Plastidic proteins

The western blotting of POR in dark-light-grown pearl millet leaf indicate that POR A and POR B proteins are present in dark-and light-grown leaves of pearl millet respectively (Fig. 4.20) which evident for their respective molecular weight. In higher plants POR consists of at least two species (Holtorf et al., 1995), of which the mechanism of enzymic action is known in detail for POR A. In barley it is a 36 kD protein and acts as a shuttling photoenzyme where NADPH and Pchl form a turnover Pchl-NADPH enzyme complex with red absorption maxima at 652 nm. During greening of tissue POR A level rapidly downregulated by proteolysis by a light-induced protease (Reinbothe et al., 1995). In daylight the Pchlide is converted primarily by the enzymic action of POR B, which is present at low level in leaf, and whose level is not regulated by light. The formation of Pchlide is also essential for import of precursor of POR from cytosol to plastid. In the absence of Pchlide in etioplasts, the POR A precursor cannot be imported into etioplasts indicating that a substrate mediated transfer takes place in etioplasts for POR. Moreover import of

POR A into plastid takes place only in etioplasts but not in chloroplasts (Reinbothe et al., 1995). The precursor of POR A can move into chloroplast only if its substrate Pchlide was present in chloroplasts. By contrast, the precursor of POR B does not depend on plastid differentiation and it is imported both in chloroplasts and also in etioplasts. (Reinbothe et al., 1995). The reason behind the susceptibility of POR A to degradation but not POR B to the difference in their location. While POR A is localized in prolamellar bodies, the POR B is present in envelope of plastids and protected from degradation (Dehesh and Ryberg, 1985; Porra et al., 1997; Sperling et al., 1998).

The examination of distribution pattern of POR A in dark-grown pearl millet leaves is in conformity with distribution of protochlorophyllide, which also show the maximal level in leaf tip (Fig. 4.21). The exposure to light induces the decline of POR A and in light-grown leaves it is present only in trace amount and leaves contain predominantly POR B. The important point which emerges from distribution of POR B in light-grown leaves (Fig. 4.22) is that, while it is at low level throughout the length of a leaf a steep increase in its level is evident in the older leaf cells, particularly near the leaf tip. This may signify the fact that leaf tip accumulates high level of both POR A and POR B and accumulation of these proteins are determined by cell position.

The distribution of LHCP protein in leaf also indicates an intimate association with gradient of cell maturity and chloroplast developmental gradient (Fig. 4.26). The LHCP protein accumulates only in light-grown leaves, but is absent in dark-grown leaves (Fig. 4.27). The distribution of LHCP protein in light-grown leaves is similar to that of POR B and POR indicating a close correlation with chloroplast development. The molecular mechanism that coordinates LHCP and chloroplast development in green leaves is not well understood, but one of the integrative element appears to be light. The analyses of chlorophyll deficient mutants revealed that in addition to light, the photosynthetic pigment particularly chlorophyll b is also needed to stabilize the LHCP and thylakoid membranes, indicating an effective post-translational mechanisms, regulating LHCP formation. It is observed that the translation of some of the plastid encoded proteins is staked on plastid ribosome in the absence of chlorophyll. The studies of Meehen et al., (1996) indicated that

transcription of *Lhcb* a member of LHCP family is positively correlated with chlorophyll content and cell size in green cells of *Arabidopsis.*

Similar to LHCP levels, exposure to light also stimulates the level of α-amylase protein in pearl millet leaf (Vally and Sharma, 1991), (Figs. 4.32 and 4.33). In fact exposure of light to pearl millet leaf stimulate synthesis of new isoform of a-amylase, which is exclusively localized in chloroplast (Vally and Sharma, 1995). The distribution of a-amylase in the pearl millet leaf also follows a distribution profile similar to that for LHCP and POR (Vally, 1994). The examination of a-amylase level of dark-and light-grown leaves of several mutant such as *early flowering* and *yellow stripe* showed that irrespective of the variety of pearl millet light stimulates a-amylase level in pearl millet leaves. The pattern was observed in leaves of mutants also (Figs. 4.33 and 4.34). Taken together all these results suggest that in pearl millet leaf, the distribution of plastidic proteins and pigment follow a profile, with a minimal level at leaf base and maximal level at leaf tip.

