Interrelationship Between Cellular Differentiation and Photoregulation of Enzymes in Maize Leaves

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By

Rupali Dutta

Department of Plant Sciences
School of Life Sciences
University of Hyderabad
llyderabad-500 046
INDIA

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Enrollment no, 91LSPH15

Department of Plant Sciences
School of Life Sciences
University Of Hyderabad

Certificate

This is to certify that the thesis entitled **"Interrelationship between cellular differentiation and photoregulation of enzymes in maize leaves"** is based on the results of the work done by Ms **Kupali Datta** 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.

28/8/96

I/c Head
Department of Plant Sciences
HEAD
Deptt. of Plant Sciences
School of Life Sciences

Prof R P Sharma
Supervisor
Prof R P. SHARMA.
HYDERABAD-500 134 INDIA

Prof A R Reddy
Dean
School of Life Sciences
Dean, School of Life Sciences
University of Hyderabad,
Hyderabad-500 134. (India)

Department of Plant Sciences
School of Life Sciences
University Of Hyderabad

Declaration

I hereby declare that the work presented in this dissertation 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:28 August, 1996
Place: Hyderabad

Rupali Datta

Prof R P Sharma
Supervisor

TO MY PARENTS

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Abbreviations

A	= absorbance
ABA	= abscisic acid
BSA	= bovine serum albumin
cop	= constitutive photomorphogenesis
d	= day
DNS	= dinitrosalicylic acid
DW	= distilled water
EDTA	= ethylene diamine tetraacetic acid
FRL	= far-red light
FW	= fresh weight
GA	= gibberellic acid
h	= height
h	= hour
hy	= elongated hypocotyl
kD	= kilo dalton
/	= length
LHCP	= light harvesting chlorophyll a/b binding protein
MES	= 2-[N-Morpholino] ethanesulfonic acid
min	= minutes
MW	= molecular weight
ND	= non-dissociating
NF	= Norflurazon
nkat	= nano katal
PAGE	= polyacrylamide gel electrophoresis
PBS	= phsphate buffer saline
PEPC	= phospho enolpyruvate Carboxylase
Pfr	= far-red light absorbing form of phytochrome
PMSF	= phenyl methyl Sulfonyl fluoride
PVDF	= polyyinylidenedifluoride
Pr	= red light absorbing form of phytochrome
RL	= red light
RUBISCO	= ribulose 1,5-bisphosphate Carboxylase oxygenase
SDS	= sodium dodecyl sulphate
TCA	= trichloro acetic acid
TBST	= tris buffere saline tween 20
TEMED	= N.N.N'.N'-tetramethyl ethylene diamine
TRIS	= (tris[hydroxymethyl]aminomethane)
tri	= temporarily red light insensitive
UV	= ultra violet
v/v	= volume/volume
w	= width
WL	= white light
WT	= wild-type
w/v	= weight/volume

CONTENTS

CHAPTER 1

Introduction

Plant development has several unique features, which have evolved from the basic need of the plants to continually monitor and interpret their ambient environment. The need for vernalization and light for the induction of flowering in many plant species is a clear example of the usage of environmental factors as cues to initiate specific developmental pathways in plants. Plant development is a continuous process. After seed germination, post embryonic development of the plant continues at several meristems located at different parts of the plant body. These meristems continue to maintain a juvenile mass of tissues, which perpetuate themselves, and at the same time, contribute the cells that differentiate into different organs of the plant body. During the post-embryonic development of plants, while the meristem contributes to the formation organs, the growth, differentiation and acquisition of biochemical pathways in many organs depend on environmental signals.

During development, plants precisely control the patterns of cell size, shape and division, position of differentiation of cell types and position and number of organs. During the recent years, progress has been made in understanding four of these five levels of control of pattern formation. These are, the specialization of cell fate by cell size in the alga *Volvox,* genetic control of cell shape in leaf hairs of flowering plants like *Arabidopsis,* precise control of cell division and enlargement in *Arabidopsis* root meristem, and control of cell number in the meristems of tobacco, maize and *Arabidopsis.* These examples indicate that both the fate and the shape of differentiated plant cells can be determined by their undifferentiated precursor, and the mutations that alter the cell fate and cell shape can affect cell size. Further, specific genes have been found that are necessary for normal pattern of cell division.

The development of both monocot and dicot leaves provide excellent examples for such environment-regulated development. In both monocots and dicots, light is required to complete leaf development, without which the leaf cannot acquire photosynthetic capability and the proplastids cannot differentiate into chloroplasts. The exposure of leaves to light triggers several morphogenetic and biochemical processes, the most prominent of which is the process of Chloroplast development. Chloroplast

biogenesis includes several processes such as the formation of chlorophyll, synthesis of plastidic proteins, and the differentiation of the internal structure of chloroplasts, along with the associated metabolic pathways. However, the responsiveness of the different cells of the leaf to light-induced Chloroplast biogenesis is in turn strictly determined by the cellular position and the developmental age of the leaf. For example, the epidermal cells of the leaf do not develop chloroplasts, but guard cells of the same layer can initiate Chloroplast biogenesis. In the leaves of many species, light induces the formation of the water soluble pigment anthocyanin, which is exclusively located in the epidermal cells. 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. The underlying mechanism for cell and tissue specific differentiation and its interaction with photoregulation of gene expression is only partially known and is currently being investigated using the techniques of molecular-genetic analysis.

The monocot leaf offers an excellent model for the study of influence of positional information on gene expression by virtue of the leaf development taking place from a basal meristem. The older cells are situated at the tip of the leaf, and younger ones are at the base, forming a continuous gradient of maturing cells from the base to the tip of the leaf. This basipetal gradient of cell maturation in the monocot leaf has been utilized to study the development of plastids, photosynthetic development and morphological development of cell types (Leech, 1985). Further, as light has a major influence on Chloroplast biogenesis, which also shows a gradient of development by the presence of proplastids at the leaf base and progressively more matured chloroplasts at the tip, the interrelationship between Chloroplast biogenesis and the expression of enzymes related to the primary function of chloroplasts such as photosynthesis and carbon fixation can be observed in these leaves.

In the present study, the maize leaf was used as a model to investigate the influence of positional information on the expression of several enzymes having important metabolic roles. The interrelationships between the gradient of cell maturation, and the photoregulation of some chloroplastic and cytosolic enzymes such as amylases, nitrate reductase and nitrite reductase was studied. Attempt was made to decipher the

correlation between cell position, cell maturation gradient, Chloroplast differentiation and photoregulation of the enzymes mentioned above. The dependence of the cell maturation gradient on Chloroplast biogenesis was also analyzed after photooxidation of chloroplasts in developing leaves using the herbicide Norflurazon. The intra- and inter-cellular distribution and the effect of stress on amylases were also studied. The coaction of light and nitrate on nitrate reductase and nitrite reductase activities was also studied in detail.

CHAPTER 2

Review of Literature

During the course of evolution, plants have evolved many ways of responding to the environment, and have incorporated several environmental signals as modulators and regulators of many developmental pathways. Light is one such environmental signal, which is used by plants not only for photosynthesis, but also to trigger several developmental pathways. Plants perceive light throughout the visible spectrum using at least 3 photoreceptors, absorbing specifically in three spectral regions, which are UV-B (280-320 nm), blue/UV-A (320-500 nm) and red/near red region (600-750 nm). These photoreceptors sense the quality, quantity, direction and duration of light and suitably modulate the growth and development of plants.

The manifestation of a normal developmental program at all stages in plant life cycle uses signals from the light environment in one or other form. In the absence of light, plants follow a developmental pathway characteristic of scotomorphogenesis or dark-induced development. Higher plants, grown in continuous darkness, are incapable of photosynthesis as Chloroplast development is obligatorily associated with the presence of light. In addition, dark-grown plants exhibit a distinct morphology which is most prominently observed in angiosperm seedlings germinating in darkness. Exposure to light triggers dramatic changes in dark-grown seedlings, initiating a series of biochemical and morphological changes such as Chloroplast biogenesis and leaf expansion. Among the several morphogenetic processes regulated by light, leaf development is one of the most prominent. Several facets of leaf development are influenced by light. For example, unrolling of leaves in monocots and expansion of cotyledons and leaves in dicots during de-etiolation are well known light-regulated processes. Light also influences several biochemical pathways necessary for leaf development by controlling the expression of several genes.

2.1 LEAF DEVELOPMENT

Leaf is one of the most important organs of angiosperms, and due to its paramount function in the process of photosynthesis, it is also one of the most well characterized organs. Over the past century, leaf development has been examined first at the anatomical level and then at the biochemical level. At present leaf development is being intensively investigated using molecular-genetic techniques.

During plant development, shoot apical meristems generally progress through the following stages: 1) vegetative apical meristems, 2) inflorescence meristems and 3) floral meristems. Among these, leaves originate by the activity of cells derived from the shoot apical meristem. The vegetative apical meristems perpetuate themselves by continually cutting new cells and forming a mass of tissues. At some distance below the apical meristem, the leaf primordia originate and differentiate into the leaf. The signal that triggers the initiation of leaf primordia on vegetative apical meristem is still unknown. The precise setting of leaf primordia on shoot apex has initiated several hypothetical explanations of the pattern formation which has mathematical connotation in some species. The hypotheses offered for the leaf positioning includes an inhibitor concept and a competition for available space on the shoot apex concept (reviewed by Held 1990).

Compared to leaf, the initiation and differentiation of inflorescence meristems and floral meristems have been investigated in greater detail. The genes determining the initiation and perpetuation of floral and inflorescence meristems have been cloned from various species such as *Arabidopsis thaliana* (Ma, 1994), *Antirrhinum majus* (Coen and Carpenter 1993), *Petunia hybrida* (Van der Krol and Chua, 1993), *Zea mays* (Veit et al., 1993) etc. These genes code for homeodomain proteins containing a conserved motif, the MADS box, which act as transcription factors (Okada and Shimura, 1994).

2.1.1 Leaf development: Genetic regulation

Unlike the floral meristem, information on genes controlling leaf initiation and development is limited. However, molecular-genetic analyses have identified a few genes regulating leaf development. Most of the genes which regulate leaf development have been analyzed in maize, as maize has a rich collection of morphological mutants. One of the well-studied genes is the *liguleless* gene in maize. A *liguleless-1* (*lg1*) mutant mosaic

leaf was developed, by inducing chromosomal breakage using X-rays to create plants genetically mosaic for *lg1*. The ligulate region of the maize leaf occurs at the junction of the leaf blade and sheath and represent the boundary region between these two developmentally distinct components of the leaf. The boundary region consists of a fringe tissue, called ligule, and two wedges of pale green tissue called auricles. Development of this region requires precise coordination of pattern forming events in different tissues and across the breadth of the leaf (Becraft et al., 1990), requiring cell-cell communication. The recessive *lg1* mutants lack ligule and auricles. Thus the wild type allele is involved in ligule and auricle development and in defining the blade-sheath boundary.

In *lg1* genetically mosaic plant, the sectors of mutant tissue have all the attributes of *lg1* mutant leaves (Becraft et al 1990). When such mutant sectors pass through the ligular region of a normal leaf, the ligule and auricles do not align on either sides of the mutant region. The ligule on the side towards the margin is displaced in a basipetal direction. This indicates that the sector of mutant tissue is interfering with the intercellular communication that normally results in a continuous ligule throughout the leaf-sheath boundary. It is believed that a signal that organizes the development of the ligular region emanates near the midrib and is transmitted towards the margin. Probably this signal is received by a cell, which then produces and passes the signal to the next cell. Sectors of *lg1* mutant tissue interrupt the transmission of this signal, resulting in delayed ligule initiation in the marginal tissue. The *LG1* gene is probably involved in the reception and transmission of this signal (Becraft and Freeling, 1991).

Mutants in atleast four genes, *Tp1*, *Tp2*, *Cg* and *Hsf1-0* in maize alter the normal overlap of the stages of shoot development. These are called the shoot stage transition mutants in which the segments higher upon the plant behave like segments more basally located, as one or more components of juvenility are prolonged during development. For example in the *tpl* mutant, vegetative leaves grow out of the axes of male inflorescence. *Tpl* encodes a soluble diffusible product which is not cell-autonomous (Poethig, 1988).

Just like the shoot, the maize leaves show morphologically distinguishable regions in the base-to-tip dimension. Mutations in atleast seven genes are known, where base-to-tip developmental boundaries are altered. In these mutants, the more basal region

called sheath occupies regions of more distal organ part called blade. Out of these, the *KNOTTED-1 (KN-J)* locus has been studied in detail. In normal plants, *KN-1* gene is expressed only in vegetative apical meristems, but in the mutant, ectopic expression of this gene disrupts normal leaf development. The gene also has a PEST motif, which acts as a signal for rapid degradation, suggesting tight regulation. Thus *KN-1* gene is similar to many animal homeobox containing genes, which regulate processes determining cell fate. The AJV-7 gene codes for a homeobox domain-containing protein which binds DNA (Vollbrecht et al 1991). In the *kn-1* mutant, cells along the lateral veins do not differentiate properly but continue to divide forming out pocketings or knots. The ligule is displaced, and is perpendicular to its normal position. The knots resulting from the cell divisions are hollow protrusions from the plane of the leaf and involve cell divisions in all layers of the leaf. It was observed that the genotype of the mesophyll, and not that of the epidermis, was important for the knotted phenotype, and the mesophyll induces the epidermis to divide (Hake and Freeling, 1986).

Rough sheath is a dominant mutation which alters cell fate and causes interrupted cell division and expansion in maize leaf (Becraft and Freeling, 1994). The cloned gene of *RS1* has similar sequence to *KN1* in the homeodomain, but an unique N-terminal region. In the shoot apical meristem, *RS1* expresses in a circular fashion preceeding leaf initiation and it is not detectable in the leaf primordium of mature plants. Therefore *RS1* expression may determine the site where the leaf would form on the meristem, i.e., determine the pattern of lateral organ formation on shoot apex. In the mutant, *rs1* is expressed in early leaf promordia, and this ectopic expression causes the mutant phenotype (Schnerberger et al., 1995).

The *hairy sheath frayed 1-0* (*Hsf 1-0*) mutant of maize, has prolonged sheath-leaf identity in the marginal regions of the leaf blade, leading to "prongs" of sheath transforming what should have been the blade. *Hsf 1-0* homozygotes display a three shoot phenotype, rarely grows more than 3 inches tall and do not flower (Bertrand-Garcia and Freeling, 1991).

Using the *KN-1* homeobox as a probe, similar genes *KNAT-1* and *KNAT-2* were pulled out from *Arabidopsis.* The KNAT-1 protein is localized in the shoot apical meristem and is down-regulated when leaf primordia are initiated. The over expression

of this gene in *Arabidopsis* causes highly abnormal morphology of leaf, including severely lobed leaves (Lincoln et al., 1994). A smilar phenotype was also observed in plants overexpressing maize *KN-1* gene.

Similar to *KNAT-1* gene, *ATK-1* gene was cloned from *Arabidopsis* using maize probe. The *ATK-1* gene is strongly expressed in the shoot apex of seedlings, while in mature plants it expresses in flower and inflorescence stem (Docky et al., 1995).

A recessive mutation *lam-1* blocks leaf blade formation in tobacco (McHale, 1993). The mutants grow to full length, but never generate leaf blade. In the leaf primordium, the blade initiation site is established at the normal time and position in the mutant. Anticlinal divisions proceed normally in the L1 and L2 layers, but the inner L3 cells fail to establish periclinal divisions, that generate the middle mesophyll core. Another mutation *fat* in tobacco results in abnormal planes of cell division in all cell layers during blade initiation and expansion, resulting in a leaf base twice the normal thickness, due to extra cell layers. *FAT* gene probably acts as a negative regulator maintaining the number of cell layers in the expanding leaf blade (McHale, 1993).

Though leaf development is a process distinct from the development of organelles, a small percentage of Chloroplast mutants shows alteration in leaf development, suggesting the existence of nuclear genes required for both Chloroplast and leaf differentiation (Reiter et al 1994). Leaf plastids of the *Arabidopsis pale cress* (*pac*) mutants do not develop further from the initial stage of differentiation, i.e., proplastids or etioplasts and contain only low levels of chlorophylls and carotenoids. Early in the development the epidermal and mesophyll cells of *pac* leaves are normal, but in later stages, the mutant leaves have enlarged inter-cellular spaces and the palisade layer of the mesophyll can no longer be distinguished. The *PAC* gene encodes a novel protein whose expression is light regulated (Reiter, 1994).

ATHB-1 gene of *Arabidopsis* was cloned by sequence homology of the homeobox region, but is of unknown function. It is presumed to code for a transcription factor. The over expression of *A THB-1* in transgenic tobacco confers de-etiolated phenotype in dark, with expanded cotyledons, true leaf development, and inhibition of hypocotyl elongation (Ayoyama et al., 1995). The other organs of the mutant remains unaffected. The results obtained indicate that Athb-1 is a transcription factor involved in leaf development.

Rev-1 mutants of *Arabidopsis* show overgrowth of leaves. The rosette leaves are abnormally large and distorted and the cauline leaves are longer and narrower with rolled under margins, curving downward along their longitudinal axis. These mutants also have defective apical meristem activity resulting in the premature termination of the shoot apex and the 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 the stem and also for the normal growth of apical meristems (Talbert et al 1995).

The genetic analysis of *augustifolia* and *rotundifolia* mutants of *Arabidopsis* demonstrated that these two genes control two different processes of leaf expansion. In the *augustifolia* mutant, cell are smaller in the leaf width direction and larger in the leaf thickness direction, leading to narrower and thicker leaves, though the number of cells in the leaf remain the same as that of the wild type. In *rotundifolia* mutant also, the same number of cells as the wild type is maintained, but the cells have reduced elongation in the leaf length direction. These two genes act independently, playing important polarity specific roles in cell elongation in leaf (Tsuge et al., 1996).

It is assumed that, during leaf development, the relative position of the cells is fixed by cell walls, which in turn is determined by the orientation of the cell division leading to a particular shape of the organ. That this assumption may not be true is shown by the *tangled-1* mutation of maize, which causes cells to divide in abnormal orientations throughout leaf development, leading to alteration of leaf shape (Smith et al., 1996). It is therefore evident that leaf shape is determined by a mechanism independent of the orientation of cell division.

In angiosperms, leaves are classified as simple leaf and compound leaf. In tomato, a range of dominant and recessive mutants are available, which turns the leaf from super compound to simple leaf. In transgenetic tomato, where the expression of *KN-1* gene was induced, the leaves turned to be super compound and the plants were dwarf and bushy. However, the effect of *KN-1* was restricted to the compound leaves, but not in the simple leaf mutant (Hareven et al., 1996). Since the ectopic expression of *KN-1* in the simple leaf of *Arabidopsis,* tobacco and maize are different than the compound leaf of tomato, it is evident that the simple and compound leaves develop via two different developmental programs. (Hareven et al., 1996).

Table 2.1 Leaf development mutants

Mutant	Phenotype	Proposed function of the gene product	Cloning method	Effect on transgenic plants
liguleless-1 (maize)	lacks ligule and auricles, blade-sheath boundary not well defined	reception & transmission of signal for development of ligule from midrib	------	------
Knotted-1 (maize)	cells along lateral veins continue to divide forming out pocketings or knots	homeodomain containing protein, binds to DNA and acts as a transcription factor	transposon tagging	constitutive expression in tobacco lead to shoot formation on leaf surface
Tp-1 (maize)	vegetative leaves grow out of unbranched male inflorescence axes	soluble protein, codes for diffusible regulator of vegetative maturation	—	------
Hsf1-0 (maize)	more juvenile stages of development found in mature regions of shoot, root, flowers.	—	—	------
bsd1-m1 (maize)	uncouples the tighly coordinated differentiation of bundle sheath & mesophyll cells	-----	------	------
lam-1 (Nicotiana sylvestris)	leaf remains bladeless	controls a common step in blade tissue initiation from L2 & L3 lineage of primordium	-----	------
fat (Nicotiana sylvestris)	abnormally thick leaf blade due to double the number of mesophyll cell layers	negetive regulator protein, repressing periclinal divisions in the blade	—	------
pale cress (pec) *Arabidopsis*	plastids do not mature, leaves have large intercellular spaces and no palisade	may encode multiple proteins which modulate leaf shape & Chloroplast development	T-DNA tagging	------
pfl Arabidopsis	pointed first leaves, reduced leaf fresh weight & retarded growth	codes for ribosomal protein S18 and is expressed int he meristems	T-DNA tagging	-----
rev-1 Arabidopsis	large leaves with rolled under margins, curving downward along the longitudinal axis	limits the growth of meristems of leaves, floral organs and stems	—	-----

In maize, six mutations that specifically disrupt bundle sheath cell differentiation have been identified. One of these, the *bundle sheath defective 1-mutable J* (*bsd1-m1*) mutation uncouples the tightly coordinated differentiation of bundle sheath and mesophyll cells (Langdale and Kidner, 1994). In these mutants, the mesophyll cells develop normally, but the bundle sheath development is disrupted, affecting the bundle sheath Chloroplast development, and the bundle sheath cell-specific C4 gene products fail to accumulate. In the second mutant *bsd2-m]*, bundle sheath chloroplasts exhibit aberrant morphology, but mesophyll chloroplasts are normal. Though the bundle sheath-specific C4 enzyme levels are reduced, the transcripts accumulate normally. In addition, the chloroplast-encoded *rbcL* transcripts accumulate ectopically in mesophyll cells, indicating that the gene plays a direct role in the post-transcriptional control of *rbcL* transcript accumulation and/or translation both in bundle sheath and mesophyll cells (Roth et al., 1996).