5.3. Peroxisomal enzymes

Since the responses described hitherto in this discussion, mainly pertains to plastid, we also examined influence of light and associated chloroplast development in leaf, by examining other cytosolic compartments such as peroxisomes. We used catalase as a marker for development of peroxisome. The information on regulation of catalase is available only for maize, where it consists of small multigene family having three genes *{cat 1, 2,* and, 5) (Scandalios, 1994). It is likely that in pearl millet too, a multigene family might encode catalase. A similarity in catalase sequence was observed between maize, rice (Higo and Higo, 1996) and barley catalase genes (Skadsen, 1995). In barley, only 2 *cat* genes are reported, which respond to light in diametrically opposite way (Acevado et al., 1996). In dark-grown leaf *cat* 1 mRNA is present, which declines on exposure to light, and at the same time the level of *cat 2* mRNA is elevated. It is likely that *cat 2* enzyme represent the peroxisomal form of catalase in barley.

In the present study the effect of light on catalase isozyme pattern and its total activity was not apparent during initial phase of leaf development but was visualized only in later phase of leaf development (Figs. 4.45-4.47). Since the studies conducted

in this laboratory indicated that maize and pearl millet seedlings show a very similar response to light (Data, unpublished results) as indicated by similar pattern of enzyme induction such as that of β-amylase, it is likely that in pearl millet catalase which is similar to maize and barley, may also have a light-inducible isoform. It is likely that the observed higher level in catalase activity in pearl millet leaf after 6-d of growth under continuous light, highlights the accumulation of light-inducible catalase isoform perhaps in B cells, similar to maize. This is particularly apparent from distribution of catalase isozymes in dark-and light-grown leaves. In light-grown leaves, the catlase isozymes follow a distinct profile of accumulation from base to leaf tip (Fig. 4.46), but in dark-grown leaves it is rather more uniformly present in all segments of leaf (Fig. 4.47).

It is also evident from the profile of catalase accumulation in the light-grown pearl millet leaf (Fig. 4.45) that the expression of catalase in light is also intimately linked to plastid development and is somewhat similar to that observed for phoshorylase distribution pattern in B cells (Vally et al., 1995). The examination of *cat 2* gene expression pattern in wild type and in chlorophyll and carotenoid mutants of maize brought forth a similar observation, that in addition to light, an other factor such as chloroplast development, also regulates *cat* gene expression in maize leaf. In maize *cat 1* gene is light independent and *cat 2* and *cat 3* are inducible by light. Out of these *cat 2* is localized in B cells and its transcripts are absent in dark-grown seedlings and requires light for its formation. The *cat 3* gene accumulates at high level in M cells under darkness. Out of these *cat 2* accumulation is dependent on chloroplast development and it fails to appear in chlorophyll less region of leaves. However, it is present in the leaf, even if the leaf lacks chlorophyll but possesses the normal carotenoid level. In contrast, the expression of *cat* 3 is independent of chlorophyll and carotenoid expression in maize leaf (Acevedo et al., 1991) and not related to chloroplast development.

In many plants organelle harboring catalase, peroxisome is intimately associated with chloroplast. It is believed that such an association is necessitated for exchange of metabolites between two organelles particularly for photorespiration in C_3 plants. The question whether the development of peroxisomes is also intimately related to the development of chloroplasts was examined by Bajracharya et al., (1987)