2.1.2 Leaf development: hormonal and light regulation.

While the initiation and differentiation of leaf shapes is genetically determined, its expansion and biochemical differentiation is regulated by light and hormones in many species. For example, in many dicots, the application of cytokinin initiates expansion of leaf (Scott and Liverman 1987). In monocots, leaf unrolling is accompanied by increase in gibberellic acid level (Virgin 1989). Application of cytokinins can promote leaf unfolding and expansion (Leech 1985). Young leaves also contain high levels of auxin (Allen and Baker 1980). Leaf growth is also altered in GA deficient mutants of tomato (Koornneef et al., 1980) and pea (Ross et al., 1993). Ethylene inhibits leaf expansion by inhibiting cell division (Scott and Possingham 1982).

Light is the most prominent environmental signal which strongly influences leaf development. In dicots, exposure of dark-grown seedlings to light results in leaf/cotyledon expansion, and hypocotyl hook opening and in monocot it leads to leaf unrolling (Shropshire and Mohr, 1983). Light influences cell differentiation in leaves, leading to Chloroplast biogenesis and induction of a number of enzymes (Kendrick and Kronenberg 1994). The developing leaf appears to possess all three major groups of photoreceptors and is sensitive to UV-B, blue and red light. These photoreceptors, independently and collectively, regulate leaf development. Among them, the role of

phytochrome in leaf development is most studied. **Photosignal** perception **by phytochrome activates** signaling pathways leading to changes in gene expression **that underlie the** physiological and developmental responses to light. The transduction process by which phytochrome relays sensory information in the cell is not known, but the studies carried out in recent years provide insight into the possible mechanisms.

Phytochrome genes encode a small family of photoreceptors. In *Arabidopsis*, phytochrome is encoded by five genes, *PHYA*,-*B*, -*C*,-*D* and -*E* (Sharrock and Quail, 1989). Biochemically, these phytochromes can be classified into two types of phytochromes: type I, which accumulates to high levels only in dark-grown plants, and is rapidly degraded on light exposure, and type II which is present in both light- and dark-grown plants and is less susceptible to light-induced down-regulation, and consists of phytochrome species other than phytochrome A (Whitelam et al 1993, Reed et al 1993). Plants lacking functional phytochrome A are not responsive to far red light-mediated inhibition of hypocotyl elongation, but have normal responses to red or white light-mediated hypocotyl elongation (Nagatani et al., 1993). The opposite phenotypes defective in red light response, but having normal far red light-mediated inhibition of hypocotyl elongation was observed in plants lacking functional phytochrome B (Reed et al., 1993). Thus phytochrome A and B play different roles in mediating plant responses to light signals, the specific roles of other phytochromes are not known.

The molecular mechanism by which phytochrome regulates leaf development is not known. However, recent studies reveal that phytochrome-mediated biochemical responses which may also include leaf expansion results from the down regulation of certain nuclear proteins. The photoactivated phytochrome molecules themselves do not translocate to the nucleus to bind to target gene promoters, but other signaling intermediates relay the information from the photoreceptor to the genome. In *Arabidopsis* two classes of mutants defective in components downstream of phytochrome have been identified. One group of mutants including *cop*, *det* and *fus* exhibit light-grown characters in complete darkness. The other class of mutants including *hy5*, *fhy1* and *fhy3* exhibit normal seedling development in darkness, but has reduced sensitivity to light. As the *cop*, *det* and *fus* mutations are recessive and their effects are pleiotropic, it is believed that the wild type gene products act negatively, early in the signaling pathway to repress photomorphogenesis in darkness, and this repression is

reversible by light (McNellis et al 1994). The *cop* and *del* mutants are believed to be at or just downstream of the convergence of phytochrome and blue light photoreceptor signaling pathways. The *COP* and *DET* genes have been cloned, and their gene products have been localized in the nucleus of dark-grown plants, but in light-grown plants, they are localized in the cytosol. These gene products probably act as suppressers of genes that are involved in light-induced leaf development.

The *hy5* mutant of Arabidopsis has impaired responsiveness to multiple wavelengths of light. Thus the *hy5* gene product may be necessary for an activity at or downstream of the convergence of phytochrome and blue light receptor transduction pathways. However, *fhy1* and *fhy2* mutants have impaired response only to far-red light, so they may lie upstream in the transduction pathway specific to phytochrome A.

Analysis of photomorphogenic mutants such as *blu3* and *hy4* of *Arabidopsis* showed that the cotyledons of these mutants did not grow like those of the wild type. However, on excision, both mutant and WT cotyledons expanded normally under blue light (Blum et al., 1994). These results have been interpreted as an evidence that in many cases, the effect of blue light is indirect, and is due to whole-plant response to light. In contrast to these mutants, *hy1*, *hy2*, *hy3* and *hy6* mutants *of Arabidopsis* showed reduced cotyledon expansion both in attached and detached conditions. These studies also demonstrated that cotyledon expansion of *Arabidopsis* seedlings under red light is mediated by phytochrome (Neff and Volkenburgh, 1994).

A strong evidence for the involvement of brassinosteroids in leaf development has been provided by the molecular-genetic analysis of the *det2* mutant. While the *det J* protein is localized in the nucleus, the sequence of *det2* protein showed a strong homology to steriod 5 α-reductase enzyme (Li et al., 1996). Brassinosteroids may constitute a distinct class of phytohormones with an important role in light-regulated development of higher plants.

2.1.3 Leaf development: Positional effects

The monocot leaf is 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). Cells at the tip are photosynthetically most

developed in a young leaf, whereas leaf segments at more basal location contain cells at progressively younger stages of differentiation (Nelson and Langdale, 1992). Many workers have taken advantage of this gradient of cell maturation in monocot leaves for studies on plastid development, photosynthetic development and morphological development of cell types. The 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 gradient of cell age upwards from the base of the graminaceous leaf has also been exploited in studies of plastid development and the role of the Chloroplast and nuclear genomes in the assembly of the photosynthetic apparatus (Viro and Kloppstech 1980; Martineau and Taylor 1985; Dean and Leach 1982 a,b). There is a reciprocal relationship between the capacity for growth and differentiation and acquisition of photosynthetic competence in the young basal tissue of expanding grass leaves (Ougham *et al* 1987). This inverse correlation is apparent in the contrasting gradients of α-tubulin (a growth related polypeptide) and LHCP-2 (a thylakoid membrane protein), along the age gradient in an expanding *Lolium temulentum* leaf tissue. There may be a common regulatory mechanism responsible for turning off growth processes and activating Chloroplast assembly (Davies, 1989) In 0.5 cm sections from the base to the tip of the *Lolium* leaves, the levels of chlorophyll and carotenoids increased with increasing distance from the base, reaching their maxima between segment 8 and 13. Appreciable amounts of chlorophyll *a* was present within 1.5 cm of the point of leaf insertion 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. Since the production of photosynthetic pigments is light dependent, this early onset of chlorophyll biosynthesis shows that there must be a substantial degree of light funneling down the leaf sheath. The gradient of trapped light extending down into the enclosed growing zone of the elongating leaf appears to be a major morphogenic influence (Vogelmann and Bjorn 1986).

In wheat leaves, a homologue of a cell cycle control protein $p34^{cdc2}$ controlling cell division showed maximum expression at the base of the leaf. The level of this protein declined gradually towards the leaf tip (John et al., 1990). In light-grown pearl millet leaves the level of the cytosolic isoform of phosphorylase is highest at the base of

the leaf, declining towards the leaf tip. In contrast, the level of the chloroplastic isoform increases from a very **low** amount at the base to reach maximal level at **the** leaf tip (Vally **et al.,** 1995).

In maize, the simple pattern of leaf development is more complicated due to chloroplastic and cellular dimorphism. Maize leaf possesses two major photosynthetic cell types; bundle sheath cells containing agranal plastids, and mesophyll cells with Chloroplast exhibiting extensive thylakoid membrane stacking (Martineau and Taylor 1985). Developmental age gradients have been exploited to characterize the appearance of C4 activities (Perchorowitz and Gibbs, 1980), proteins (Martineau and Taylor, 1985) and mRNAs (Martineau and Taylor, 1985). These studies reveal that while the levels of C4 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 C4 protein accumulation is always associated with a mature vein and bundle sheath anatomy, even in relatively young (basal) regions of a developing maize leaf (Nelson and Langdale, 1992).

In C4 plants like maize, gene expression depends on positional signals. Both light and cell position relative to veins influence C4 photosynthetic gene expression. Light induces a switch from C3 type to C4 type of gene expression pattern in etiolated tissue. However, cell position is more important than light induction in the regulation of C4 photosynthesis. Cells that are distant from veins do not accumulate C4 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 C3 type of gene expression patterns in cells more distant from a vein.

A positional effect on the expression of enzyme pattern in monocot leaf was obtained for the regulation of phosphorylase in pearl millet. Pearl millet leaf has two isozymes of phosphorylase, one localized in the cytosol, and the other in the Chloroplast. The plastidic isozymes is present ibnthe bundle sheath cells, whereas, the cytosolic isozymes is present int eh mesophyll cells. In etiolated leaves, both the isozymes are present in all segments of the leaf, with activity increasing towards the leaf tip. In light-grown leaf, the distribution pattern was altered. While light stumulated 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 shows that **cell position** in the monocot leaf plays an important role in determining the expression pattern of light-regulated enzymes. Similarly, the photostimulation of β-amylase activity is higher near the leaf base, and light alters the pattern of distribution in these leaves (Vally and Sharma, 1991).

Ribulose 1,5 bis-phosphate carboxylase-oxygenase (RUBISCO) is developmentally programmed to accumulate in all photosynthetic cell types. In *CA* plants, light is required to suppress the expression of RUBISCO protein in mesophyll cells and induce the expression of other photosynthetic genes (Langdale, 1988). In C4 plants, while PEP Carboxylase (PEPCase), malate dehydrogenase, and pyruvate phosphate dikinase act in the mesophyll cells, RUBISCO and maleic enzyme act in the bundle sheath cells. Thus the expression of the above genes is light dependent and cell specific (Langdale et al., 1987). In contrast to the expression of chloroplastic genes, the gradient of mitochondrial gene expression in wheat leaf shows that these genes are expressed maximally at the leaf base, and decrease in expression gradually towards the leaf tip (Topping and Leaver, 1990).

To define the cellular processes that control morphogenesis, it is important to have detailed information regarding the number of cells and their fate in a primordium and an accurate picture of cell division, differentiation and expansion in the primordium at different stages of its development. Clonal analysis identifies cell lineages and determines the distribution, rate and orientation of cell division within an organ (Poethig, 1987). Clonal analysis data is available in maize (Johri and Coe 1983), and also in wheat, barley, *Arabidopsis,* rice and sorghum (Balkema, 1971). Clonal data from both monocots and dicots indicate that leaves develop from a large number (100-250) of initial cells without the involvement of localized meristems (Poethig, 1984). Plant cell lineage does not limit cell fate, and plant cells retain the capacity to switch from one fate to another even after cell division has ceased. As a result, higher plant cells acquire their identity entirely by interpreting their position in the organism (Poethig, 1989).

2.2 AMYLASES

Amylases are starch hydrolyzing enzymes which hydrolyze (1→4) a-D linked glucosidic bonds by the transfer of a glucosyl residue to water. Amylases have been

classified into α-amylase and β-amylase; a- and (3- specifying the stereochemical configuration of the products (Kuhn, 1925; Thoma and Koshland 1960). α-Amylase is an endo-enzyme and can attack any internal (1→4) α-D linked glucosidic bonds; whereas β-amylase is an exo-enzyme and acts on alternate α-(1→4) linkages of starch chains starting from the non-reducing end.

2.2.1 a-Amylase

a-Amylase (E.C.3.2.1.1) is a very widely distributed enzyme and occurs in all groups of living organisms. In higher plants, a-amylase is predominantly present in tissues which are involved in the active metabolism of starch, like germinating seeds and young leaves (Gates and Simpson 1968). Compared to leaf, the seed a-amylase has been more extensively studied, especially in cereals. a-Amylase of cereals like barley, rice, wheat etc consists of several isoforms, which have been classified into two groups, low pI and high pI isozymes, based on their isoelectric points. In barley seeds, the isoelectric points of high pI amylases range from pH 5.7 to 6.2, and in wheat fron pH 6.3 to 7.5. The low pI isozymes have isoelectric points ranging from pH 4.4 to 5.2 in barley and from pH 4.9 to 6 in wheat. In barley, the genes encoding high pI amylases are located on chromosome 6 and those encoding low pI, on chromosome 1 (Muthukrishnan et al 1984). In wheat, the amylase isozymes have been divided into 3 groups, the genes encoding group 1 amylases being located on chromosome 6, group 2 on chromosome 7 and group 3, a class expressed only in immature grains, on chromosome 5 (Lazarus et al, 1985; Baulcombe et al 1987). In comparison to the high pI group, isozymes of the low pI group are more stable at low pH, more sensitive to sulfhydryl reagents and heavy metal ions and also less sensitive to chelating agents. The low pI isozymes have lower pH optima and lesser requirement for calcium ions. The two groups differ immunologically and in proteolytic fingerprints.

In comparison, the information available on leaf a-amylase is very meagre. In contrast to cereal seeds, where α-amylases are secreted from the scutellum and the aleurone layer, leaf a-amylases are non-secretory in nature. Moreover, the a-amylases of leaf are likely to be localized in the chloroplasts, where its substrate starch is stored. However, the localization studies on leaf amylases have shown that a-amylases in leaf are located predominantly in the extra-plastidic fraction. The major pea leaf a-amylase is

extra-chloroplastic, however, at low levels it is detectable in isolated chloroplasts. <x-Amylase has also been localized to the apoplast of pea stems. Beers and Duke (1990) reported the amylase from pea cotyledon and shoot to be identical. Pea shoot a-amylase is a secreted enzyme present in the apoplast. Chao and Scandalios (1972) found that two genetically identical amylases were present in all organs (endosperm, scutellum, shoot, root) of maize seedlings. Jacobsen et al., (1986) reported an extra-chloroplastic barley leaf amylase corresponding to a low pI group aleurone a-amylase. The synthesis of this isozyme was induced by water stress in the leaf tissue.

While the principal function of amylase in leaf is to mobilize transitory starch generated during photosynthesis, evidences regarding the presence of chloroplastic a-amylase are very limited, and have been observed in only a few species. α-Amylase has been localized in chloroplasts of spinach (Okita and Preiss, 1980). In sugar beet leaves, five endo-amylases were detected, of which four a-amylase isozymes (a-2,-3,-4, and -5) were localized in the Chloroplast and α-amylase-1 in the cytosol. a-Amylase was localized in chloroplasts of sugar cane (Bourne et al 1970). Ziegler and Beck (1986) found a-amylase of wheat, pea and spinach are located in chloroplasts.

The major difficulty in assigning a definite chloroplastic location to a-amylase is the cross-contamination of cytosolic a-amylase in cell fractionation studies. An elegant evidence for the localisation of a plastid-specific a-amylase was provided by Vally and Sharma (1995) in pearl millet. The aquisition of photosynthetic capacity in pearl millet leaves was accompanied by the appearance of a new a-amylase isozyme in light-grown leaves. The photooxidation of chloroplsts led to the disapperance of the plastidic a-amylase demonstrating it to be localized in the chloroplasts.

2.2.1.1 a-Amylase Regulation

The seed a-amylase genes are mainly under two modes of regulation, hormonal and metabolic. While the regulation of seed α-amylases has been extensively studied, information available on the regulation of leaf a-amylase is limited. Leaf a-amylases have been reported to be regulated by hormones, light, stress and positional effects. In detached cotyledons of pea, incubation with auxin led to the stimulation of a-amylase activity (Hirasawa, 1989). In maize, while gibberellin caused an increase in the activity

of a-amylase in germinating seeds, it had no effect on leaf a-amylase. On the other hand, leaf a-amylase was controlled by light. In excised cotyledons of chick pea, light acting via phytochrome-induced a-amylase activity (Casado et al 1991). Similarly in pearl millet leaves, light induces a *de novo* synthesis of Chloroplast specific a-amylase (Vally and Sharma, 1995).

In barley leaf a-amylase activity increased with decreasing leaf water potential and increasing ABA. This stress-induced a-amylase was extra-chloroplastic in nature. Increase in a-amylase activity was found to involve synthesis of the enzyme (Jacobsen et al 1986). Defense related proteins are found in plants developing a resistant reaction against pathogen attack. Such proteins which accumulate in large amounts under pathological conditions are named PRs (pathogenesis-related). Tobacco has two a-amylases which are apoplastic, and are strongly induced by tobacco mosaic virus infection. The tobacco α-amylases show several PR specific characteristics, such as acidic isoelectric points, apoplastic localization resistance to protease action, and similar kinetics of induction during hyper sensitive reaction (Heitz et al., 1991). Like PRs, a-amylase can be induced by water stress (Jacobsen et al 1986), by plant hormones (Hirasawa 1989; Jacobsen and Higgins 1982) and by senescence (Saeed and Duke 1990b). Apoplastic α-Amylase is also induced several folds by treatment with norflurazon (Saeed and Duke, 1990a) a-Amylase might be involved in the degradation of substrates released by necrotizing cells, the hydrolytic products being then available to the neighboring cells, which develop defense reaction (Heitz et al 1991).

2.2.2 P-AMYLASE

P-Amylase (E.C.3.2.1.2) is a 1,4 a-D glucan-maltohydrolase. It is present in a few species of higher plants and microbes (Thoma et al 1971). P-Amylase is predominantly localized in the extra-chloroplastic fraction of leaves. Furthermore, in these species, almost all of the β-amylase activity is localized in the extra-plastidic fraction. In broad bean leaves, the enzyme is localized in the cytosolic fraction of the epidermal cell layer (Chapman et al 1972). In pea and mustard, the enzyme is cytosolic in localization (Levi and Preiss 1978, Manga and Sharma 1990). This phenomenon has also been observed in wheat, barley and *Chenopodium rubrum* (Lin et al 1988).

The compartmentalization of P-amylase in chloroplasts has been observed in **the** leaves of *Stellaria* and spinach (Bourne et al., 1970, Haapala 1969, Okita et al., 1979, Pongratz and Beck 1978). In sugarcane, P-amylase has been localized in the chloroplasts (Echeverria and Boyer 1986). β-Amylase has also been localized in the chloroplasts of *Arabidopsis* (Lin et al., 1988) and pea (Kakefuda et al., 1986). p-Amylase has beep localized in vacuoles of pea, wheat (Ziegler and Beck 1986) and *Arabidopsis* **(Monroe** and Preiss 1990). In case of both a- and p-amylase, there is no report showing **the** presence of a signal sequence at the N-terminus directing their translocation to chloroplasts. Therefore, the reports on the plastidic localization of p-amylase are suspect and could be artifactual.

A tissue specific localization of P-amylase has been reported in *Streptanthus tortuosus* (Brassicaceae), where high level of p-amylase was found in the phloem cells of vegetative organs of *Arabidopsis thaliana* by immunofluorescence microscopy. Monoclonal antibodies against *S. tortuosus* β-amylase showed similarity with the major form of P-amylase in *A. thaliana* (Wang et al., 1995).

In seeds, p-amylase has been extensively characterized. Five isoforms of P-amylase in barley were identified (Shewry et al., 1988). P-Amylase in barley is synthesized in the developing endosperm, where it is stored till germination, in contrast to a-amylase which is synthesized *de novo* in germinating aleurone and embryo (Briggs 1973). However, in rice seeds, p-amylase is synthesized *de novo* in the scutellum during germination, and in the later stages, the inactive form stored in the starch granules in the endosperm, is activated. In later stages of germination, this activated form becomes dominant (Okamoto and Akazawa, 1980). In maize, a single isozyme of p-amylase was synthesized *de novo* and secreted into the medium (Subba Rao, 1992).

A single locus on chromosome 5 in rye encodes mature grain P-amylase (form II) while P-amylase located in shoots, roots and pericarp of developing seeds (form I) are controlled by another locus on chromosome 2 (Sharp et al., 1985; Daussant et al., 1991). In maize, almost no p-amylase is found in the mature caryopsis, but the activity increases again and reaches much higher values during seedling development. Both the aleurone layer and the scutellum produce and secrete P-amylase during germination, **the** secretion

being stimulated by calcium (Subba Rao, 1992). The enzyme from caryopsis is similar to that in the leaves and roots of maize seedlings (Laurière et al., 1992).

2.2.2.1 fi-Amylase Regulation

Exposure to light during greening caused a stimulation in p-Amylase activity. In mustard cotyledons, phytochrome induced *de novo* synthesis of p-Amylase by elevating its mRNA level (Sharma and Schopfer, 1982, 1987, Subbaramaiah and Sharma, 1987). Similarly, in pearl millet leaf also, exposure to light significantly stimulated p-Amylase activity in leaf, which was independent of Chloroplast development and was localized mostly near the leaf base (Vally and Sharma, 1992).

P-Amylase in the vegetative organs is mainly regulated metabolically by sucrose, and also by various forms of stress like water stress, light stress and mechanical wounding. In the tuberous roots of sweet potato, β-amylase constitutes about 5% of the total protein, but is absent, or present in extremely low levels, in other vegetative organs. However, when leaf petiole cuttings are kept in sucrose solution, P-amylase is induced along with starch and sporamin accumulation. The accumulation of both p-amylase and sporamin showed similar dependence on sucrose and 6% solution gave the highest induction when assayed after 7 d of treatment. Glucose and fructose acted like sucrose but not sorbitol and mannitol. Continuous light also stimulated P-amylase, probably due to the photosynthetic accumulation of sucrose. Thus p-amylase gene expression in sweet potato appears to be under metabolic control (Nakamura et al., 1991).

In sweet potato, p-amylase is not only induced by sucrose, but also by mechanical wounding. When leaf petiole cuttings are dipped in a solution of polygalacturonic acid, sporamin and P-amylase genes are induced. Polygalacturonic acid and Chitosan mediate the induction of wound inducible defense reactions. P-Amylase and sporamin are also induced by abscisic acid and repressed by GA. However, methyl jasmonate does not cause significant induction of sporamin or p-amylase (Ohto et al., 1992).

The level of p-amylase activity and it's mRNA in rosette leaves of *Arabidopsis thaliana* also increased significantly, with concomitant accumulation of starch, when whole plants or excised mature leaves were supplied with sucrose, glucose and fructose but not mannitol or sorbitol. This expression is repressed in the absence of light. In

transgenic *Arabidopsis* a fusion gene composed of the promoter of p-amylase and β-glucuronidase coding sequence showed the sugar inducible expression in a light dependent manner. Also, it was shown that the expression of the sugar-inducible gene for β-amylase in light is regulated at the level of transcription. The sugar inducible expression of the gene in the presence of light was not inhibited by DCMU, an inhibitor of photosynthetic electron transport. Therefore, light might be required for a process other than photosynthesis (Mita et al., 1995).

The mutants of *Arabidopsis thaliana* with altered starch metabolism have high levels of leaf P-amylase activity when grown in a 12 h photoperiod (Caspar et al., 1989). In these mutants, p-amylase is induced by soluble sugars that accumulate during the photoperiod.

In NF-treated and *Albostrians* mutant barley leaves, 3 isozymes of P-amylase were present. Illuminated white tissue developed 3-5 fold higher activity of all 3 isozymes when compared to illuminated green tissue. NF treatment had no effect on dark-grown plants. Maximum induction was observed in NF-treated illuminated tissues which were completely photobleached. This increase in amylase activity is interpreted as stress response to high light intensity. The p-amylase activity increased by other stresses such as 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 carotenoids and chlorophyll (Dreier et al., 1995).

2.3 NITRATE REDUCTASE AND NITRITE REDUCTASE

Nitrogen is the mineral nutrient needed in greatest abundance by plants. Plants assimilate inorganic forms of nitrogen such as nitrate and ammonia and organic forms, such as urea. Some plants such as legumes, can fix dinitrogen gas in association with symbiotic bacteria (Crawford, 1995). Once taken up, nitrate is either stored in the vacuole or reduced to nitrite by nitrate reductase (NR) in the cytosol. Thereafter Nitrite enters the Chloroplast (or plastid in the root) and is then reduced to ammonia by nitrite reductase (NIR). The energy for reduction is provided in the form of NAD(P)H for NR and reduced Ferridoxin for NIR. The resultant ammonia is then fixed into carbon by the action of glutamine synthase.

2.3.1 NITRATE REDUCTASE

Nitrate reductase is a key enzyme involved in the first step of nitrate assimilation in plants. NR is found in most plant organs, specially where nitrate is the source of nitrogen. In eukaryotes, three forms of NR have been found. The most common form of NR in higher plants is NADH-specific NR. In several plants a NAD(P)H bispecific NR has been reported, which either occurs as a second isoform along with NADH-specific NR isoform as in maize, barley and soybean, etc. (Campbell and Smarelli, 1986), or appear as a sole isoform in *Betula pendula* (Freimann et al., 1991). The third form of NR is NADPH-specific, but has not been found in higher plants, and is present in fungi and mosses (Paddiam et al., 1991).

Among the NRs, NADH:NR has been extensively studied. It has been purified from several plants such as barley, spinach, squash and maize. NR has been cloned from tobacco, barley, *Arabidopsis,* maize, etc. (Cheng et al., 1986; Calza et al., 1987; Campbell 1988a; Crawford et al., 1988). NR in all these plants is very similar to 80% homology. The polypeptide chain of NR has a molecular weight of 105-115 kD. However the native form of the enzyme is a homodimer with a molecular weight of 210-230 kD. Each subunit consists of three prosthetic groups: FAD, heme-Fe and Mo-pterin; with one each of these cofactors per subunit. Several lines of evidence support the concept that NR has two active sites, one site for the electron donation by NADU where the flavin of the enzyme is reduced to $FADH_2$ and a second site where Mo reduces nitrate (Campbell and Smarelli 1986).

2.3.1.1 NR-Regulation

The regulation of NR has been intensively studied for the past several years. These studies have revealed that multiple control mechanisms are involved in NR regulation. NR is regulated by atleast five factors such as its substrate nitrate, light, plastids, sugars and reversible phosphorylation.

NR is a substrate inducible enzyme. On addition of nitrate to plants, after a lag period, NR activity rapidly increases for some hours and then levels off to a steady state. The above nitrate-mediated NR induction is due to the *de novo* synthesis of NR protein (Somers et al., 1983). Moreover, when the supply nitrate to plants is terminated, the NR

activity and the protein level decrease. Studies on the regulation of NR in most species showed that the induction of NR occurs primarily at the level of mRNA. In maize, experiments with nitrate induction after pretreatment of seedlings with cycloheximide showed that inhibition of protein synthesis does not block NR mRNA in leaves, indicating that nitrate signal transduction system is expressed constitutively in maize (Gauri et al., 1992).

NR is also regulated by the levels of hormones like abscisic acid and cytokinin. In etiolated barley leaves, the accumulation of NR mRNA is enhanced by the cytokinin and benzyladenine (BA) and suppressed by abscisic acid (ABA). In barley leaves the NR mRNA levels were influenced mainly by the BA/ABA concentration ratios. Nuclear runoff transcription studies showed that the NR transcription was suppressed by ABA, and the addition of an equal concentration of BA partially reversed the ABA action (Lu et al., 1992).

The light effect on the substrate-inducible NR activity in etiolated maize seedlings is mediated through phytochrome (Rao et al., 1983). Recently, components of the signal transduction chain between phytochrome and maize leaf NR was examined. It was shown that NR can be induced in the etiolated leaves of maize by phorbol myristate acetate (PMA) treatment to the same extent as light (Chandok and Sopory, 1992), suggesting the involvement of a protein kinase in the light-mediated NR stimulation. PMA mimics light effect and follows similar kinetics as that of light-induction in a nitrate-dependent manner. PMA also inhibits phytochrome A transcript accumulation in a manner similar to red light, indicating a protein kinase-C type enzyme involvement in mediating light effect in both cases. Serotonin or 5-hydroxytryptamine, a stimulator of phosphoinositide turnover, also mimics light effect and enhances phytochrome A transcript accumulation. These results indicate that the phosphoinositide cycle may generate second messengers for the regulation of both phytochrome A and NR genes in maize (Raghuram and Sopory, 1995).

In a mutant *of Nicotiana plumbaginifolia,* lacking an active NR gene, which was transformed with tobacco NR cDNA fused to a constitutive promoter, though NR mRNA was produced constitutively, light was still needed for the accumulation of NR protein (Vincentz and Caboche 1991). Thus light besides promoting transcription of wild type

NR genes, also regulates the transcription of NR mRNA and/or the stability of the protein.

The nitrate/phytochrome induced appearance of NR activity in mustard cotyledons can be attributed exclusively to *de novo* synthesis of the protein. Activation of an inactive precursor protein by nitrate does not play a role (Schuster and Mohr, 1990). Strong synergistic action of nitrate and phytochrome which was observed at the enzyme level was also seen at the level of NR mRNA.

A third regulator of NR level in green tissues is the functional plastids. When plastids were damaged by photooxidation with Norflurazon, the action of nitrate and phytochrome on the appearance of cytosolic NR was abolished and the level of NR mRNA was strongly reduced. Since NR is a cytosolic enzyme, the level of NR is indirectly affected by photooxidation of the plastids. Rather, after the onset of a photooxidative treatment with RL, the NR level continued to increase unimpaired for several hours. The NR level decreased only once the mRNA level became limiting for the synthesis of the enzyme. Positive control of NR gene expression by the plastidic factor was clearly indicated by several other studies. Apparently, the plant cell regulates NR gene expression as if NR were a plastidic protein (Mohr et al., 1992).

Similar results have been obtained in a parallel study using squash cotyledons (Oelmiiller and Briggs 1990), where the dependence of NR activity on the state of the plastids reported to be determined at the level of it's mRNA and was not attributable to an inactivation of the enzyme. Thus intact plastids are a prerequisite for induction of NR activity by light and nitrate (Oelmüller et al., 1988) and for mRNA accumulation (Oelmüller and Briggs, 1990). The plastidic control may be mediated by a signal molecule originating from the plastids (Susek et al., 1993).

Several studies have shown that diurnal variation in NR activity is linked with photosynthesis and the byproducts of carbon fixation. In the dark, glucose, fructose and sucrose supplied to detached green leaves of dark-adapted *Nicotiana plumbaginifolia* plants resulted in. NR mRNA and protein accumulation and the loss of circadian rhythmicity in the size of the transcript pool. This carbon metabolite control takes place at the transcript level (Vincentz et al., 1993).

Since NR is located in the cytosol, it obtains its reductant not from photosynthesis but from carbohydrate catabolism. Sucrose can replace light in eliciting an increase of NR mRNA accumulation in dark-adapted green *Arabidopsis* plants. It was shown further that sucrose alone is sufficient for the full expression of NR genes in etiolated *Arabidopsis* plants (Cheng et al., 1992).

Photosynthetic fixation of CO_2 is often necessary for maximal NR activity (Kaiser and Bendel-Behnisch, 1991). In some experiments, increase in NR activity and mRNA was observed during night (Lilo 1984, Deng et al., 1989). Starch degradation and mobilization from the chloroplasts could cause such increases. The light induction of NR mRNA is apparently mediated through products of CO_2 fixation, since it has been shown that sucrose and glucose can replace light in the light induction of NR mRNA in Arabidopsis leaf. The sucrose/glucose response was confined to a 2.5 kilobase region of the 5' flanking sequence of NR gene (Cheng et al., 1992).

In plants grown with a normal day/night cycle, NR activity has been shown to have a diurnal rhythm (Srivastava 1980). NR mRNA has been shown to exhibit a diurnal rhythm in both leaves and roots of tomato, tobacco, *Arabidopsis* and maize grown in the presence of nitrate and a light/dark regime (Galangau et al., 1988; Deng et al., 1990; Bowsher et al., 1991; Cheng et al., 1991). In tobacco and tomato, NR mRNA levels decrease progressively during the day to virtually undetectable levels, and increase during the dark period, reaching a maximum during the end of the dark period. The fluctuations in the NR protein reflect with a 3-5 hour lag the changes in the mRNA level.

In maize seedlings that were grown in a light/dark regime for 5 days, followed by induction with 5 mM nitrate for 24 h, the decline in NR activity and NR mRNA in dark was immediate in the shoot, whereas in the root, there was a 4 h lag in the loss of NR activity, protein and mRNA. Return to light after a 2 h dark period led to rapid increase in NR activity, which reached 92 % of the original level within 30 min of illumination; suggesting that NR message production requires light, and the light stimulated NR protein is inactivated in dark (Li and Oaks, 1994).

NR undergoes rapid fluctuations in activity following changes in the photosynthetic rate, caused by changes in external CO_2 or water stress. The rapid modulation in NR activity involves ATP hydrolysis. Under photosynthetic conditions,

the cytosolic ATP/AMP ratio is low, and NR is active. When stomata close, there is depletion in CO_2 levels, and increase in cytosolic ATP/AMP leads to phosphorylation and inactivation of NR (Kaiser and Spill, 1991).

One important finding in recent years is the light regulate NR activity also at the post-transcriptional level. When spinach NR was assayed in the presence of Mg^{+2}, a reversible inactivation of NR can be seen when plants are subjected to water stress or transferred to dark (Mackintosh 1992) or to low CO_2 (Kaiser and Brendle-Behnisch, 1991).

Activity of NR matches the amount of NR protein. With changing light conditions or CO_2 levels, very rapid changes in the NR activity are observed (Kaiser and Brendle-Behnisch, 1991). NR is regulated by alteration in the cytosolic metabolite concentration such as changes in ATP and AMP levels, generated by changing rates of photosynthesis (Kaiser et al., 1992). It was reported that in spinach leaves, NR in illuminated plants is inactivated by a protein kinase. NR is phosphorylated in the dark and is activated upon illumination due to dephosphorylation by either PP1 or PP2A. It was also shown that PP2A is the major phosphatase in spinach leaves which dephosphorylates and activates NR (Mackintosh 1992).

Posttranslational modulation by reversible phosphorylation allows a rapid response to environmental conditions. In leaves, NR is inactive under aerobic conditions in dark, but is activated by anerobiosis (Kaiser et al., 1992). It is active in light in the presence of CO_2, but is inactivated when CO_2 is removed (Kaiser and Brendle-Behnisch 1991). The rapid modulation, with a half-life of 2-20 minutes occurs due to Mg^{+2} and ATP dependent NR protein phosphorylation, and activation by dephosphorylation (Huber et al., 1992). This posttranslational modulation of NR by protein phosphorylation and dephosphorylation permits rapid adjustments of nitrate reduction rates to fluctuations in carbohydrate availability (Kaiser and Huber 1994).

In mature green leaves, NR activity increases at the beginning of the photoperiod, due to increased protein level and protein dephosphorylation. Phosphorylation of NR was reported to be more in the dark, and reduced when leaves are illuminated (Huber et al., 1994). Primarily, light is responsible for the diurnal variation in NR mRNA levels. The

overall regulation of NR activity in response to light involves the control of gene expression, protein synthesis and reversible protein phosphorylation (Huber et **al.,** 1994).

2.3.2 NITRITE REDUCTASE

Nitrite reductase (NIR; ferridoxin: nitrite oxidoreductase; EC 1.7.7.1) is **the** second enzyme in the nitrite assimilation pathway, which catalyzes the reduction of nitrite to ammonia. NIR was first identified as a ferridoxin-dependent Chloroplast enzyme which catalyses the six-electron reduction of nitrite to ammonia. NIR is a chloroplastic enzyme and in non-photosynthetic tissues, it is localized in the plastids. NIR is nuclear encoded and the appearance of this enzyme in plant material is controlled by nitrate and light (Rajasekhar and Oelmüller 1987; Redinbaugh and Campbell 1991), and requires a positive control by a plastidic factor (Schuster and Mohr 1990; Seith et al., 1991).

It is well documented that two isoforms of NIR (NIR1, NIR2) exist in most higher plants (Kutscherra et al., 1987). In physiological experiments with *Triticum aestivum* and *Gelium aparine* it was found that NIR1 showed significant activity only during the first days of plant development while NIR2 was the main isoform in later stages of development. Only the latter was found to be directly influenced by nitrate and light (Kutscherra et al., 1987).

2.3.2. J NIR- Regulation

Like NR, NIR activity is also substrate-inducible, and is strongly induced by nitrate in a variety of plants. In spinach, using a cDNA clone coding for spinach NIR, it was demonstrated that the level of NIR mRNA increases in the presence of nitrate (Back et al., 1988). An *in vitro* translation assay has also been used to show that the level of translatable NIR mRNA increases with the addition of nitrate in wheat (Small and Grey 1984) and pea (Gupta and Beevers 1987).

Induction of NIR in etiolated excised maize leaves was more in nitrate than in nitrite both in dark as well as in light. Though nitrite induced some nitrite reductase activity, the nitrate activity could not be induced at all. The appearance of NIR activity was independent of NR activity in light. This was shown by using tungstate which at 2

mM concentration completely inhibited NR activity in this system (Rao et al., 1981). Red light and kinetin (20 μM) increased NIR by 85 and 47% respectively in excised leaves of etiolated maize.

NIR activity is increased by phytochrome in etiolated maize leaves (Rao et al., 1981), similar to NR activity. It was shown that nitrate is required for *in vivo* synthesis of NIR and this synthesis is stimulated by light. While nitrate appears to regulate NIR synthesis by triggering transcription, light may control the level of transcription and translation (Gupta and Beevers, 1985). In spinach cotyledons the NIR transcript level was unaffected by light but determined by nitrate. The strong action of light on NIR synthesis was multiplicatively superimposed on the action of nitrate, consistent with the conclusion that nitrate affects the transcript level while light controls enzyme synthesis (Seith et al., 1991).

It appears that phytochrome strongly stimulates the level of NIR2 mRNA while significant enzyme synthesis takes place only in the presence of relatively large amounts of nitrate. Coarse control of the appearance of translatable NIR2 mRNA operates through phytochrome while fine tuning of gene expression, i.e., the actual appearance of the NIR2 protein is controlled by nitrate. Photooxidative treatment of the plastids abolished the level of NIR2 mRNA, i.e. in the absence of the plastidic factor, the gene expression was blocked and phytochrome was totally ineffective.

In tobacco seedlings the NIR transcript level was determined by a synergistic action between nitrate and light e.g., both factors coact at the level of transcription. There was no effect of light on NIR synthesis in the absence of nitrate, while a strong action of light on protein synthesis was observed in the presence of nitrate. Thus, in tobacco a coaction of nitrate and light (phytochrome) is required to bring about a high NIR transcript level, while in mustard the NIR transcript level was determined by phytochrome alone and in spinach by nitrate alone. It is obvious that in different plants phytochrome and nitrate control NIR gene expression differently. Only the dependence on the plastidic factor appears to be the same in all cases studied so far (Mohr et al., 1992).

The NIR induction differs from that of NR in the fact that light induction of NIR mRNA cannot be mimicked by adding sugars to the incubation medium of detached

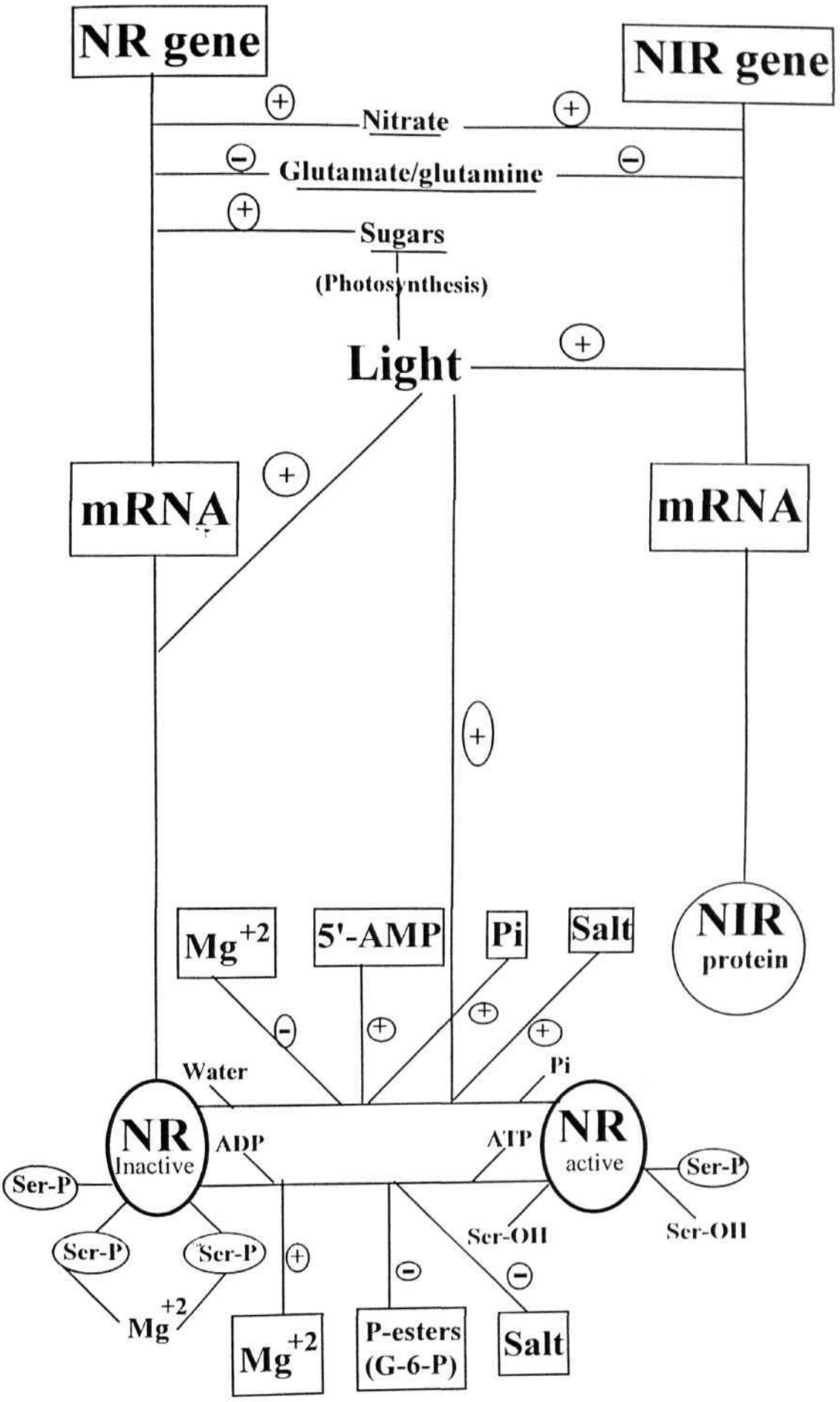


Figure 2.1 Regulation of Nitrate Reductase and Nitrate reductase enzymes.