in mustard by studying the development of enzyme activity of peroxisomes in normal light-grown seedlings and seedlings treated with NF. In NF-treated seedlings they observed that elimination of functional chloroplasts either by NF mediated photooxidation or by blocking plastid protein synthesis reduces the development of peroxisomal enzymes glycollate oxidase and hydroxypyruvate reductase. Interestingly loss of chloroplasts stimulated the activity of glyoxysomal enzymes such as isocitrate lyase and malate synthase. Moreover the loss of chloroplast by NF-treatment also drastically reduced the frequency of peroxisome chloroplasts association as visualized by electron microscopy. However NF-treatment had no effect on morphology of peroxisomes as observed under electron microscope, though the chloroplast structure was severely damaged. Taken together, the data obtained by Bajracharya et al., (1987) in mustard, observations of mutants in maize (Avecedo et al., 1991), and developmental correlation seen in pearl millet, it is clearly evident that the observed profile of catalase enzyme in light-grown leaf shows an intimate link to chloroplast development in leaf (Figs. 4.45 and 4.46).

The plastid does not exist in leaf cells as a 'stand alone' organelle and requires a close co-ordination with other cellular compartments. It is essential to have this co-ordination to regulate metabolism between different cellular compartments of plants. The studies on catalase induction highlighted such a coordinated development between catalase level in peroxisome and chloroplast development. Since pearl millet is a C_4 plant which also has a cytosolic fixation of CO_2 using PEP-carboxylase enzyme, the link between PEP-carboxylase and chloroplast development was examined during pearl millet seedling development (Fig. 4.30).

PEP carboxylase is the enzyme responsible for the primary fixation of CO_2 in C_4 photosynthesis and crassulacean acid metabolism. The activity of PEP-carboxylase is regulated by light (Karabourniotis et al., 1983; Huber et al., 1986) and through a reversible phosphorylation (Nimmo et al, 1984, Jiao and Chollet, 1988) or a dimer and tetramer interconversion (Wu and Wedding, 1985; Wu et al., 1990). Profile of PEP-carboxylase distribution in green leaves has a strong resemblance to that of POR B and LHCP in green leaves, with levels increasing towards the leaf tip. Evidently, chloroplast development also strongly influences cytosolic PEP-carboxylase distribution in maize leaves suggesting a close interdependence of these two

processes.

Another representative enzyme of cytosolic compartment is peroxidase. Peroxidases basically consist of several distinct proteins, but all share a common catalytic activity. Since peroxidase consists of several distinct enzymes, **the** quantification of total activity of this enzyme in pearl millet leaf at best represents **the** potential of cells for scavenging peroxide radicals generated in leaf. Though peroxidase are localized in all cellular compartments of leaf cells, in maize leaves maximal activity was found associated with cytosolic fraction (Sharma et al, 1979) indicating that major part of peroxidase activity in pearl millet leaf is cytosolic in nature. Though a precise function can not be ascribed to this enzyme, in the present study analysis of peroxidase activity during leaf elongation in seedling revealed that the younger seedlings have high level of peroxidase activity in darkness (Fig. 4.40). By contrast the level of peroxidase activity is low in light-grown leaf but increases with age. Thus, while peroxidase activity in dark-grown leaves decline with age, and a reverse pattern was observed for light-grown seedlings.

The analyses of activity profile and isozyme profile of peroxidase along the leaf length highlighted a drastic different pattern of distribution, (Figs. 4.40, 4.43 and 4.44) compared to plastidic proteins as described above. In dark-grown leaves, the amount of peroxidase activity was localized in gradient fashion similar to that of LHCP or PEP-carboxylase, with maximal activity in leaf tip. However, exposure to light altered this profile of distribution, with maximal activity present in the leaf base. Such shift in distribution profile in pearl millet leaf has been found for other cytosolic proteins too.

5.4. Cytosolic proteins

In detailed studies carried out by Vally, (1994) it was found that exposure of light to etiolated pearl millet seedlings shifts the β-amylase distribution with maximal activity of β-amylase located near the leaf base due to the photostimulation of (3-amylase. The above distribution profile was not restricted to (3-amylase alone but also was observed from cytosolic phosphorylase whose distribution in pearl millet leaf also showed a similar shift in its distribution with maximal activity near the leaf base in light-grown leaves (Vally et al., 1995). In another investigation on maize seedlings

similar profile of β-amylase distribution was observed in leaf under dark-and light-growth condition respectively (Datta, 1996). Taken together, the results of peroxidase distribution clearly indicates that cytosolic enzyme distribution in pearl millet leaf followed two distinct profiles one specific for dark-grown plants which is similar to that of plastidic proteins in light-grown seedlings. The second profile is typical of light-grown seedling, where the maximal amount of β-amylase is localized in the cell near the leaf base. The reason for the above specific distribution and mechanism initiating the process is not known but can only be speculated.

One possibility is that the level of (3-amylase and also of peroxidase may be negatively determined by the presence of chloroplast in a leaf. Since in dark-grown leaves, only etioplasts are present the level of β-amylase and peroxidase follows a normal profile with increasing level towards the leaf tip. However, in light-grown leaves the formation of chloroplast specifically suppresses the (3-amylase and peroxidase induction, but at the same time light promotes the induction of these proteins in leaf. Consequently, the effect of light on level of proteins is only visualized in younger leaf cells, which has under-developed chloroplasts. However, it is also likely that other mechanisms may also operate regulating these distribution profiles.

The possibility of such interaction between chloroplast development and (3-amylase induction was examined by using chloroplast developmental mutants in pearl millet particularly *zebra* and *yellow stripe* mutants (Figs. 4.38 and 4.39). The mechanism causing these phenotypes are not known, however it is likely that pearl millet mutants may be similar to few known maize mutants that also show similar phenotypes. Since there is no detailed information on pearl millet mutants other than genetic analysis and evidence that plastids of these mutants may have a block in protein synthesis, it may be appropriate to examine properties of comparable maize mutants where detailed information is available.

Similar to pearl millet *yellow stripe* mutant maize also have at least 3 *yellow stripe* mutants *ys1*, *ys2, ys3* located on different chromosomes (Neuffer et al., 1997). The *yellow stripe* on maize resembles phenotype caused by deficiency of micro-nutrient iron and for *ys-3* can be corrected by iron supplementation to the plants. In *yellow stripe* phenotype in maize results due to ineffectiveness of mutant plants to

utilize ferric form of iron (Bell et al., 1962). It is observed that leaves can regreen if seedlings with cut roots were kept in Fe-EDTA solution. The above defect is now known to be caused by the lack of efficient uptake of iron from the medium. It is now known that though *yellow stripe ys1* mutant release phytosiderophore to complex iron for uptake, it is defective in uptake of Fe phytosiderophore, therefore causing the defective *yellow stripe* phenotype (von Wiren et al., 1994). It is likely that pearl millet mutant may have a similar defect causing *yellow stripe* but it is still to be examined. By contrast another maize *yellow stripe* mutant *ys3* is defective in secretion of phytosiderophore to the medium (Basso et al., 1994) and therefore can not obtain iron from soil.

However, in a maternally inherited *yellow stripe* mutant of maize, it was reported that this defect is caused by a defective mitochondrial cytochrome oxidase subunit 2 gene (Newton et al., 1990). The genetic analysis of *zebra* mutation in pearl millet leaf has not yet been conducted. In a recent publication Neuffer et al., (1997) listed at least *zebra* like mutations in maize, for few of which their location on chromosomes is known e.g., *zb4* and *zb7* are on short and long arm of chromosome respectively, *zb6* on chromosome 4L, *zb8* on chromosome 9s and *zb3* on chromosome 8L. On comparison of the phenotypes described above the pearl millet *zebra* mutant is similar to maize *zb6* and *zb4*, where yellow cross band on leaves are promoted by cooler temperature. While several other mutant loci from maize have been cloned the molecular lesion (Neuffer et al., 1997) causing *zebra* phenotype is not known.