Nicotiana plumbaginifolia leaves (Vincentz et al., 1993). In the transgenic tobacco, in which the NIR-promoter from spinach was fused to a reporter gene (GUS) and introduced into tobacco, response to nitrate and light was seen in accordance with the host, i.e. tobacco (Neininger et al., 1992; Neininger et al., 1993).

Other environmental factors also affect the levels of NIR in plants, such as water stress (Heuer et al., 1979) and treatment with cytokinins (Sharma and Sopory 1987).

2.3.3 COUPLED REGULATION OF NR AND NIR

Coupled regulation of nitrate and nitrite reductase genes is controlled primarily by the substrate nitrate and by a number of environmental stimuli that modulate their expression. As for NR, Back et al., (1991) have shown that nitrate-inducibility of the NIR gene is under transcriptional control. GUS reporter gene with a spinach NIR promoter when introduced into tobacco, GUS was found to be nitrate inducible. Since both NR and NIR genes are regulated by nitrate, it is expected that they share a common regulatory element within their respective promoter regions that is responsible for nitrate induction.

Just like NR, NIR mRNA shows diurnal fluctuations, but the enzyme activity remains fairly constant throughout the day. This may be because NIR protein is more stable than NR (Bowsher et al., 1991). In *Nicotiana plunbaginifolia* plants, NR and NIR mRNA levels fluctuate according to a circadian rhythm, with similar timings of maximal and minimal transcript accumulation (Faure et al., 1991). This suggests that there is a corregulation of NR and NIR gene expression (Faure et al., 1991). Tight coordination of NR and NIR is necessary, so that nitrite, which is extremely toxic, is never allowed to build up.

CHAPTER 3

Materials and Methods

3.1. Plant material

Maize seeds *(Zea mays* L. cv. Ganga-5) were obtained from Andhra Pradesh State Seed Corporation, Hyderabad, India. The seeds were first soaked for 12 h in distilled water at room temperature and then sown on moist germination papers in plastic trays (40 cm / x 30 cm *b* x 7 cm *h).* The trays were covered with transparent glass plates for 4 days, then left open. The seedlings were watered daily with distilled water. Seedlings were grown under continuous red light (RL) *(X* max 650 nm, 2.8 μ mole sec^{-1}) or in complete darkness at 25°C. Rice, wheat, pearl millet and oat were also grown in a fashion similar to maize.

3.2. Light sources

Red light (0.67 Wm^{-2}) was obtained by filtering the light from two cool white fluorescent tube lights (40 W) through two red plexiglass sheets (*X* max 650 nm). Long wavelength far red light (FRL) (6.0 Wm^{-2}) was obtained by using Schrott interference filters *(X* max 756 nm). The green safe light (<0.01 Wm^{-2}) 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 LI-COR radiometer.

3.3 Norflurazon Treatment

For Norflurazon (NF) treatment, the seeds were soaked in a 0.4 mM solution for 12 h and then sowed on germination papers presoaked with Norflurazon solution. The Norflurazon solution was prepared by dissolving Norflurazon in minimum amount of methanol, and making up the volume with the requisite amount of distilled water. The seedlings were grown in either in red light or in darkness and irrigated with Norflurazon.

3.4 Stress Treatment

4-d-old seedlings grown in distilled water for were subjected to water stress. Seedlings were transferred to various concentrations (100-500 mM) of sorbitol or NaCl solution and grown under white light or darkness for four more days. The seedlings were

irrigated with the respective solutions. Control seedlings were grown in distilled water for 8 d.

3.5 Enzyme Assay

3.5.1 Amylase

One g of tissue was homogenized in 2 ml of 100 mM sodium acetate buffer (pH 5.0) containing 4 mM $CaCl_2$ and 500 mg of quartz sand in a prechilled mortar on ice. The homogenate was diluted to 4 ml with extraction buffer and centrifuged at 7,600 *g* for 10 min at 4°C. The clear supernatant was assayed for enzyme activity.

Total amylase activity was assayed in a reaction mixture (final volume 4 ml) consisting of 100 mM sodium acetate buffer (pH 5.2), 4 mg/ml soluble starch, 4 mM $CaCl_2$ and 1 mM NaF. The assay was initiated by adding 100 µl of the crude enzyme extract to the reaction mixture. The assay was carried out at 30°C. Aliquots of 500 µl were withdrawn at 0, 15 and 30 min from the beginning of the assay and were mixed with 500 µl of dinitrosalicylic acid reagent (Bernfeld,1955). The resulting mixture was heated for 5 min on a boiling water bath. After cooling, the mixture was diluted to a final volume of 6 ml with distilled water. The increase in reducing sugars was determined by measuring absorbance at 540 nm using maltose as standard (Fig 3.1). Since the linearity of the standard curve is lost for maltose concentration above 1000 |ig/ml, the enzyme extracts were always suitably diluted so that amylase activity fell within the range of linearity of the standard curve.

3.5.2.Preparation of β-limit dextrin:

P-Limit dextrin was prepared by digesting amylopectin by β-amylase. 10 g of amylopectin was dissolved in 2.5 L of 100 mM sodium acetate buffer (pH 5.2) and was incubated with 200 µl (5500 units) of sweet potato P-amylase (Sigma). The increase in reducing sugars was monitored by taking aliquots at different time intervals by the procedure of Bernfeld (1955). The incubation was continued till no further increase in the level of reducing sugars was detected. After 48 h of incubation, when the values of reducing sugars remained constant, the total digest was boiled for 3 min to inactivate the enzyme. After cooling to room temperature, ß-limit dextrin was precipitated by adding

chilled methanol and incubating the mixture on ice for 1 h. The precipitated ß-limit dextrin was collected by decanting the supernatant and was dried in oven at 37°C to remove methanol. The dried ß-limit dextrin was stored at room temperature. Its purity was confirmed by incubating a 4 mg/ml solution of it with sweet potato β-amylase; which did not release reducing sugars confirming the purity ofβ-limit dextrin.

3.5.3 α-amylase assay :

α-amylase assay was performed after inactivation of P-amylase present in the crude extract by heating equal volumes of the crude extract and 20 mM $CaCl_2$ solution in a water bath at 70°C for 10 min. The assay mixture for a-amylase in a final volume of 4 ml consisted of 100 mM sodium acetate pH 5.2, 1 mM NaF and 4 mg/ml 6-limit dextrin. To initiate the assay, 500 µl of crude enzyme extract was added to the reaction mixture. The a-amylase activity was assayed as described earlier for total amylase (3.5.1).

The enzyme activity of amylases was expressed in katals as recommended by Enzyme Commission. One katal is defined as the amount of enzyme required to produce 1 mole of product under standard assay conditions in one second. After assaying total amylase activity and a-amylase activity, P-amylase activity was calculated by subtracting the value a-amylase activity from that of the total amylase activity.

3.5.4 Nitrate Reductase Assay

Nitrate reductase assay was performed according to the procedure of Whitelam et al (1979). One g of tissue was homogenized in 2 ml of 50 mM potassium phosphate (pH 8.8) buffer consisting of 1 mM EDTA, 25 mM cystein and 3% (w/v) bovine serum albumin (BSA, fraction V). The assay mixture consisted of 200 µl enzyme extract, 100 µl KNO_3 (100 mM) and 600 µl of 100 mM potassium phosphate buffer (pH 7). The assay was started by adding 100 µl of 2 mg/ml freshly prepared NADH solution. The assay was carried out at 30°C for 30 min. Thereafter, the reaction was terminated by transferring the assay mixture into 0.5 ml of boiling 0.3 M $ZnSO_4$ solution. The mixture was boiled further for 1 min and cooled to room temperature. 200 µl of 1 N NaOH was added to the mixture and centrifuged at 5000 *g* for 5 min at room temperature. The supernatant was used to estimate the amount of nitrite released. The amount of nitrite

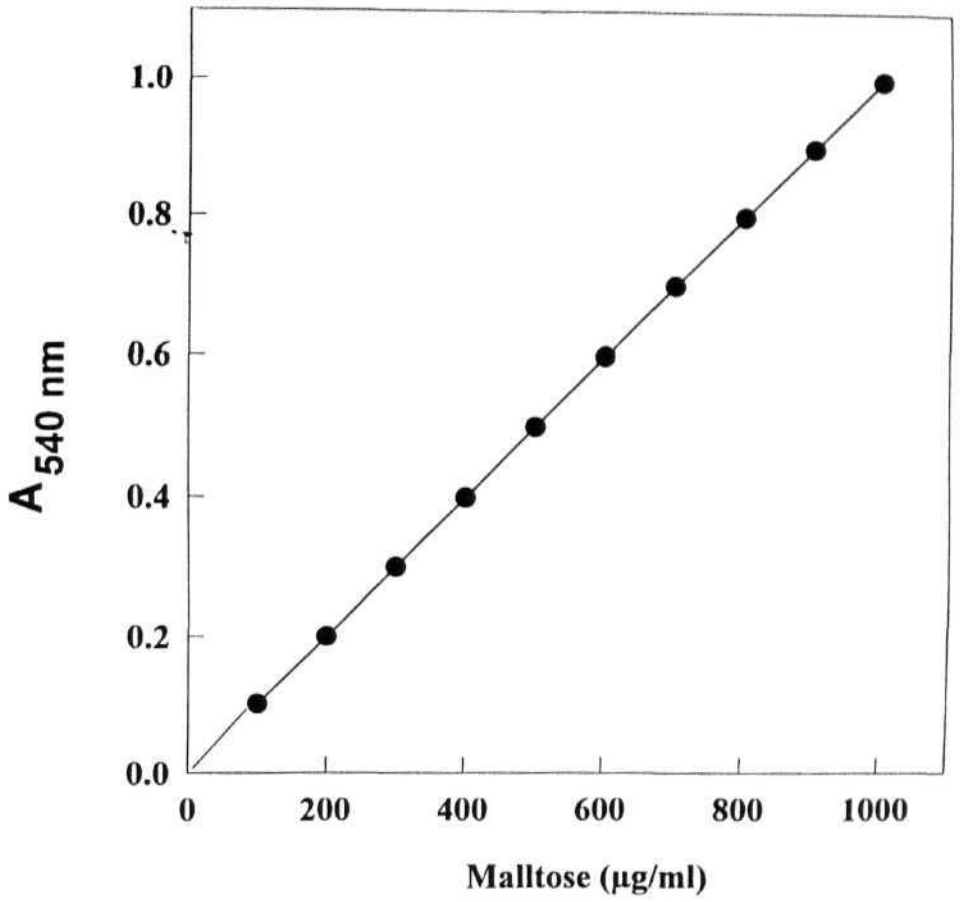


Figure 3.1 Standard curve for the estimation of maltose by the method of Bernfeld (1955)

released was estimated by adding 1 ml of sulfanilamide (1% (w/v) in 3 N HC1) and 1 ml of 0.05% (w/v) N-(l-naphthyl) ethylene diamine dichloride (NED) solution. The nitrite released was estimated by incubating the above solution at 30°C for 30 min and the absorbance was read at 540 nm. The amount of nitrite released was read from a standard curve of nitrite (Fig 3.2). The entire assay was completed within 1 h after homogenization of the sample.

3.5.5 Nitrite Reductase Assay

The nitrite reductase assay was performed according to Ramirez et al, (1966). One g of tissue was homogenized in 2 ml of 50 mM potassium phosphate (pH 8.8) buffer consisting of 1 mM EDTA, 25 mM cystein and 3% (w/v) BSA. The assay mixture consisted of 1.4 ml of 100 mM potassium phosphate buffer (pH 7.5), 100 µl of 5 mM KNO_2, 100 µl of enzyme extract, 100µl of methyl viologen (2 mg/ml). The volume was made upto 1.8 ml with distilled water. To start the assay, 200 µl of sodium dithionite (25 mg/ml in 290 mM $NaHCO_3$ solution) was added and incubated for 30 min at 30°C. At the end of the incubation period, 100 µl of the assay mixture was added to 1.9 ml of water and vortexed immediately to oxidize the dithionite. The amount of nitrite used up by nitrite reductase was estimated by adding 1 ml of sulfanilamide (1% (w/v) in 3 N HC1) and 1 ml of 0.05% (w/v) N-(l-naphthyl) ethylene diamine dichloride (NED) solution. The above solution was incubated at 30°C for 30 min and the absorbance was read at 540 nm. The amount of nitrite used up by nitrite reductase was estimated from a standard curve of nitrite (Fig 3.2). The nitrate reductase and nitrite activities were expressed in katals.

3.5.6 PEP Carboxylase assay

Phosphoenolpyruvate Carboxylase (PEP) activity was estimated according to Nott and Osmand (1982), using a coupled enzyme assay with malate dehydrogenase and measuring NADH oxidation at 340 nm. The Chloroplast extract was subjected to this assay to check for cytosolic contamination. The reaction mixture in a final volume of 1 ml consisted of 25 mM Tricine-KOH (pH 7.8), 5 mM $MgCl_2$, 10 mM $NaHCO_3$, 2 units malate dehydrogenase (MDH), 0.2 mM NADH, and 2.5 mM Phosphoenolpyruvate. The

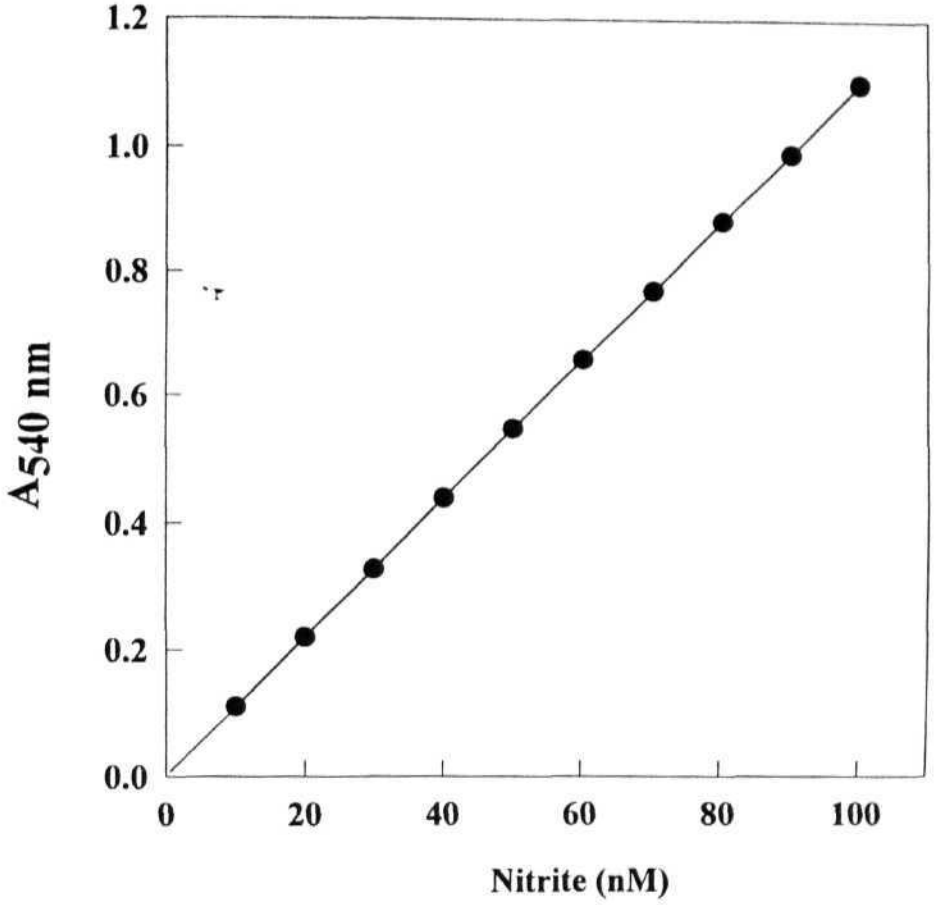


Figure 3.2 Standard curve for the estimation of nitrite by the method of Snell and Snell (1949).

reference cuvette contained the above reaction mixture minus PEP. PEP Carboxylase activity was measured using the extinction coefficient of NADH E = 6.2 mM^{-1} cm^{-1} and expressed in katals.

3.5.7 Acid Phosphatase Assay

Acid phosphatase assay was carried out according to the procedure of Boiler and Kende (1979). The assay mixture consisted of 50 µl of vacuolar extract, 25 µl of 100 mM succinic acid (pH 5) and 350 µl of distilled water. The assay was started by adding 75 µl of p-nitro phenol phosphate (final concentration 0.3 µM). The mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 800 µl of 1 M Na_2CO_3 solution. The absorbance of the p-nitro phenol released was read at 405 nm. The amount of p-nitro phenol released was estimated from a standard curve (Fig 3.3). Acid phosphatase activity was expressed in terms of µM of p-nitrophenol released.

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% (v/v) trichloroacetic 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. 3.4). 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 80% acetone at room temperature. The chlorophyll content of the isolated chloroplasts was estimated by diluting the Chloroplast suspension with 5 volumes of acetone. The chlorophyll was estimated according to the extinction coefficient derived by Bruinsma (1961), by reading the absorbance of the samples at 652 nm and the chlorophyll concentration was estimated by the formula µg chl/gm FW = A_{652} x 27.8 x dilution factors.

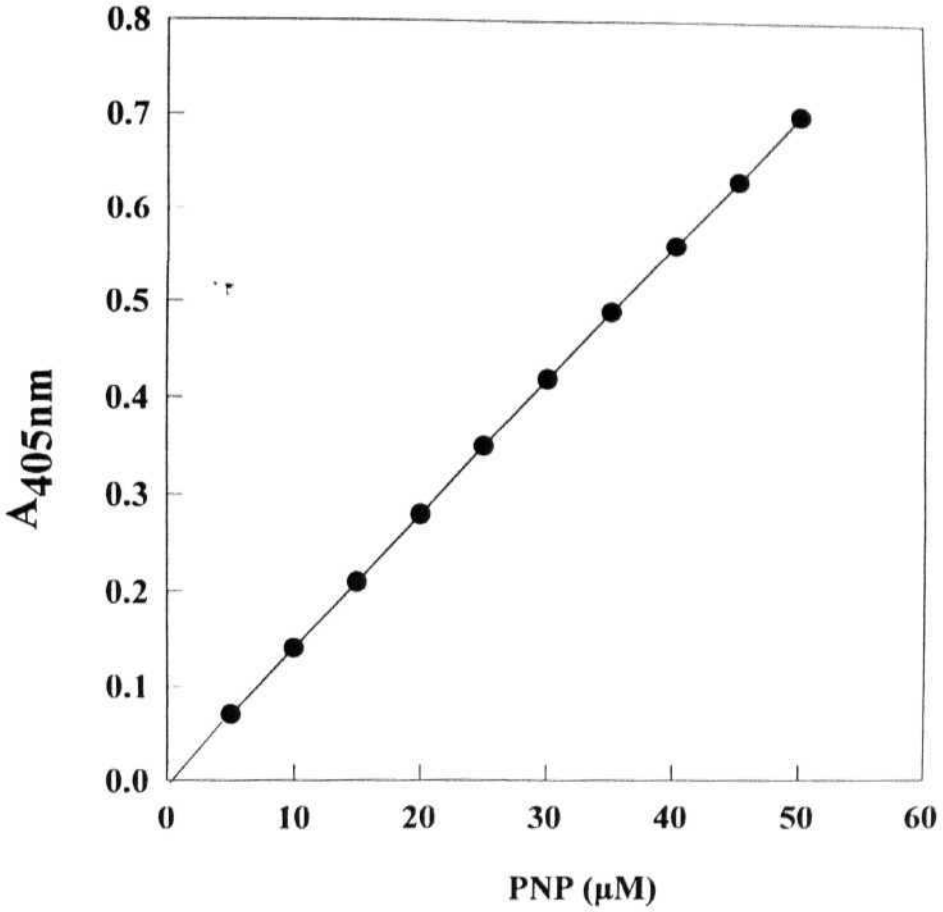


Fig. 3.3 Standard curve for the estimation of p-nitrophenol (PNP).