The examination of defect in chloroplast development in the pearl millet mutants was first conducted by examining the capacity to perform photosynthesis by excising yellow and green tissues from mutant leaves and examining the rate of O_2 photosynthetic evolution. Compared to green leaf tissue the light-mediated O_2 evolution in yellow region of *zebra* mutant was nearly less than three fold differences indicating diminished activity of chloroplasts in yellow region of leaf (Fig. 4.31). The quantification of POR proteins in mutant leaves again reconfirmed this fact, the yellow region of leaf has very low level of POR B protein, compared to adjacent green segments (Fig. 4.23), and white stripe leaf POR was altogether absent in white region (Fig. 4.24). The examination of LHCP levels in yellow and green region, confirms the observation that yellow region have very low level of chloroplast

activity. The level of LHCP was very low in yellow region of mutant tissue was also observed in green region (Figs. 4.27 and 4.28). The quantification of carotenoid, chlorophylls and protochlorophylls level further confirms that this region has severely reduced functional activity of chloroplasts (Figs. 4.19, Tables. 4.4 and 4.5).

The examination of β-amylase level in yellow and green region of leaf revealed that loss of chloroplast in yellow region stimulated P-amylase activity by atleast two folds difference (Figs. 4.38 and 4.39). It can be assumed that loss of chloroplast may have caused increase in P-amylase level, which was confirmed by treating seedling with NF, which blocks the chloroplast development in leaf. In dark-grown leaves P-amylase level increases from base to the leaf tip (Fig. 4.36), while in light-grown leaves its activity is maximally localized in leaf base (Fig. 4.37). The treatment with NF stimulated p-amylase to a high level in all segments of leaf to a great extent (Fig. 4.35). Such high regulations of enzyme activity by loss of chloroplast have also been observed also for chalcone synthase in barley (Hess et al., 1994)

The mechanism by which such upregulation of enzyme levels for P-amylase and peroxidase and loss of chloroplast is brought about, needs to be investigated. However, few experiments conducted in our lab (Datta, unpublished results) have indicated that P-amylase is upregulated under the condition of stress (Drier et al, 1995). In maize and pearl millet leaves photooxidation of chloroplasts by NF drastically upregulates the P-amylase activity (Fig. 4.35), indicating that loss of chloroplast is taken as a stress signal by leaf resulting in an enhancement of β-amylase activity. A similar increase in P-amylase is also observed when leaves are exposed to water stress or salt stress. The upregulation of P-amylase by loss of chloroplast by mutation appears to be part of same phenomena, which is a general response to stress. It is likely that the upregulation of peroxidase activity in basal segment (Fig. 4.41) was also dependent on loss of chloroplasts.

The question what could be the signal for such response is still to be deciphered. Recently using *chlamydomonas*, an evidence was obtained that plastid-derived chlorophyll precursors may replace light in induction of two nuclear heat shock genes and therefore can be considered as putative plastidic signal (Kropat et al., 1997). Imagining a similar situation in higher plants it is tempting to speculate that

block in chloroplast development by mutation may cause excess buildup of chlorophyll precursors that it in turn would upregulate β-amylase activity in tissues in absence of chloroplasts. Alternatively regulation of β-amylase may be negatively with chloroplast development and loss of chloroplast itself may act as a trigger for upregulation of P-amylase and peroxidase activity.

In pearl millet leaf peroxidase activity like P-amylase activity is also inversely associated with chloroplast development. It has been reported in several species that the loss of chloroplasts induces the synthesis of proteins/enzymes that may help the plants to overcome the damaging effects of stress. For example in barley, photobleaching by NF induces accumulation of the ELIP (Adamska et al., 1992), a nuclear-encoded protein known to protect chloroplasts against photooxidative stress. Similarly, the loss of chloroplasts stimulates the synthesis of phenylpropanoids by increasing the levels of chalcone synthase in barley (Echmann and Schafer, 1988) in albostrians mutant of barley (Hess et al., 1994), and in mustard (Rebeiz et al., 1983). In tobacco, the loss of chloroplasts stimulated mRNA levels of plastidic Cu Zn-superoxide dismutase (Kurepa et al., 1997). A role for extra-plastidic amylases being a stress-induced protein has also been suggested for pea, where NF-treatment stimulates p-amylase activity by 80 fold in the apoplast (Saeed and Duke 1990a). In pearl millet leaves, the level of P-amylase appears to be inversely related to the presence of functional chloroplasts. Similarly in wheat, P-amylase level was under negative control of the chloroplast (Ziegler et al., 1994). In pea also, an inverse relationship was observed between chloroplast function and the level of apoplastic P-amylase and vacuolar P-amylase activity (Saeed and Duke, 1990a,b). In barley, P-amylase activity in NF bleached leaves was higher compared to green leaves, and this induction by NF was interpreted as a stress response to the absence of light (Drier et al., 1995). However, results obtained in this study indicate that the role of light is rather indirect, and it only amplifies the effect of stress on the amylase level.

The molecular events determining selective photostimulation of P-amylase and peroxidase activity in the basal region of the leaf are not known; and can only be speculated. Analysis of p-amylase activity between 5-10-d old pearl millet seedling shows that during this period highest p-amylase activity is invariably localized near the leaf base, 1-2 cm (Vally, 1994). Such a fixed distribution pattern indicate that

either a gradient of cell maturity, or of a metabolite moving along the length of leaf determines observed pattern of β-amylase and peroxidase distribution. Since in monocot leaves, the basal region contains young cells undergoing division and differentiation (Kemp, 1980; Sylvester et al., 1990), these enzyme distribution may be linked to the gradient of cell maturity. For example, in wheat leaves, the distribution of cell cycle control protein, $p34^{cdc2}$ shows a pattern similar to that of P-amylase (John et al., 1990). Its level is maximal at the leaf base and declines gradually to a low and constant level after 4 cm. It is possible that young cells near the leaf base are more responsive to photoinduce p-amylase, as a consequence of which high levels of P-amylase is observed near the base of light-grown leaves. Such a selective response of cells has been observed with respect to hormone-promoted cell division in wheat leaf, where the cells which are distant from the basal meristem are unable to resume cell division, even after the administration of auxins, although all cells of the leaf possess equal rates of uptake and metabolism of auxins (Wernicke and Milkovits, 1987a, b).

The possibility that the observed P-amylase distribution pattern may be determined by a metabolite moving in the leaf is also suggested by stimulation of P-amylase activity by sugar (Datta et al., Unpublished results) The dipping of excised leaf bases in sugar solution shifted P-amylase distribution in dark-grown leaves of pearl millet and maize to a pattern induced by light exposure. It is therefore possible that the light mediated induction of P-amylase may involve either sugar as a part of the signal transduction chain or light and sugar act on a common signal pathway. The interaction between sugar and light has also been examined with reference to P-amylase in other plant systems. In wild-type *Arabidopsis,* similar to maize and pearl millet, light further enhanced the sugar inducible expression of P-amylase gene (Mita et al., 1995). It may be assumed that, in light-grown leaves, the generation and transport of sugar towards the leaf base in maize and pearl millet may cause the observed P-amylase distribution where high activity of P-amylase at leaf base results due to accumulation of sugar.

Considering the likely *in vivo* function of p-amylase as a starch degrading enzyme, the observation that loss of chloroplasts, which is the site of starch synthesis,

increases its expression rather than inhibiting it appears anomalous. However, recent studies have revealed that β-amylase *in vivo* may function differently than its presumed enzymatic function, such as, it may be a vegetative storage protein in sweet potato tuber (Nakamura et al., 1991) or a stress inducible protein in barley (Drier et al., 1995). Incubation of potato tubers at low temperature induces sugar accumulation and induction of a new isoform of p-amylase. β-Amylase is also induced by light-stress and other stresses like water stress and high temperature in barley (Drier et al., 1995) suggesting that it may act as a marker protein for stress.