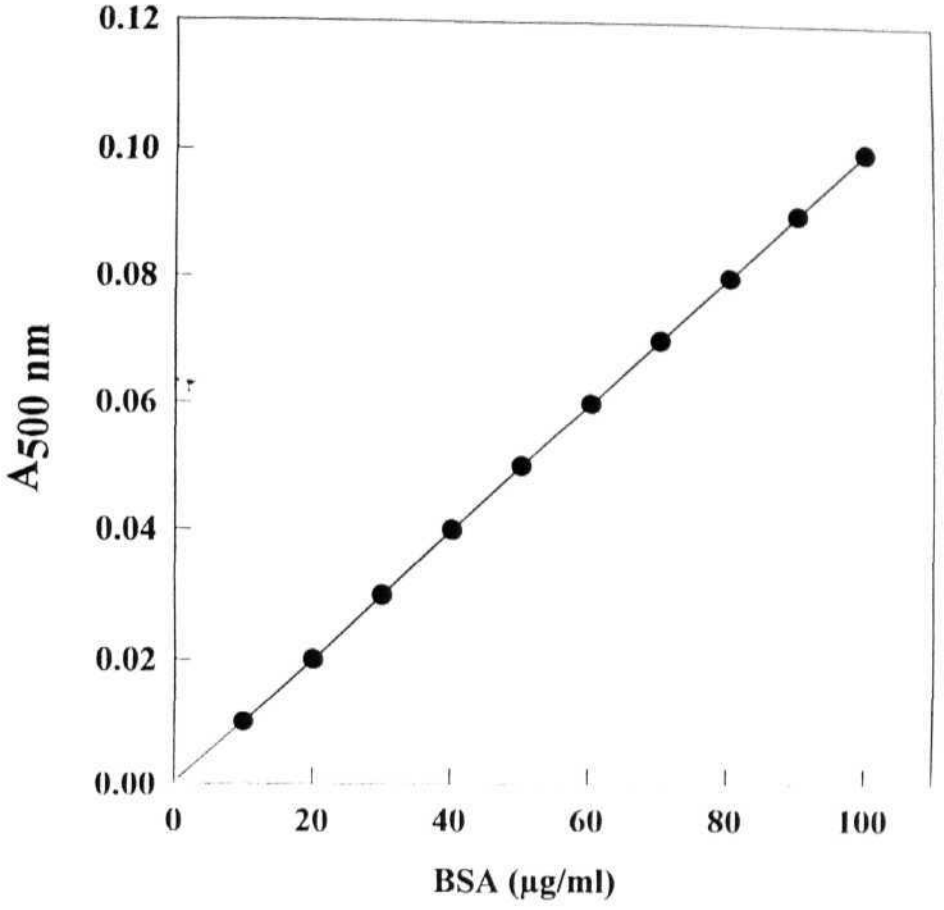


Figure 3.4 Standard curve for the estimation of protein by the method of Lowry et al., (1951).

3.8 Amylase Purification

3.8.1 Sample extraction

Amylase purification from maize seeds was done according to the procedure outlined by Subba Rao (1992). One kg of 6-day-old de-embryonated maize seeds was homogenized in 1 L of extraction buffer consisting of 100 mM sodium-acetate (pH 5), 5 mM $CaCl_2$, 4 mM NaCl, 4 mM phenylmethylsulfonyl fluoride (PMSF) and 80 mM ß-mercaptoethanol. The homogenate was filtered through three layers of cheese cloth and centrifuged at 10,000 *g* for 20 min at 4°C.

3.8.2 Ammonium Sulfate precipitation

Ammonium Sulfate precipitation was done according to the method of Stanley and Matteo (1971). To the supernatant solid ammonium Sulfate was added slowly with constant stirring at low speed to a final concentration of 20% (w/v). The solution was stirred for a further period of 30 min and centrifuged at 15,000 *g* for 20 min at 4°C. The pellet was discarded and to the supernatant ammonium Sulfate was added to a final concentration of 65% (w/v) with constant stirring. The solution was stirred for a further period of 1 h and centrifuged at 20,000 *g* for 30 min at 4°C. The pellet was resuspended in 200 ml of extraction buffer and dialyzed against the same buffer with several changes to completely remove the remaining ammonium Sulfate.

3.8.3 Amylose Chromatography

The dialysate was clarified by centrifugation at 10,000 *g* for 10 min and loaded onto a potato amylose column (20 cm *h* x 2 cm dia.), preequilibrated with extraction buffer. The unbound amylase was collected and stored for glycogen precipitation. The column was washed with 8 volumes of extraction buffer and then eluted at 25°C with 6 column volumes of saturated white dextrin dissolved in extraction buffer with a flow rate of 8 ml/h. Five ml fractions were collected. The fractions containing amylase activity were pooled and dialyzed against 50 mM Tris-HCl (pH 8.5), 5 mM $CaCl_2$, 2 mM PMSF and 5 mM ß-mercaptoethanol to eliminate white dextrin.

3.8.4 DEAE Sepharose Chromatography

The dialysate was clarified by centrifugation and loaded onto a DEAE-Sepharose column (15 cm *h* x 1.2 cm dia.), which was preequilibrated with 50 mM Tris-HCl (pH 8.5) consisting of 5 mM $CaCl_2$. The column was washed with the equilibration buffer until A_{280} of the effluent from the column was close to zero. The column was then eluted with a 100 ml linear gradient of 0-1 M NaCl at a flow rate of 20 ml/h. One ml fractions were collected. Individual fractions were assayed for amylase activity as well as for protein by SDS-PAGE. Two peaks of amylase activity were as judged by amylase assay were pooled separately, concentrated by precipitation with 75% (w/v) ammonium Sulfate, dialyzed against extraction buffer and stored in 20% (v/v) glycerol at -20°C.

3.8.5 Glycogen precipitation

The unbound fraction of the amylose column was subjected to glycogen precipitation according to Schramm and Loyter (1966). The sample was made up to a final concentration of 40% (v/v) ethanol. Ethanol was added drop wise with continuous stirring. The mixture was centrifuged at 10,000 *g* for 20 min. The pellet was discarded and the supernatant was made up to a final concentration of 20 mM PO_4 with 0.2 M phosphate buffer (pH 8.0). Glycogen solution (2% (v/v)) at 9.5 ml per 100 ml of sample was added, followed immediately by ethanol to readjust the final concentration in the mixture to 40% (v/v). The mixture was stirred for 30 min and centrifuged at 10,000 *g* for 20 min. The pellet was washed 3 times with 10 mM phosphate buffer (pH 8.0) containing 40% (v/v) ethanol. The supernatant was discarded and the tubes were drained by inverting them on tissue paper. The pellet was dissolved in extraction buffer and left standing at 30°C for 3 h to degrade the glycogen in the pellet by amylases. The solution was dialyzed against 50 mM Tris-HCl (pH 7.0), 5 mM $CaCl_2$.

3.8.6 DEAE-Sepharose Chromatography of glycogen precipitate

The dialysate was clarified by centrifugation and loaded on to a DEAE-Sepharose column (15 cm *h* x 1.2 cm dia.) preequilibrated with 50 mM Tris-HCl (pH 7.5) containing 5 mM fl-mercaptoethanol. The column was washed till A_{280} of the effluent was close to zero and then eluted with 100 ml of a linear gradient of 0-1 M NaCl in

column equilibration buffer at a flow rate of 20 ml/h. One ml fractions were collected. Fractions were assayed for amylase activity and also for protein by SDS-PAGE. Two peaks of amylases were obtained, which were pooled separately, concentrated by precipitation using 75% (v/v) ammonium Sulfate, dialyzed against extraction buffer and stored at -20°C in 20% (v/v) glycerol.

3.9 *Coupling of β-cyclodextrin (cycloheptaamylose) to epoxy Sepharose*

The coupling of Cycloheptaamylose (CHA) to sepharose was done according to the procedure of Vretblad (1974). One g of epoxy Sepharose was soaked in distilled water in a G 3 sintered funnel and washed thoroughly with water to remove preservatives. 3 ml of cycloheptaamylose solution (25 mg/ml in 0.1 N NaOH) was added to the pre-swollen epoxy Sepharose and agitated on a reciprocal shaker at 45°C for 16 h. The coupled Sepharose was washed thoroughly with distilled water to remove unbound cyclodextrin, followed by washing with 25 mg/ml of glucose solution. The Sepharose was further washed with distilled water, followed by 100 mM sodium acetate buffer (pH 5) and packed into a column.

3.10 Purification of Chloroplastic α-Amylase

3.10.1 Sample Extraction

Five hundred g of 9-d-old maize leaves were harvested, chopped into small pieces and homogenized in 500 ml extraction buffer consisting of 100 mM sodium acetate buffer (pH 5), 5 mM $CaCl_2$, 4 mM PMSF and 80 mM ß-mercaptoethanol. The homogenate was filtered through three layers of cheese cloth and centrifuged at 15,000 *g* for 20 min. The supernatant was subjected to ammonium Sulfate fraction.

3.10.2 Ammonium Sulfate Precipitation

Solid ammonium Sulfate was added to a final concentration of 40% (w/v) with constant stirring. The solution was centrifuged at 15,000 *g* for 20 min and the supernatant was made upto 70% (w/v) ammonium Sulfate. After centrifugation for 30 min at 20,000 *g*, the pellet was resuspended in 60 ml of extraction buffer and dialyzed against the same buffer at 4°C to completely remove the remaining ammonium Sulfate.

3.10.3 CHA-Sepharose Chromatography

A 3 ml cycloheptaamylose-Sepharose column was packed and pre-equilibrated with extraction buffer. The dialyzate after ammonium Sulfate precipitation was loaded on to the column after clarification by centrifuging at 10,000 *g* for 10 min at 4°C. The sample loading was done at a flow rate of 8 ml/h. The unbound fraction was collected and passed through the column once again. The column was washed extensively with extraction buffer and eluted using 10 mg/ml solution of cycloheptaamylose at a flow rate of 8 ml/h. Two ml fractions were collected, pooled and dialyzed to remove excess cycloheptaamylose. The dialyzed sample was concentrated by lyophillization and checked for protein by SDS-PAGE and Western blotting.

As a control, 6-d-old maize endosperms were also homogenized, concentrated by ammonium Sulfate precipitation and subjected to CHA-Sepharose chromatography under similar conditions as that of leaf. Seed α-amylases purified by the above column was subjected to DEAE-Sepharose chromatography as described in section 3.8.3.

3.11 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). One 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. 5% (w/v) stacking gel 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 sample 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) fl-mercaptoethanol. The samples were boiled for 4 min for an uniform coating of detergent. After cooling, the samples were loaded and electrophoresis was carried out at room temperature at a current of 10 mA and 20 mA in stacking and 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.

3.12 Silver staining

The gels were stained according to the method of Blum et **al.,** (1987). After electrophoresis, the gels were fixed in a solution containing 10% (v/v) methanol, 12% (v/v) acetic acid and 0.0185% (v/v) formaldehyde for 1 h. Then the gels were washed three times with 50% (v/v) ethanol for 20 min each, and pre-fixed with 0.02% (w/v) sodium thiosulfate for 1 min. Prefixing was followed by three washes with distilled water for 20 sec each. The gels were impregnated with 0.2% (w/v) silver nitrate solution containing 0.028% (w/v) formaldehyde for 20 min. The gels were then washed twice with distilled water for 20 sec each. The protein bands were visualized by developing the gels with 6% (w/v) sodium carbonate solution containing 0.0185% (v/v) formaldehyde. The reaction was stopped by washing the gels with several changes of water and gels were stored in fixer at 4°C.

3.13 Molecular weight determination

The molecular weight of the purified amylases was determined by SDS-PAGE in 10% gels. The following molecular weight markers were used as standards: γ-Lactalbumin (14.2 kD); Trypsin inhibitor, soybean (20.1 kD); Trypsinogen, PMSF treated (24 kD); Carbonic anhydrase, Bovine erythrocytes (29 kD); Albumin, egg (45 kD); Albumin, bovine (66 kD).

3.14 Non-dissociating PAGE

Non-dissociating polyacrylamide gel electrophoresis (ND-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) ammonium persulphate, 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. Following polymerization, 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.

3.15 Activity staining of native gels

After ND-PAGE, the gel was washed three times in distilled water and incubated for 2 h at 37°C in l% (w/v) soluble starch, 0.1 M sodium-acetate pH 5 and 10 mM $CaCl_2$. The gel was then rinsed in distilled water to remove excess starch and incubated for a further period of 30 min in 0.1 M sodium acetate buffer (pH 5) containing 10 mM $CaCl_2$. The amylase bands on the gel were visualized by staining it in 0.2 M HCl containing 5.7 mM I_2 and 43.3 mM KI (Lin et al, 1988). The amylase bands were visible as clear areas against deep blue background within 1 min. The gels were immediately photographed.

3.16 Contact Printing of non-dissociating PAGE

One percent (w/v) agar solution containing 4 mg/ml ß-limit dextrin was prepared by boiling it in 0.1 M sodium acetate buffer (pH 5). The agar solution was poured uniformly on a glass plate and the plate was kept at 4°C for 30 min to solidify agar. After the electrophoretic run, the polyacrylamide gel was washed with distilled water followed by 0.1 M sodium acetate buffer (pH 5) and carefully laid on the agar gel. A thin layer of 0.1 M sodium acetate buffer (pH 5) was poured on top of the gel. Precaution was taken to avoid trapping of any air bubbles in between the two gels. The gels were incubated at 37°C for 6 h in a humid chamber. At the end of the incubation period, the polyacrylamide gel was carefully removed from the top of the agar gel and the agar gel was stained for amylase activity as described in section 3.15.

3.17 Renaturation of denatured amylase protein

Seed and leaf extracts were first subjected to SDS-PAGE in 10% acrylamide gels and amylolytic activity was recovered as per the procedure described by Segundo et al (1990). After SDS-PAGE, the gel was washed in a solution of 5% (v/v) Triton X-100 and SDS was allowed to exchange with Triton X-100 for 6 h with three changes at room temperature with 3 changes. At the end of the incubation period, the gel was washed thoroughly with distilled water and the amylase activity was visualized as described in section 3.15.

3.18 Antibodies production

200 µg of pure amylase protein was mixed with 0.5 ml of phosphate buffer **saline** (PBS), containing 0.8% (w/v) NaCl and 0.02% (w/v) KCl in 10 mM phosphate **buffer (pH 7.4).** This sample was mixed thoroughly with 0.5 ml of Freund's complete adjuvant. Using this mixture, multiple injections were made subcutaneously to a **rabbit.** Subsequently, two booster injections were given at intervals of 15 days using 100 µg of amylase each mixed with an equal amount of Freund's incomplete adjuvant. The rabbit was bled during the 5th week and the antiserum was collected and stored in aliquots with 0.001% (w/v) sodium azide at -20°C. Pre-immune serum was collected from the same rabbit prior to immunization and was used as a control.

3.19 Ouchterlony double immunodiffusion

Double immunodiffusion was performed on agar plates following the procedure of Ouchterlony (1949). One percent (w/v) agar solution was prepared in PBS buffer (pH 7.4). The agar solution was mixed with sodium azide at a final concentration of 0.001% (w/v) and poured on glass plates to a thickness of about 2 mm and allowed to solidify on a horizontal surface at room temperature. A central well and four peripheral wells were punched in the gels with a steel template. The central well was loaded with amylase antiserum and the peripheral wells were loaded with seed and leaf extracts from light and dark-grown samples. After loading the wells, the agar gels were incubated at 4°C in a humid chamber to avoid dehydration of the gel. The precipitin lines were visualized after 24 h against a dark field illumination and were photographed.

3.19.1 Activity staining of agar plates

After development of the precipitin lines, the agar plates were extensively washed with saline for 2 days with several changes. The agar plates were then washed with distilled water and stained according to the procedure of Work and Work (1972). Th plates were incubated in 1% (w/v) starch solution in 50 mM sodium phosphate buffer (pH 7.4) for 1 h. The plates were washed with phosphate buffer to remove the excess starch. Amylases were visualized by staining with iodine solution (section 3.15).

carried out in a custom made apparatus. Both anode and cathode graphite plates were washed thoroughly with distilled water. On the anode plate three sheets of Whatman papers were layered carefully avoiding trapping of any air bubbles between the sheets. The membrane was layered over the sheets, and a small cut was made at the left bottom corner of the membrane to coincide with the gel. The gel was carefully layered on top of the membrane on which three more sheets of Whatman papers were layered. Finally on the top sheet of Whatman paper, the cathode plate was placed. The blotting unit and the whole sandwich consisting of the plates, papers and gels were tightened using clamps. The blotting unit was connected to power supply which was set to 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.

3.23. Western blotting

Immunoblotting was done following the procedure of Towbin et al., (1979) at room temperature. After electroblotting, the membranes were blocked for three min with blocking buffer containing 2% (v/v) polyoxyethylene sorbitan 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 µl of nitro blue tetrazolium (50 mg/ml) and 33 µl 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.

3.24 Gel documentation

The relative amounts of protein in the immunoblots were measured by scanning the bands using gel documentation (Molecular Dynamics) system. 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 resulting values were used to plot graphs.

3.25 Isolation of mesophyll and bundle sheath protoplasts

3.25.1 Enzymatic Isolation

Isolation of mesophyll and bundle sheath protoplasts was done according to the procedure of Leegood et al., (1982). Ten-d-old leaves were cut into 0.5 mm sections with a razor blade and washed in the wash medium containing 0.5 M sorbitol and 1 mM calcium chloride in 5 mM MES (pH 6). The tissue was digested with digestion medium containing 0.5 M sorbitol, 1 mM calcium chloride 2% (w/v) Onozuka 3S (cellulase), 0.1% (w/v) Macerozyme R-10 in 5 mM MES (pH 5.5) for 2 h at 25°C in a petridish. The medium was removed using a pipette and the tissue washed with 20 ml wash medium and filtered through a 500 urn nylon mesh (a tea stainer). The washing was repeated three times. The filtrates of all three washes were pooled and passed through a 80 μm nylon mesh. The filtrate contained mesophyll protoplasts and the residue contained the bundle sheath strands. The filtrate was centrifuged at 100 *g* for 5 min. The pellet was resuspended in 5 ml of sucrose medium containing 0.5 M sucrose, 1 mM $CaCl_2$, 5 mM MES buffer (pH 6.0). 5 ml sucrose medium containing protoplasts was transferred to a centrifuge tube and over it 1 ml of wash medium was layered and centrifuged at 250 *g* for 5 min. The protoplasts at the interface of the two solutions were collected using a wide bore pipette and centrifuged at 100 *g* for 5 min to obtain mesophyll protoplasts.

The residue left after filtration through 80 μm nylon mesh contains the bundle sheath cells. The residue was incubated in the digestion medium at 25°C for 2 h. The procedure used to isolate bundle sheath protoplasts was the same as that used for the isolation of mesophyll protoplasts.

3.25.2 Mechanical Isolation of Bundle Sheath Strands

For large scale preparation, bundle sheath strands were isolated using the mechanical method followed by Chollet and Ogren (1973) with a few modifications. 20 g of leaves were cut into 5 cm segments and homogenized in a pre-cooled blender at 4°C at low speed for 20 sec in a 100 ml isolation medium containing 50 mM Tris-HCl (pH 8), 0.35 M sorbitol, 10 mM Na-ascorbate, 1 mM $MnCl_2$, 5 mM EDTA, and 0.2% (w/v) BSA. The homogenate was filtered through a 350 µm nylon mesh and the fragments that were retained on the mesh were rehomogenized for 30 sec at full speed in 100 ml of isolation medium. After filtration, the above process was repeated once more. All the three filtrates were pooled and passed through a 80 µm nylon mesh. Bundle sheath strands retained on the nylon mesh were washed with wash buffer containing 50 mM Tris-HCl, pH 8, 0.35 mM sorbitol, 20 mM EDTA, 1 mM $CaCl_2$.

Protoplasts were isolated by incubating the bundle sheath strands obtained for 2 h in a digestion medium. The digestion medium and the protocol for isolating protoplasts were essentially similar to the one used for isolation of mesophyll protoplast (section 3.25.1).

3.26 Isolation of intact chloroplasts

3.26.1 Isolation of Mesophyll Chloroplasts by Mechanical Method

The isolation procedure for chloroplasts was adapted from Palmer (1986). 100 g leaf tissue was homogenized in a pre-cooled blender (5 sec homogenization X 5 times) in four volumes of isolation buffer containing 50 mM Tris-HCl buffer (pH 8.0), 0.35 M sorbitol, 5 mM ethylenediamine tetra acetic acid (EDTA), 0.1% (w/v) BSA and 0.1% (v/v) ß-mercaptoethanol. The homogenate was centrifuged at 1000 *g* for 15 min and the pellet was washed 3 times with wash buffer containing 50 mM Tris-HCl (pH 8.0), 0.35 M sorbitol and 25 mM EDTA. Thereafter, the pellet was suspended in 8 ml of the wash buffer and layered on top of a step gradient of 31% and 54% (w/v) sucrose. The gradient was centrifuged at 25,000 rpm in a SW-28 (Beckman) rotor for 60 min at 4°C. The intact chloroplasts obtained at the interface of 31% and 54% (v/v) sucrose were

diluted 5 times with the wash buffer and were pelleted by centrifugation at 1000 *g* for 15 min. The washing step was repeated three times to remove contaminations.