SUMMARY

In this study, we used the pearl millet leaf as a model system to examine the effect of light on regulation of cytosolic and chloroplastic enzymes and levels of photosynthetic pigments in relation to the cellular position and cell maturity gradient in developing leaves of pearl millet seedlings. We also used chloroplast developmental mutants of pearl millet to study the nuclear-plastidic interaction on photoresponse in developing pearl millet leaf. Since pearl millet is a C_4 plant the development of leaf and chloroplast biogenesis is directly depend on the availability of light. The magnitude of the light effect on induction of enzyme and pigment is dependent on the cell position, cell age gradient and is different for different proteins located in different cellular compartments.

In WL-and RL-grown pearl millet seedlings the amount of chlorophyll continually increased in the leaf with age, with greater level of chlorophyll accumulation under WL exposure. The accumulation of sub-species of chlorophylls: chlorophyll a and chlorophyll b also followed an accumulation pattern similar to that of total chlorophyll. Dark-grown seedlings possessed very low level of carotenoid in leaf however WL-or RL-grown seedlings showed greatly enhanced accumulation of carotenoid in the leaf. Among the two light treatment used, WL was more efficient in stimulating chlorophyll and carotenoid accumulation in the leaf.

The fact that leaf cells possess full complement of chlorophyll and carotenoid synthesis in dark but need exposure of light to express it, was demonstrated by transferring dark-grown seedlings to light. The transfer of etiolated seedlings to light stimulated increase in both chlorophyll and carotenoid levels in a coordinated fashion. While transfer of etiolated seedlings to light elicited a rapid induction of both chlorophyll and carotenoid, the magnitude of induction was directly related to the cell maturity. The younger cells at the leaf base, on exposure to light showed only a slight stimulation of chlorophyll and carotenoid levels, whereas the older leaf cells near the leaf tip showed maximal increase of chlorophyll and carotenoid levels. These results highlight the fact that magnitude of light induced photosynthetic pigment accumulation in pearl millet leaf is determined by an innate cell maturity gradient, with older cells being more responsive to light.

The examination of dark-grown pearl millet leaf revealed that influence of cell maturity on accumulation of chlorophyll is also evident at the level of accumulation of its precursors. The distribution of chlorophyll precursor protochlorophyllide followed a profile similar to that of chlorophyll with a minimal level of protochlorophyllide at the leaf base, and maximal level of protochlorophyllide near the leaf tip. These results indicated that the mature cells near the leaf tip have higher potential to develop photosynthetic pigments than juvenile cells near the leaf base.

The distribution profile of protochlorophyllide, showing increasing level towards the leaf tip, similarly matched with distribution profile of protochlorophyllide oxidoreductase enzyme (POR) responsible for conversion of protochlorophyllide to chlorophyllide. Analysis of relative level of POR using western blot analysis indicated that the dark-grown leaves that predominantly accumulate POR A, possess low level of POR A protein near the leaf base, but show high level of POR A protein near leaf tip. Even in light-grown leaves, which possess another isoform of POR: POR B the level of POR B was maximal in the leaf tip, indicating a close correlation between chlorophyll formation and POR protein level.

The distribution of another protein that binds chlorophyll, light harvesting chlorophyll binding protein (LHCP) also showed a distribution profile similar to POR. In dark-grown leaf LHCP can not be detected by western blot as it is below the level of detectability. Exposure of leaf to light induced the accumulation of LHCP with a distribution profile in the leaf similar to that of chlorophyll accumulation.

Similar to LHCP, the level of cytosolic enzyme phosphoenolpyruvate (PEP) carboxylase is below the detectability limit in the dark-grown leaf. The exposure of dark-grown leaf to light induced accumulation of PEP carboxylase in the leaf, with a distribution profile similar to LHCP and chlorophyll. Evidently, though light induced PEP carboxylase level, its accumulation was determined by cell maturity gradient and also by the presence of chloroplasts. These results also indicated that though PEP carboxylase is a cytosolic enzyme, its photoinduction is coupled to chloroplast differentiation in M cells.