3.26.2 Isolation of Chloroplasts from Mesophyll and Bundle Sheath Protoplasts

Mesophyll and Bundle Sheath protoplasts were resuspended in a medium containing 50 mM Tris-HCl (pH 8), 0.35 mM sorbitol, 20 mM EDTA, and 1 mM $CaCl_2$ and disrupted by repeatedly passing through a 30 µm nylon mesh fitted to the nozzle end of a 2 ml disposable syringe. This procedure broke the protoplasts but left chloroplasts intact. The released intact chloroplasts were centrifuged at 250 *g* for 1 min to obtain a Chloroplast pellet largely free of the cytosolic fraction. The Chloroplast pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 0.35 M sorbitol, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM EDTA, and 5 mM β-mercaptoethanol.

3.26.3 Ferricyanide Reduction Test

The intactness of chloroplasts was confirmed by the ferricyanide reduction test outlined by Lilley et al (1975). Isolated chloroplasts suspended in 50 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM $MnCl_2$, 2 mM EDTA and 0.35 M sorbitol were subjected to the ferricyanide reduction test. Potassium ferricyanide solution (3 mM) was prepared in Chloroplast wash buffer. The intact Chloroplast suspension (100 µl) containing 100 µg of chlorophyll was either mixed with 1 ml of Chloroplast suspension buffer (intact chloroplasts) or was lysed. Thereafter it was mixed with 1.9 ml of potassium ferricyanide solution and was assayed for Hill activity. The reduction of ferricyanide was measured by reading A520nm at 0 and 30 min from the start of the reaction.

3.27 Isolation of etioplasts

Seven-day-old dark-grown maize leaves were cut above the coleoptile into 2 cm pieces. One hundred g of leaf material were homogenized for 8 seconds with 4 vol of isolation medium consisting of 0.067 M phosphate buffer (pH 8), 0.5 M sucrose, 1 mM $MgCl_2$ and 2% (v/v) BSA. The homogenate was filtered through cheese cloth and 25 µM nylon mesh. The filtrate was centrifuged for 90 sec at 3000 *g* and the centrifuge was

stopped quickly using break, so that it came to rest in 25 sec. The pellet was resuspended in 5 ml of isolation medium and layered over a 3 cm height of sucrose cushion consisting of 0.6 M sucrose in 0.067 M phosphate buffer (pH 8), 1 mM $MgCl_2$ and 2% (v/v) BSA. After centrifugation for 15 min at 440 *g*, the etioplast pellet was resuspended in isolation medium containing 0.6 M sucrose.

3.28 Isolation of vacuoles

Isolation of vacuoles was done according to the procedure of Ziegler and Beck (1986). Two g leaf sample was cut into thin strips under buffer A consisting of 5 mM MES (pH 6), 1 mM $CaCl_2$ and 0.5 M sorbitol. The strips were incubated in dark for 3 h in 30 ml of buffer A supplemented with 2% (w/v) cellulase and 0.1% (w/v) macerozyme. The pH of the digestion medium was adjusted to 5.5 after addition of the enzymes. The mixture was tapped and shaken, and the solution was decanted. The strips were washed three times with buffer A and the solutions were pooled. The solution was filtered through a 100 µM nylon mesh and centrifuged for 5 min at 165 *g*. The pellet was resuspended in 50 ml buffer A and recentrifuged. The pellet was suspended in 7 ml buffer B consisting of 5 mM MES (pH 6), 1 mM $CaCl_2$, 0.5 M sucrose and 20% (v/v) percoll. Over this suspension in a centrifuge tube, 2 ml buffer C consisting of 5 mM MES (pH 6), 1 mM $CaCl_2$, 0.4 M sucrose, 0.1 M sorbitol and 10% (v/v) percoll, and 2 ml buffer A was layered. Intact protoplasts at the interphase of buffer C and A were collected after centrifugation at 1000 *g* for 10 min. The protoplasts were lysed in buffer A by mixing 4.5 ml buffer D consisting of 25 mM HEPES (pH 7.8), 2 mM EDTA and 20% (w/v) Ficoll (400) to every 3 ml protoplast suspension and shaken by hand occasionally. Two ml buffer E consisting of 10 mM HEPES (pH 7.6), 2 mM EDTA, 0.5 mM sorbitol and 5 % (w/v) ficoll (400) and 2 ml buffer F consisting of 10 mM HEPES buffer (pH 7.6), 2 mM EDTA and 0.5 M sorbitol were layered on the mixture and centrifuged for 10 min at 1000 *g*. The vacuoles banded at the interphase of buffer D and E and the chloroplasts sedimented at the bottom.

3.29 Enzyme extraction from isolated orgenelles

3.29.1 Osmotic Shock

The protoplasts were lysed by osmotic shock to yield an intact chloroplasts preparation and a cytosolic fraction. Protoplasts suspended in 0.6 M sorbitol medium were centrifuged at 100 *g* for 3 min. The protoplasts were osmotically lysed by resuspending in a medium containing 100 mM sodium acetate (pH 5), 1% (w/v) BSA and 1 mM $CaCl_2$ for 10 min. The sample was centrifuged at 250 *g* for 2 min and the supernatant was used for assaying cytosolic enzyme activity. The pellet containing intact chloroplasts was suspended in 100 µl of above resuspension medium and was used for assaying plastidic enzyme activity. Etioplasts and vacuoles were lysed in a similar manner.

3.29.2 Sonication

The protoplast, chloroplasts, etioplasts and vacuoles were also disrupted by sonication, at 4°C. After suspending in 2 ml 100 mM sodium acetate buffer (pH 5) for amylases extraction, the samples were sonicated in glass tubes surrounded with ice, by eight 20 sec bursts in a MSE 150 Watt sonicator at 40% of its maximum amplitude (60 W). An interval of 30 sec was maintained between each sonication burst. After sonication, the samples were centrifuged at 8,000 *g* for 5 min and the supernatants were collected.

CHAPTER 4

Results

In this study, the light mediated induction of amylase, NR and NIR activity were analyzed with respect to the gradient of cellular differentiation in maize leaves. The dependence of this gradient on Chloroplast biogenesis was examined by photooxidizing chloroplasts in developing leaves by Norflurazon (NF). The distribution and photoregulation of amylases were also investigated by immunoblotting using polyclonal antibodies raised against purified seed amylases.

4.1. Morphological features of maize seedlings

Fig 4.1 shows the difference in morphology between 7-d-old light- and dark-grown maize seedlings. The light-grown seedlings had green expanded leaves and a short mesocotyl, whereas dark-grown seedlings had long mesocotyl, but short and tightly rolled leaves. Norflurazon treatment right from the time of sowing, led to the formation of albino leaves in continuously light-grown seedlings. Dark-grown seedlings were also white in color due to the NF-mediated block in caroteniod biosynthesis. However, the morphological features of NF-treated seedlings were essentially similar to control seedlings, indicating that the NF-treatment did not block the scotomorphogenic or photomorphogenic developmental pathways. However, the NF-treated leaves were shorter than the DW-grown leaves in both light- and dark-grown seedlings.

4.2. Isozyme pattern of amylases in maize seed and leaf

The ND-PAGE of crude endosperm extracts from 7-d-old maize seed revealed the presence of five major amylase bands on staining of the gel using starch as the substrate (Fig 4.2). The analysis of the maize leaf extract showed an isozyme pattern nearly identical to that present in seeds. The isozyme bands were classified as α-amylase or p-amylase based on their action on β-limit dextrin. β-amylase cannot degrade β-limit dextrin, whereas a-amylase can degrade P-limit dextrin. At the same time, both a- and β-amylase can degrade starch (Thoma et al 1971).

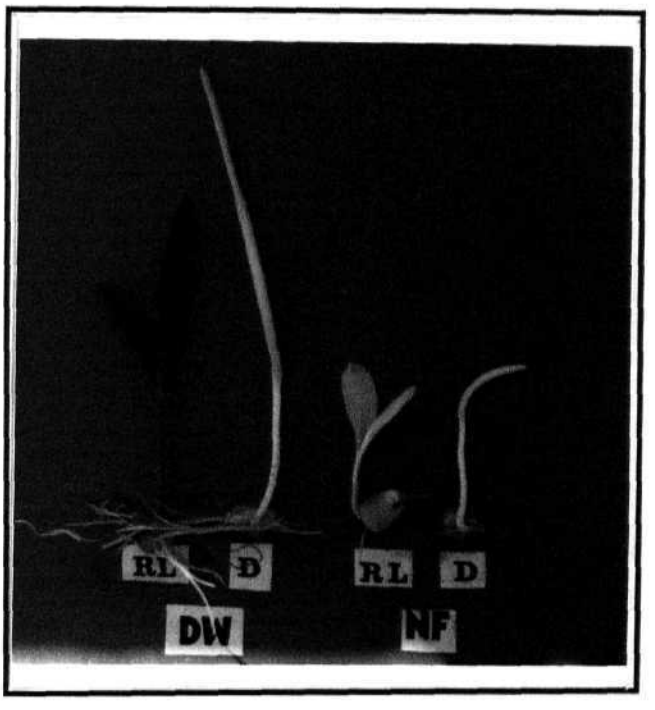


Figure 4.1 Morphology of 7-d-old maize seedlings grown under various treatments. Maize seedlings were grown in red light (RL) or darkness (D) using either distilled water (DW) or norfluazon (NF).

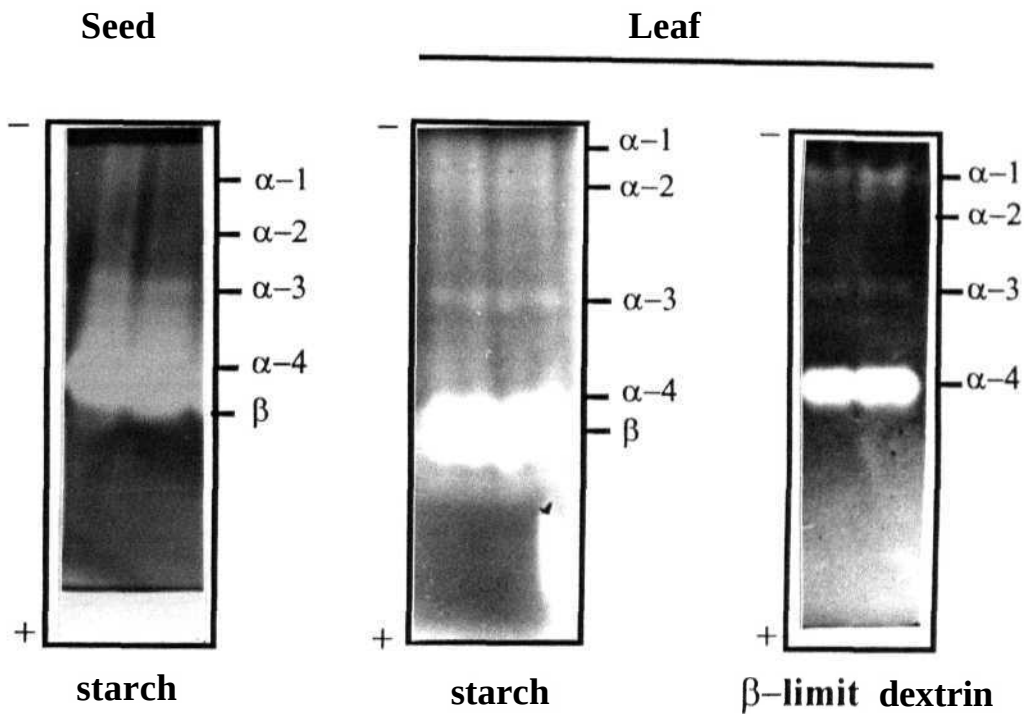


Figure 4.2 Zymogram of seed and leaf amylase. The crude extracts from 7-d-old maize seed and leaf were subjected to non-denaturing PAGE. Amylases were visualized by activity staining using starch as the substrate. Leaf a-amylase was distinguished by contact printing on a ß-limit dextrin agar gel. The symbols α- and **ß-** indicate the positions of a- and ß-amylase isoforms respectively. The a-amylase isoforms arc numbered in an order based on their increasing mobility.

Since ß-limit dextrin solution is too viscous and difficult to incorporate into polyacryl amide gel, the amylase bands were visualized by contact printing the gel on ß-limit dextrin containing agar gel. To check for the effectiveness of the transfer of amylases onto the agar gel, another gel was contact printed on a separate agar gel containing starch. Fig 4.2 shows that while the starch-agar gel reveals five amylase bands, the 6-limit dextrin-agar gel showed only four bands. The lower most amylolytic band was absent in β-limit dextrin-agar gel and was concluded to be a p-amylase band. The four amylase bands observed on the ß-limit dextrin agar gel were classified as α-amylases.

The above four α-amylase bands possessed Rf values of 0.068, 0.22, 0.32 and 0.46 respectively. Based on their respective mobility on the gel, these α-amylases were numbered as α-amylase-1,-2,-3 and -4. The topmost band (Rf 0.068) was designated as α-amylase-1, and the other 3 bands as α-amylase-2,-3 and -4, in an increasing order of their mobility. The fastest moving band (Rf 0.55) which was detected only when starch was used as the substrate, but not with ß-limit dextrin, was classified as p-amylase. Since the P-amylase band was relatively thick, though it was assumed to consist of a single isozyme, it may also consist of two closely migrating bands.

4.3. Purification of amylases from maize endosperm

The amylase purification followed a protocol similar to one standardized by Subba Rao (1992), cf Fig 4.3. The fractions obtained at different steps of purification were analyzed by ND-PAGE (data not shown) and also by SDS-PAGE (Fig 4.4, 4.5). Amylases were purified by affinity chromatography on an amylose column and eluted using white dextrin (Table 4.1). The 20-65% ammonium Sulfate fractionation pellet was resuspended in extraction buffer, dialyzed to remove remaining ammonium Sulfate, and loaded onto a potato amylose column. About two thirds of the applied amylase activity bound to the amylose column, which could be recovered by elution with white dextrin. All four isozymes of a-amylase was present in the white dextrin eluate (data not shown). β-Amylase did not bind to the amylase column and was recovered in the unbound fraction of the starch column. Thus, the amylose affinity chromatography purified a-amylase from all other proteins, and also separated a-amylase from P-amylase.

Figure 4.3 Protocol for the purification of amylases from endosperm of 7-d-old maize seedlings.

Table 1: Purification ofAmylases from Maize Endosperm

Step	Volume (ml)	Activity (nkat x10^3)	Protein (mg)	Specific Activity (nkat/mg)	Yield (%)
Crude extract	1,270	1,450	1,219	1,189	100
AS	300	890	283	3,145	61
Amylose column (unbound)	300	322	262	1,224	22
Glycogen precipitate	70	98	8.6	11,395	6.8
β-Amylase	3	35	2	17,500	2.4
Dextrin eluate	90	75	31	1,555	5.2
α-Amylase 1/2	7	2.8	7.2	388	0.2
α-Amylase 3/4	13	24.2	10.1	2,394	1.7

Since the dextrin eluate consisted of 4 isozymes of a-amylase, separation of these isozymes into 2 groups, i.e., α-amylase-1,2 and a-amylase-3,4 was achieved by ion exchange chromatography on a DEAE-Sepharose column. The DEAE-Sepharose column was eluted with a linear gradient of 0-1 M NaCl. α-Amylase-1,2 was eluted early fractions, whereas later fractions consisted of a-amylase-3,4. a-Amylase-1,2 migrated as a single band on SDS-PAGE, whereas, a-amylase-3,4 were seen as two closely migrating bands (Fig 4.4).

β-Amylase did not bind to the amylose column, and was recovered in the unbound fraction. The unbound fraction was subjected to glycogen precipitation. The glycogen precipitation step purified β-amylases and a small amount of a-amylase-3,4 to near homogeneity, and all other proteins were eliminated (Fig 4.5). Purified P-amylase was separated from a-amylase by DEAE-Sepharose chromatography. The amylases were eluted using a linear gradient of 0-1 M NaCl. P-Amylase was eluted in the early fractions and a-amylase-3,4 in the later fractions of the DEAE-sepharose column (Fig 4.5).

The above purification method yielded three groups of purified amylases, a-amylase-1,2; a-amylase-3,4 and P-amylase. Further separation of α-amylase-1,2 and a-amylase-3,4 could not be achieved. Therefore, both the α-amylase-1,2, a-amylase-3,4 and mixture of all four a-amylase isozymes were used for further characterization and antibodies raising.

4.4. Molecular weight of the purified amylases

The molecular weights of the purified amylases were estimated by SDS-PAGE using standard molecular weight markers. Fig 4.6 shows a standard graph of the Rf values of the molecular weight markers plotted against their log molecular weights. The Rf values of the purified a- and β-amylases were determined and their molecular weights were estimated from the standard graph. On SDS-PAGE, α-amylase-1,2 migrated as a single band and their molecular weights were estimated to be 46 kD. However, a-amylase-3,4 was resolved into two proteins with molecular weights of 44 kD and 47 kD. OnNDPAGE, a-amylase-4 migrated faster than a-amylase-3, whereas, SDS-PAGE revealed that a-amylase-4 has higher molecular weight than a-amylase-3 (Subba Rao, 1992). The molecular weight of p-amylase was 56.8 kD.

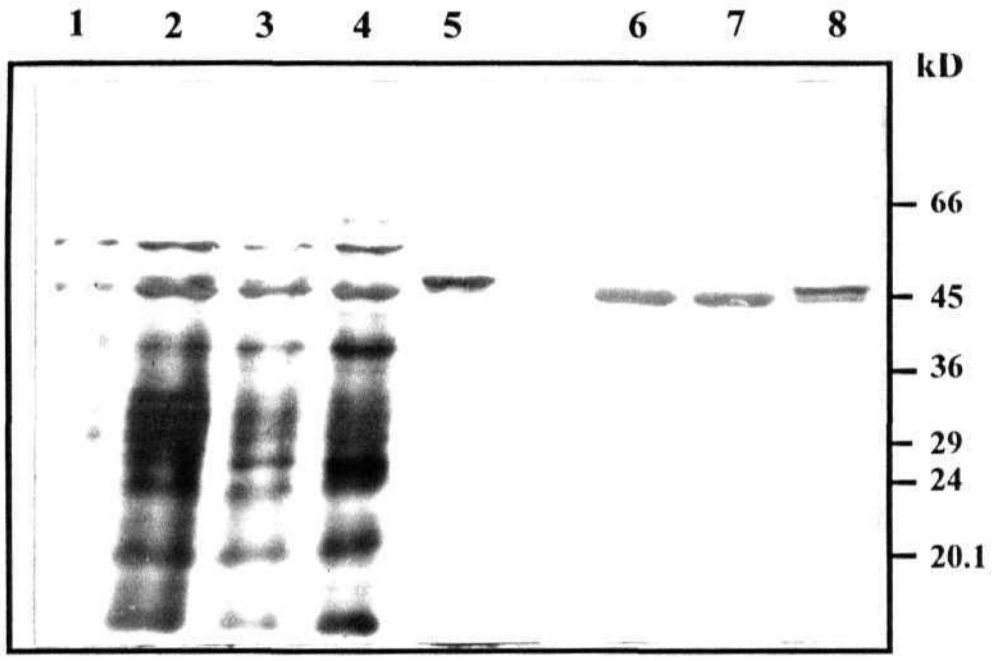


Figure 4.4 Purification of α-amylase from endosperms of 7-d-old maize seedlings. Aliquots from different stages of purification were analyzed by SDS-PAGE. The protein bands were visualized by silver staining. Lane 1, Crude Extract; Lane 2, ammonium Sulfate pellet; Lane 3, Ammnium Sulfate pellet after dialysis; Lane 4, Unbound fraction of the amylose column; Lane 5, Eluate of amylose column; Lane 6, Eluate of amylose column after dialysis; Lane 7, Eluate of DEAE-Sepharose column containing α-amylase-1,2; Lane 8, Eluate of DEAE-Sepharose column containing α–amylase-3,4.

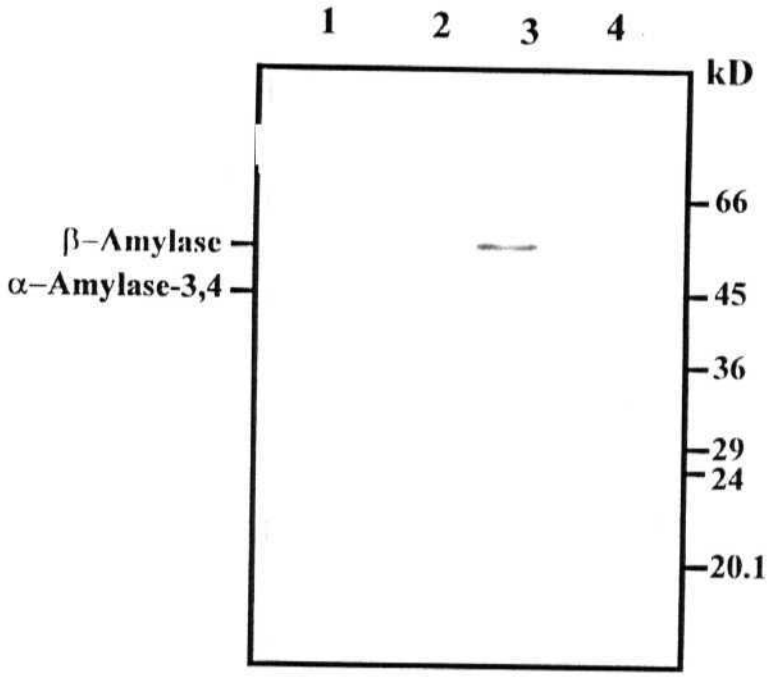


Figure 4.5 Purification of ß-amylase from endosperms of 7-d-old maize seedlings. Aliquots from different stages of purification were analyzed by SDS-PAGE. The protein bands were visualized by silver staining. Lane 1, Glycogen precipitate of the unbound fraction of the amylose column; Lane 2, Eluate of DEAE-Sepharose column containing a-amylase-3,4 and β-amylase; Lane 3, Eluate of DEAE-Sepharose column containing β-amylase; Lane 4, Eluate of DEAE-Sepharose column containing a-amylase-3,4.

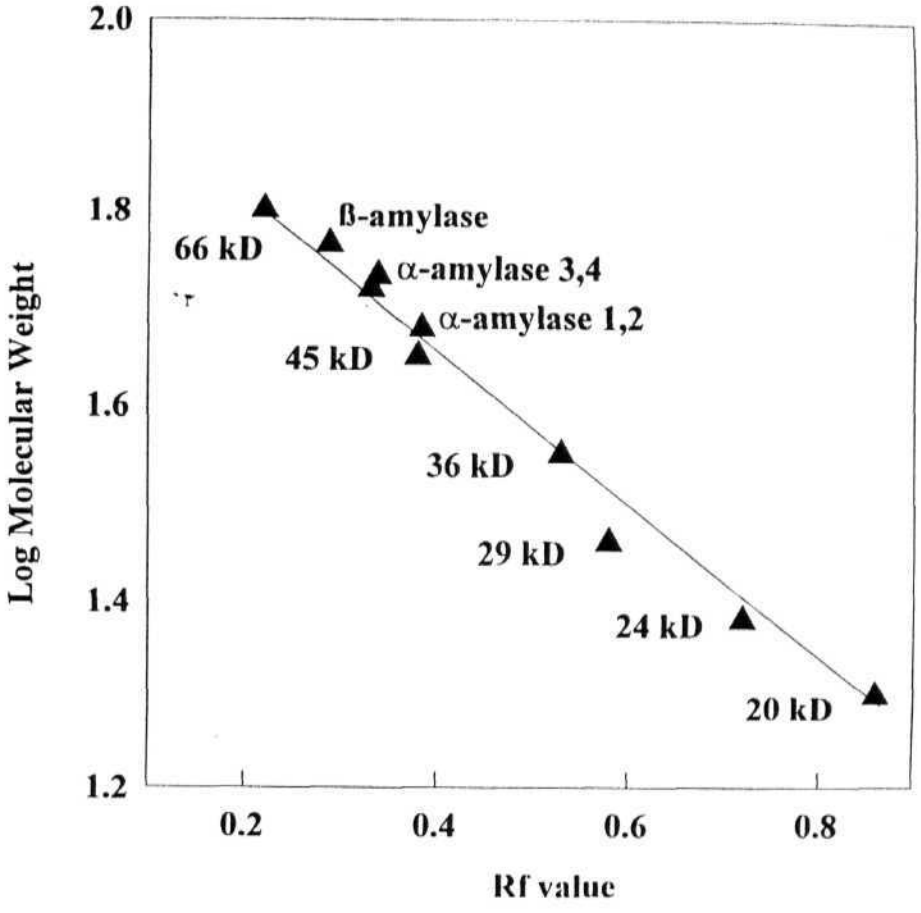


Figure 4.6 Determination of the molecular weights of amylases by SDS-PAGE. The molecular weights of the purified a- and tt-amylase isozymes was determined against a standard curve obtained by plotting the Rf values of standard molecular weight markers against their log molecular weights. The molecular weights of the standards used were a) Albumin (bovine)-66 kD, b) Albumin (egg)-45 kD, c) Glyceraldehyde-3-phosphate dehydrogenase (rabbit muscIe)-36 kD, d) Carbonic anhydrase (bovine erythrocytes)-29 kD, e) Trypsinogen-24 kD and f) Soybean trypsin inhibitor-20.1kD.

4.5. Immunological characterization

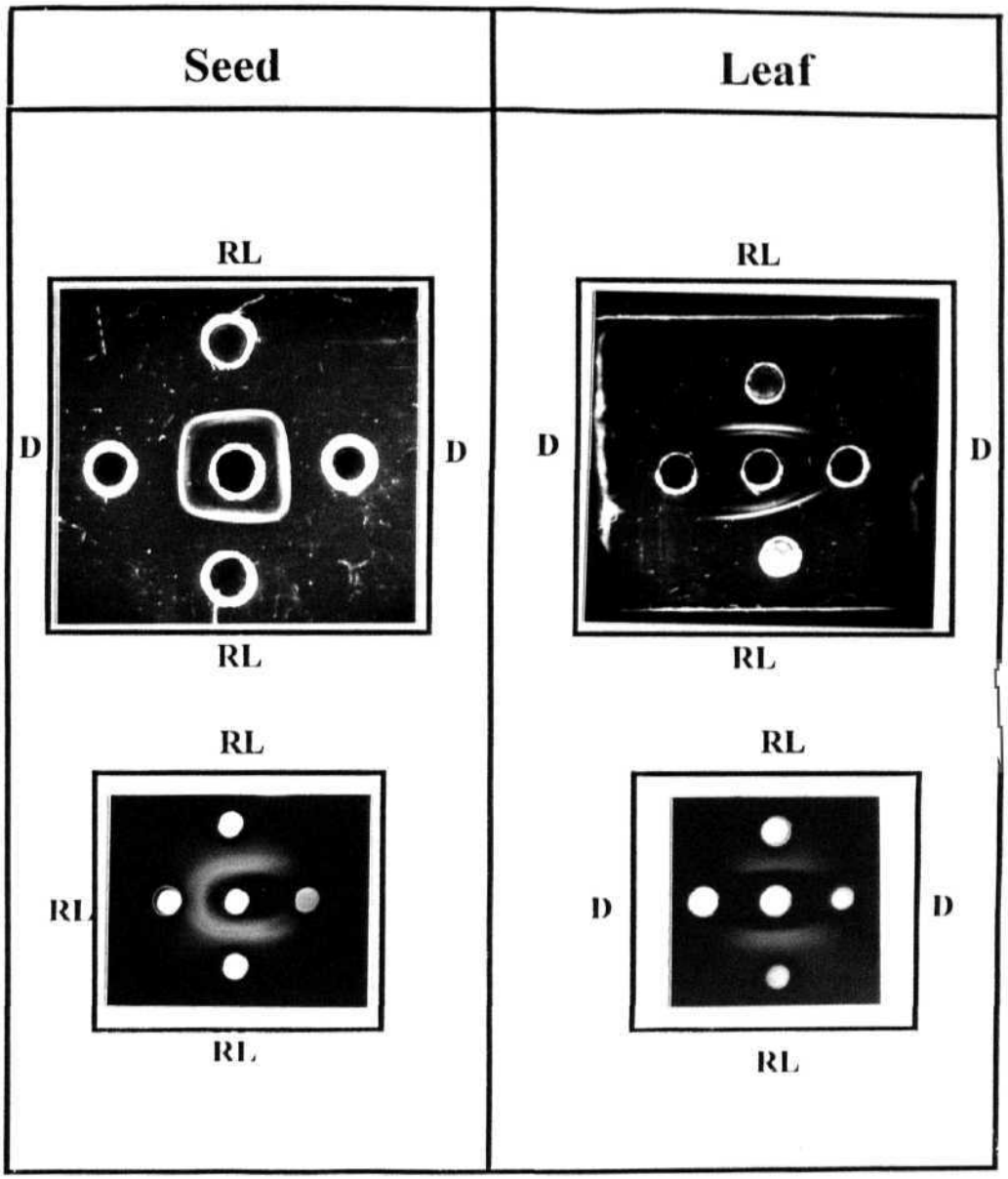
Polyclonal antibodies were raised against the purified a- and β-amylases in rabbit. Four groups of antibodies were used in this study. Antibodies were raised against α-amylase-1,2; α-amylase-3,4; mixed α-amylase-1,-2,-3,-4 and P-amylase. The specificity of these antibodies against seed and leaf amylases was checked by various immunological techniques like Ouchterlony double immunodiffusion, immunoprecipitation and western blotting.

4.5.1. Ouchterlony double immunodiffusion

Extracts from seed and leaf were subjected to Ouchterlony double immunodiffusion. In case of a-amylase (Fig 4.7), when antibodies against a mixture of all four a-amylase isozymes was loaded in the central well, and seed extracts from RL and dark-grown seedlings in the peripheral well, a double precipitin line was observed. The same was observed in the case of leaf. However, while in the case of leaves, RL-grown leaves show a strong stimulation in the a-amylase, in dark-grown plants, almost no a-amylase precipitin line was observed. This is due to the low amount of a-amylase in dark-grown seedlings, which is below the level of detectability of Ouchterlony double immunodiffusion technique. However in seeds, light had no effect on the amount of a-amylase (Fig 4.7). Since the amount of a-amylase in the leaf is very low compared to seeds, about 50-fold more protein had to be loaded in the case of leaf to observe a precipitin line.

Double immuno-diffusion gels showed a single precipitin line against P-amylase antibodies both in seed and leaf extracts (Fig 4.8). While the seed p-amylase level was not influenced by light, in case of leaf, light-grown leaf showed a thick precipitin line due to higher level of P-amylase in light. In dark-grown samples, no precipitin line was observed due to very low amount of P-amylase in these tissues. For leaf, about 10 times more protein was loaded when compared to seed, in order to observe precipitin line.

In order to confirm that the observed precipitin lines are indeed formed by cross-reaction between amylases and amylase-specific antibodies, the agar gels, after precipitin lines had developed, were activity stained for amylases. Both a- and P-amylase



igure 4.7 Ouchterlony double immunodiffusion of seed and leaf α-amylase. The central wells were loaded with anti α-amylase antibodies mixture against all the four a-amylase isozymes. The peripheral wells were loaded with crude extracts from seed and leaf of 7-d-old maize seedlings. **RL** and **D** indicate red light and dark grown samples respectively. The lower panel of photographs represent the activity staining of the seed and leaf α-amylase precipitin lines.

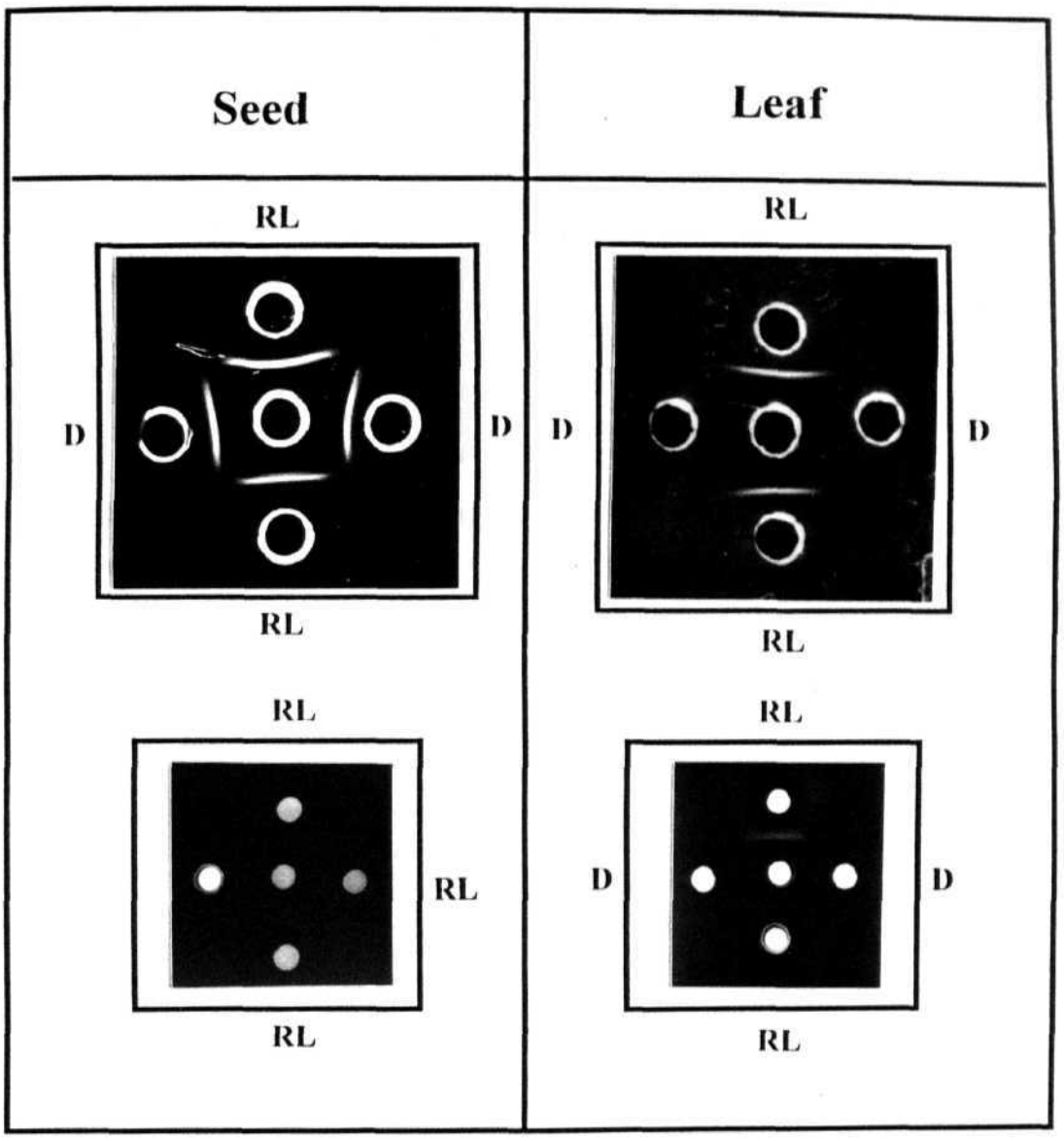


Figure 4.8 Ouchterlony double immunodiffusion of seed and leaf ß-amylase. The central wells were loaded with anti β-amylase antibodies. The peripheral wells were loaded with crude extracts from seed and leaf of 7-d-old maize seedlings. RL and D indicate red light and dark grown samples respectively. The lower panel of photographs represent the activity staining of the seed and leaf ß-amylase precipitin lines.

precipitin lines show amylase activities (Fig 4.7 and 4.8) confirming that the antigens precipitated by the antibodies are amylases.

4.5.2. Immunoprecipitation

The specificity of antibodies raised against leaf a- and β-amylases was also checked by immunoprecipitation of the amylases in leaf extracts and examining the residual amylase activity in the supernatant. The antigen-antibody complex formed by the cross-reaction was pelleted by binding to protein A Sepharose beads. In a control experiment using pre-immune serum, the entire activity of amylases remained in the supernatant. In contrast, on cross-reaction against α-amylase antiserum, α-amylase activity was immunoprecipitated proportionally with the amount of antibody used. Using different dilutions of antibody, it was observed that with 1/50 dilution of a-amylase antiserum, 86% of the a-amylase was pelleted. On further dilution, the magnitude of immunoprecipitation decreased and with 1/6400 dilution, almost the entire a-amylase activity remained in the supernatant. This confirmed that a-amylase antiserum specifically bound to leaf a-amylase and precipitated it, whereas, pre-immune serum was unable to precipitate a-amylase (Fig 4.9).

A similar result was obtained using p-amylase antiserum. While pre-immune serum could not immunoprecipitate P-amylase activity, anti-β-amylase antiserum specifically precipitated p-amylase activity. Similar to a-amylase, 50-fold diluted antiserum precipitated 92% of the activity, however, on further dilution, most of the amylase activity was observed in the supernatant (Fig 4.9).

4.5.3. Cross-reactivity of maize antibodies against other monocot species

The cross-reactivity of seed extracts from various monocots against maize antibodies was checked. Maize antibodies recognized a- and p-amylases in rice, wheat, oat and pearl millet with molecular weights in the same range as that of maize. This confirms that maize antibodies can recognize amylases from various other monocots (Fig **4.10).**

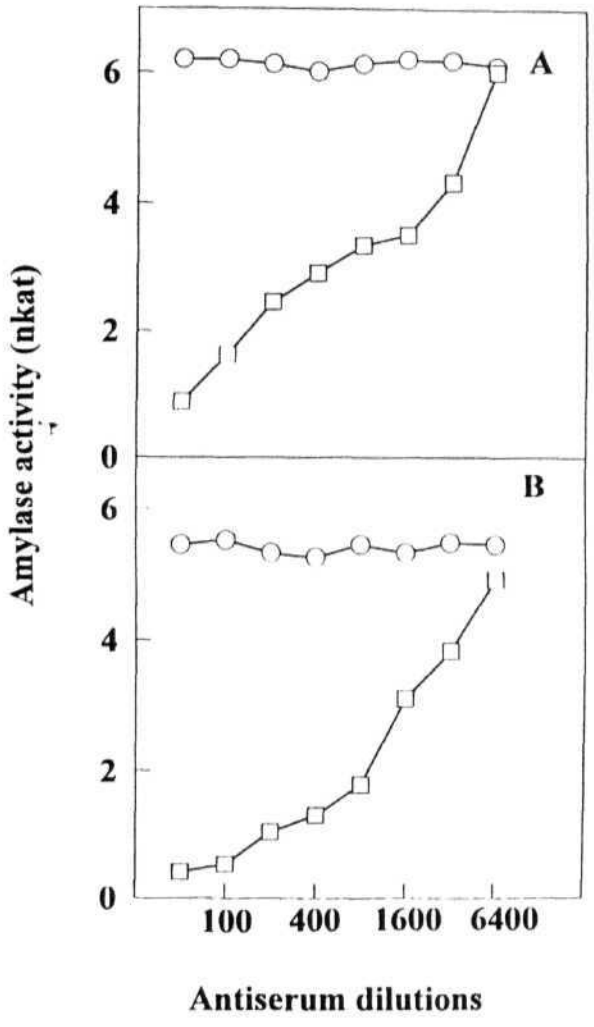


Figure 4.9 Immunoprecipitation of amylasc using different dilutions of a- (A) and 6- amylase **(B)** antisera. Control experiments were carried out using different dilutions of preimmune serum. The immunoprecipitated proteins were pelleted with protein A sepharose and a- and β-amylase activities were assayed in the supernatant.

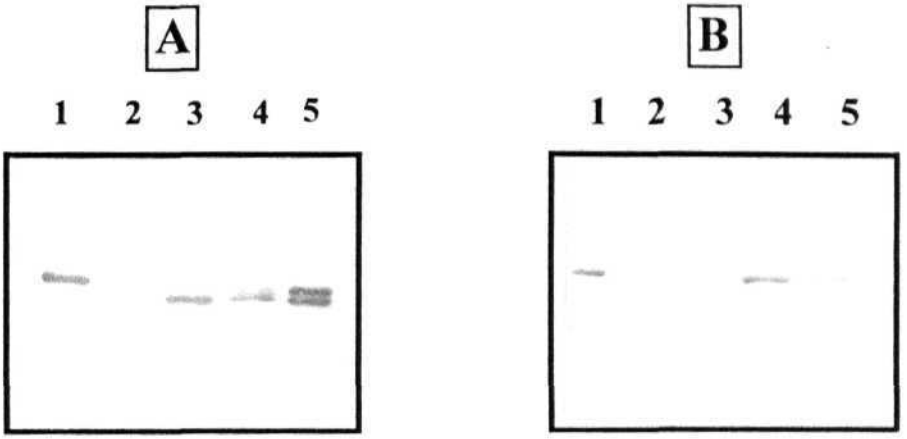


Figure 4.10 Detection of amylase in seeds of different monocot species using antibodies raised against purified maize a- (A) and β-amylase **(B)**. 5 μg of total proteins from crude extracts of seeds were subjected to SDS-PAGE and Western blotting. A) Lane 1, Rice; Lane 2, Pearl Millet; Lane 3, Wheat; Lane 4, Oat; Lane 5, Maize. **B)** Lane 1, Rice; Lane 2, Maize; Lane 3, Wheat; Lane 4, Oat; Lane 5, Pearl Millet.