The coupling between plastid maturity and activity profiles of enzymes in peroxisomal compartment was examined by examining the distribution of catalase in the pearl millet leaf. It is known that in the cytosol peroxisomes are closely associated

with chloroplasts, and have exchange of metabolites between them. The profile of catalase distribution in pearl millet leaf also showed a profile similar to that of chlorophyll, LHCP and PEP carboxylase.

The expression of all light induced proteins in the cell was not tightly coupled to chloroplast gradient in pearl millet leaf. The distribution profile of β-amylase, a cytosolic enzyme showed two distinct accumulation profiles in dark-and light-grown leaves. In dark-grown leaves its level increased from leaf base to leaf tip. The exposure of light totally altered this profile, and unlike plastidic protein, the younger cells near the leaf base showed maximal level of p-amylase accumulation in light-grown leaves. Apparently in this case the light effect on P-amylase activity was maximal in younger cells located at the leaf base and it declined towards the leaf tip. A profile similar to that of p-amylase was also observed for another cytosolic enzyme peroxidase. In dark-grown leaves the peroxidase activity increased from leaf base to leaf tip. The exposure of light altered the distribution of peroxidase enzyme, with maximal peroxidase activity being near the leaf base.

Apparently the effect of light in the pearl millet leaf on different enzyme/pigment levels differed with respect to their cellular location and functions. The plastidic enzyme/pigments and associated protein PEP carboxylase and catalase showed a distribution pattern with low or little activity in dark, but with maximal level near the leaf tip. The light stimulated the accumulation of this protein with maximal induction near the leaf tip. By contrast the accumulation profile of cytosolic protein p-amylase and peroxidase showed different profile. In dark-grown leaves the accumulation profile was similar to that of plastidic proteins with increasing level towards the leaf tip. But on exposure to the light maximal induction was observed in younger cells near the leaf base.

In addition to influence of cell position on accumulation of cytosolic protein P-amylase, it was found that the loss of chloroplast also stimulated the activity of cytosolic P-amylase. The pearl millet *zebra* mutant has alternate yellow and green band on leaves respectively. The tissue excised from these yellow and green band shows that compared to green tissue, tissue from yellow band has reduced level of photosynthetic O_2 evolution, reduced level of LHCP and also plastidic α-amylase enzyme. Interestingly the loss of chloroplast in yellow tissue elevated the level of p-

amylase activity by three fold difference over the green tissue. The yellow and green tissue excised from leaves of *yellow stripe* mutant also showed results similar to that of *zebra* mutant indicating that loss of chloroplast stimulates P-amylase activity. It was also found that loss of chloroplast by NF mediated photooxidation in control plants also strongly stimulated P-amylase activity in albino leaves.

In summary pearl millet leaf the magnitude of the light effect on induction of enzyme and pigment is dependent on the cell position cell age gradient and is different for different proteins located in different cellular compartments. For plastidic enzymes/pigments, it follows a pattern with the light mediated accumulation of pigment/enzyme is maximal in older leaf cells near the leaf tip. It is evident from our observations that dark-grown leaves display a distinct distribution profile of protochlorophyll and carotenoid with increasing level towards the leaf tip. Similarly the maximal effect of light on increase in chlorophyll and carotenoid level is also observed in older leaf cell near the leaf tip. Similar to chlorophylls, the light-grown leaves also show increasing levels of POR B, LHCP, PEP-carboxylase proteins towards the leaf tip. Interestingly the peroxisomal enzyme catalase also shows a distribution pattern similar to plastidic enzyme indicating clear coordination between the two compartments. By contrast, the distribution profile of cytosolic enzyme follows different pattern in dark-and light-grown leaves. In dark-grown leaves, the level of both cytosolic enzymes p-amylase and peroxidase showed increases towards leaf tip. However, exposure to the light lowers the levels of these enzymes in older cells near the leaf tip and significantly stimulates the level in younger cells near the leaf base. For cytosolic enzyme P-amylase was also found to be under negative regulation of chloroplast, and loss of chloroplast further induced P-amylase level in cells.

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