4.5.4. Western blotting of non-denaturing gel

The presence of a- and P-amylase isozymes in different organs of maize seedlings was examined by western blotting after non-dissociating (ND) PAGE. For all the organs, 50 μg of protein was loaded in the respective tracts except for seed, for which only 5 μg of protein was sufficient to detect α-amylase isozymes. Comparatively, seed and leaf possessed considerably higher amounts of a-amylase protein, whereas in root, mesocotyl and coleoptile, a-amylase protein level was below detectability limit. In the crude seed extract, four isozymes of a-amylase was detected. The Rf values of the a-amylase isozymes were identical to those calculated after native PAGE followed by activity staining. This shows that the mixture of antibodies raised against a-amylase are specific to α-amylases and can detect all the isozymes of a-amylase (Fig 4.11).

However, the western blotting of leaf extract after ND-PAGE in fact revealed that the respective a-amylase bands in leaf may consist of several closely migrating isozymes- Apparently, a-4 amylase comprises 3 bands, whereas a-2 amylase appears to possess a single band. This is in contrast to seeds, and the reason for the multiplicity of bands in leaves is not apparent. Moreover, while on activity staining, a-4 amylase is the most prominent band, on Western blotting, a-2 amylase is the most prominent. This discrepancy may arise because of the difference in enzyme activity and protein level. While a-2 amylase protein level may be higher, its enzyme activity could be low.

β-Amylase blot afterNDPAGE showed a single isozyme with Rf value of 0.55 that is identical to that obtained by activity staining. Maximal amount of P-amylase was detected in seed and leaf, whereas very low amount of p-amylase was detected in the root, mesocotyl and coleoptile (Fig 4.12).

4.5.5. Western blotting of SDS-PAGE

Seed and leaf extracts were subjected to SDS-PAGE and electroblotted on PVDF membranes. The membranes were probed against α-amylase-1,2, α-amylase-3,4 and β-amylase antibodies. Fig 4.13 shows that the anti-α-amylase-1,2 antibodies recognized a band with a molecular weight of 46 kD in seed. Anti-α-amylase-3,4 recognized two closely migrating bands of 45 kD and 47 kD and P-amylase antibody recognized a 56.8 kD protein in seed.

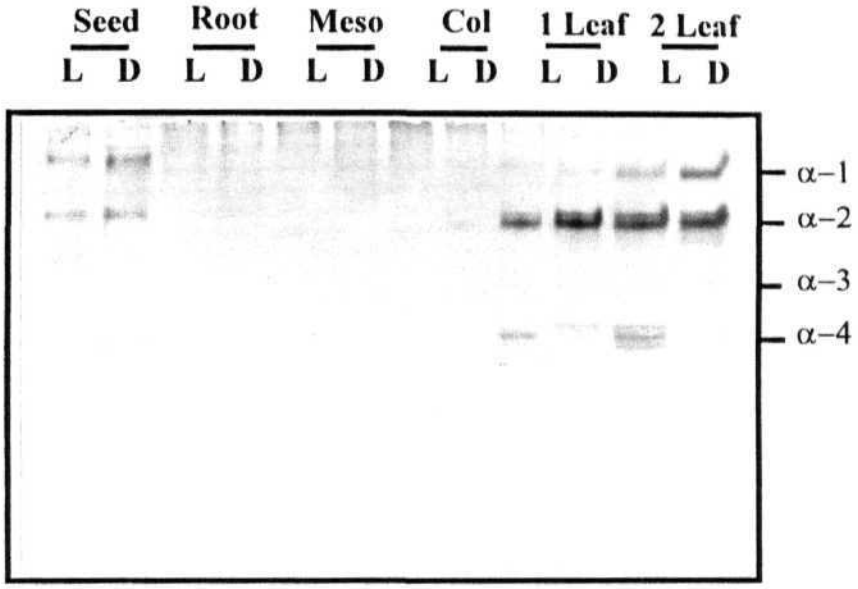


Figure 4.11 Distribution of various isoforms of a-amylase in different organs of 7-d-old maize seedlings. Red light (L) and dark-grown (1)) seedlings were excised into **seed, root,** mesocotyl **(meso),** coleoptile **(col),** first leaf **(1 Leaf)** and second leaf **(2 Leaf).** Crude extracts were subjected to immunoblotting after non-denaturing PAGE and probed with anti α-amylase mixture antibodies. The symbol a- indicates the positions of a-amylase isoforms which arc numbered in an order based on their increasing mobility.

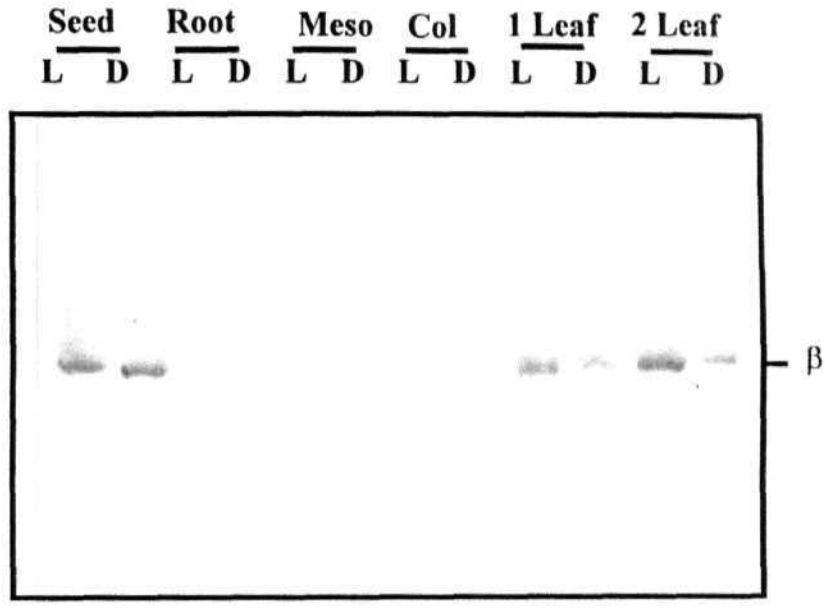


Figure 4.12 Distribution of p-amylase in different organs of 7-d-oId maize seedlings. Red light (L) and dark-grown (D) seedlings were excised into **seed, root,** mesocotyl **(meso),** coleoptile **(col),** first leaf **(1 Leaf)** and second leaf **(2 Leaf).** Crude extracts were subjected to immunoblotting after non-denaturing PAGE and probed with anti p-amylase antibodies. The symbol p- indicates the position of the p-amylase isoform.

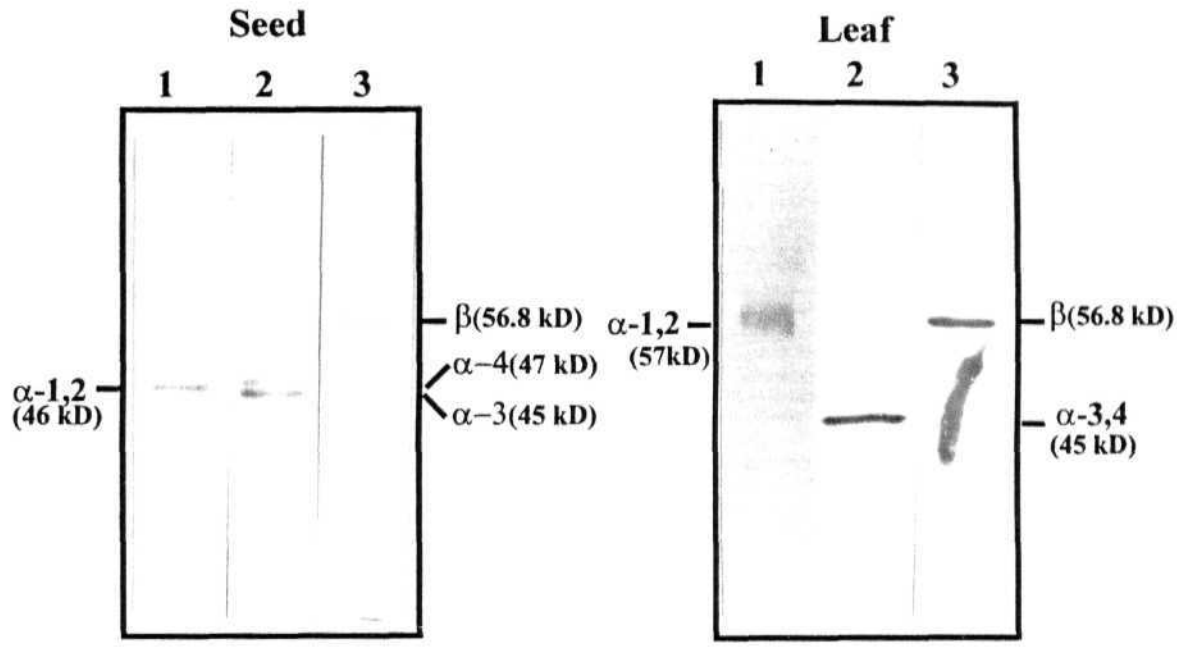


Figure 4.13 Characterization of various isoforms of a- and β-amylase in maize seed and leaf by immunoblotting. Crude extracts of seed and leaf after SDS-PAGE and electro-blotting were probed against anti a-amylase 1/2, anti cx-amylase 3/4 and anti β-amylase antibodies. The symbols a- and β- indicate the positions of a- and β-amylase isoforms, with their respective molecular weights in brackets.

In the case of leaf, α-amylase-1,2 recognized a 57 kD protein in isolated chloroplasts. α-Amylase-3,4 recognized a single band with molecular weight of 45 kD and P-amylase recognized a band with molecular weight of 56.8 kD. Thus the molecular weights of seed and leaf P-amylase and α-amylase-3,4 are similar, but the chloroplastic a-amylase has different molecular weight, though it is antigenically similar to a-amylase-1,2 of seed.

4.6. Spatial and temporal expression of amylases

The spatial and temporal distribution of a- and β-amylases in maize seedlings was studied in detail. Attempts were made to decipher the interrelationship between cell position and photoregulation of amylases in the first leaf of 7-d-old maize seedlings. During germination, the coleoptile appears on the 2 d followed by the leaf on the 3 d. The first leaf is about 7 cm in length on the 7 d.

4.6.1. Temporal expression of amylases in maize leaf

The time course of appearance of a- and P-amylase activity was checked by both enzyme assay and activity staining after ND-PAGE. The protein levels of both a- and β-amylases were also studied by western blotting. Analysis of time course of a-amylase activity in 5 to 10-d-old light-grown maize leaf showed that a-amylase activity increased gradually from a low level on the 5 d to almost 4-fold higher activity on the 10 d. In case of p-amylase, the highest activity was detected in 7-d-old seedlings, followed by a decline. In dark-grown seedlings, the activity pattern of both a- and p-amylase was similar but consistently lower than that of light-grown leaves (Fig 4.14).

When extracts from 5-10-d-old dark-grown seedlings were electrophoresed and stained for activity, a similar pattern was observed (Fig 4.15). While no a-amylase activity could be observed on the 5 d, there was a gradual increase in activity upto the 10 d. Fig 4.15 shows the time course pattern for α-amylase-4, which is the most prominent band observed after activity staining of ND-PAGE. Other isozymes also showed a similar pattern as that of a-amylase-4. Similarly, β-Amylase activity pattern closely followed the enzyme activity pattern obtained by amylase assay. 7-d-old leaves showed the highest P-amylase activity, followed by a gradual decline upto the 10 d. Dark-grown

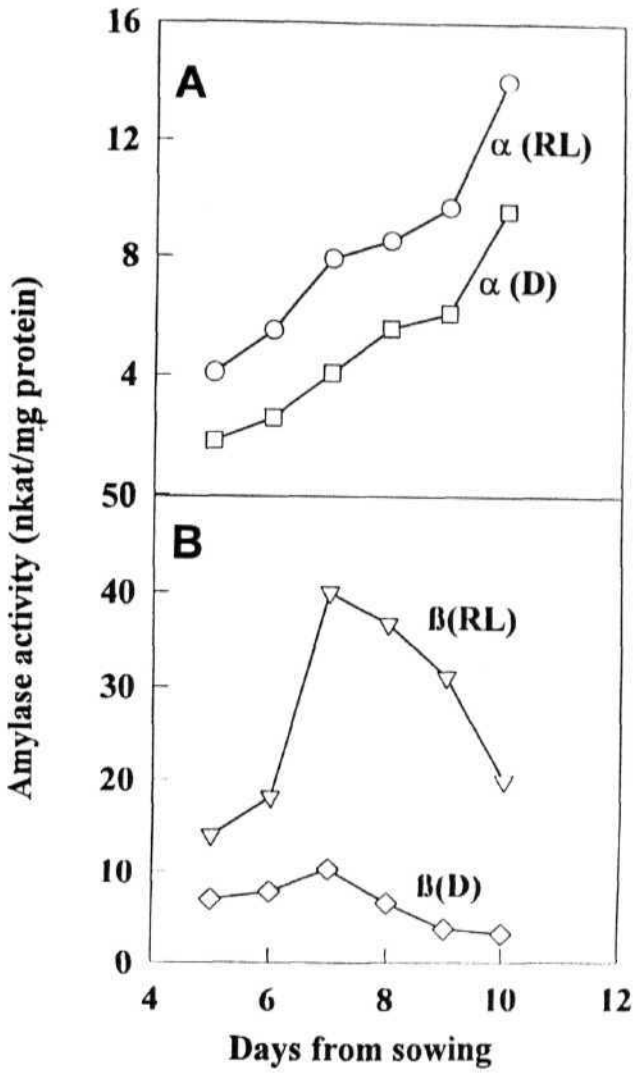


Figure 4.14 Time course of amylase activity in primary leaf of maize. Seedlings were grown either in red light (RL) or in darkness (D). a- (A) and fl-amylase (B) activities were assayed from 5-d to 10-d after sowing and expressed as nkat/ mg protein.

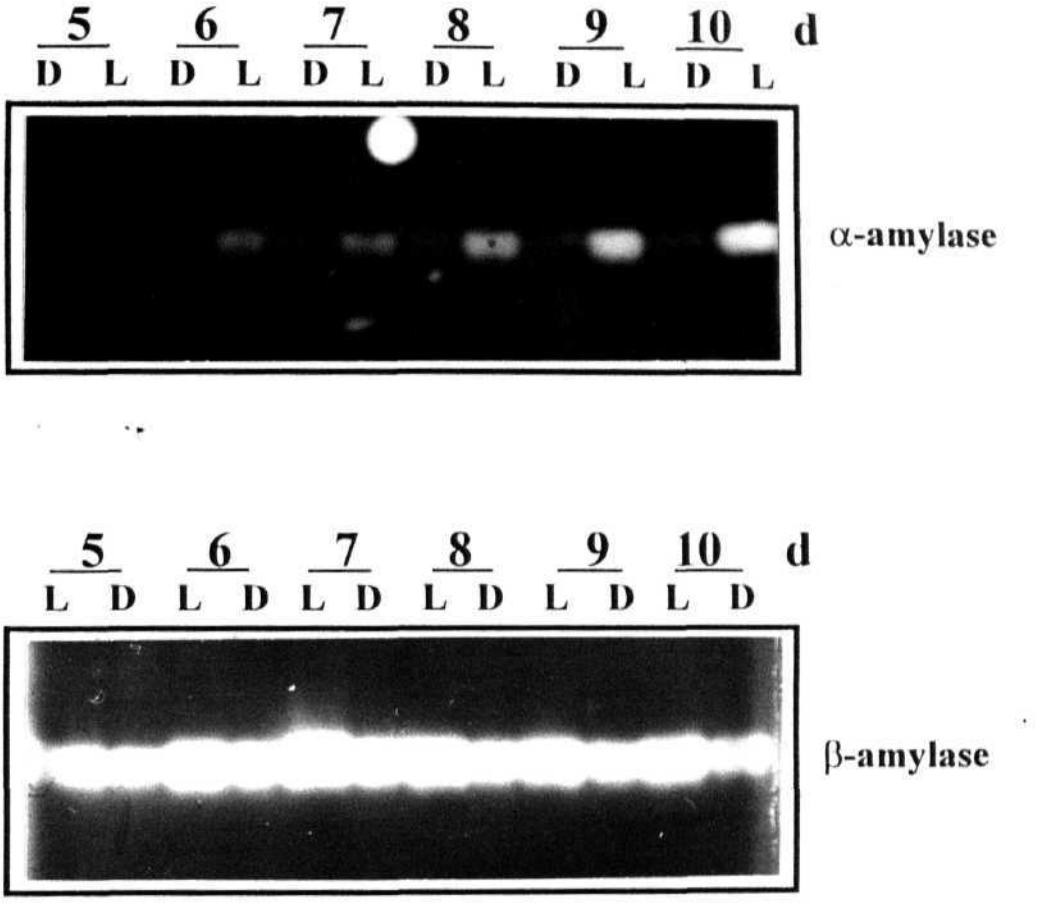


Figure 4.15 Time course of amylase activity in the first leaf of 5-10 d-old red light (L) and dark (I)) grown maize seedlings. a-Amylase bands were visualized by contact printing of the gel after non-denaturing PAGE on a ß-limit dextrin agar gel. B-Amylase bands were visualized using amylose as the substrate.

leaves follow a similar pattern to that of light-grown leaves, but the level of amylase activity is much lower.

Western blotting of the same extracts confirm the above pattern, indicating that the increase in the amylase activity with age is due to the increase in the protein level of of both a- and p-amylase protein which could be due to either increased synthesis or reduced degradation of the protein (Fig 4.16). Since 7-d-old leaves were found to contain the highest level of p-amylase and reasonably high level of α-amylase, subsequent experiments on spatial expression of amylases were done using 7-d-old seedlings.

4.6.2. Distribution of amylases in different organs

The distribution of amylases was studied in all the organs of 7-d-old seedlings by amylase assay and Western blotting using a- and P-amylase antiserum. Root, mesocotyl and coleoptile had very low levels of a-amylase which was barely detectable by amylase assay and Western blotting. Leaves possess appreciable levels of a-amylase which is more in the second leaf when compared to the first leaf. Seeds have the highest level of a-amylase. Dark-grown seedlings have consistently lower levels of a-amylase than light-grown leaves. Induction by light is, however, not observed in the seeds, and both dark and light-grown seedlings show equal amounts of amylases in seed. Light-induction of amylases is observed in all other organs of the 7-d-old seedlings (Fig 4.17).

On immunoblotting, the highest level of P-amylase was observed in the seeds, followed by the leaves. Other organs like the root, mesocotyl and coleoptile also showed P-amylase which could be detected by both enzyme assay and Western blotting. Similar to a-amylase, in p-amylase also, photoinduction was not observed in seeds, but was apparent in all other organs of the seedlings (Fig 4.18).

4.6.3 Spatial distribution of amylases in the first leaf

The first leaf from 7-d-old seedlings were excised at the mesocotyl junction and cut into 1 cm long segments from the base to the tip. Amylase activity was estimated in each of these segments by enzyme assay.

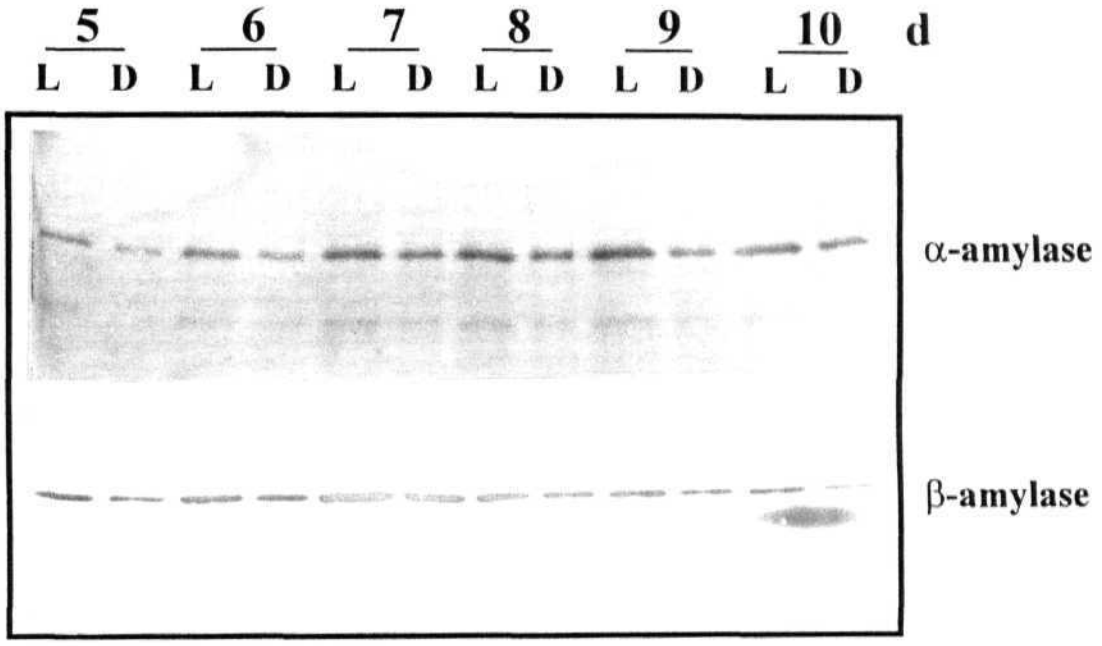


Figure 4.16 Time course oramylasc level in the first leaf of 5-10 d-old red light **(L)** and dark (1)) grown maize seedlings. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti a- and p-amylase antibodies.

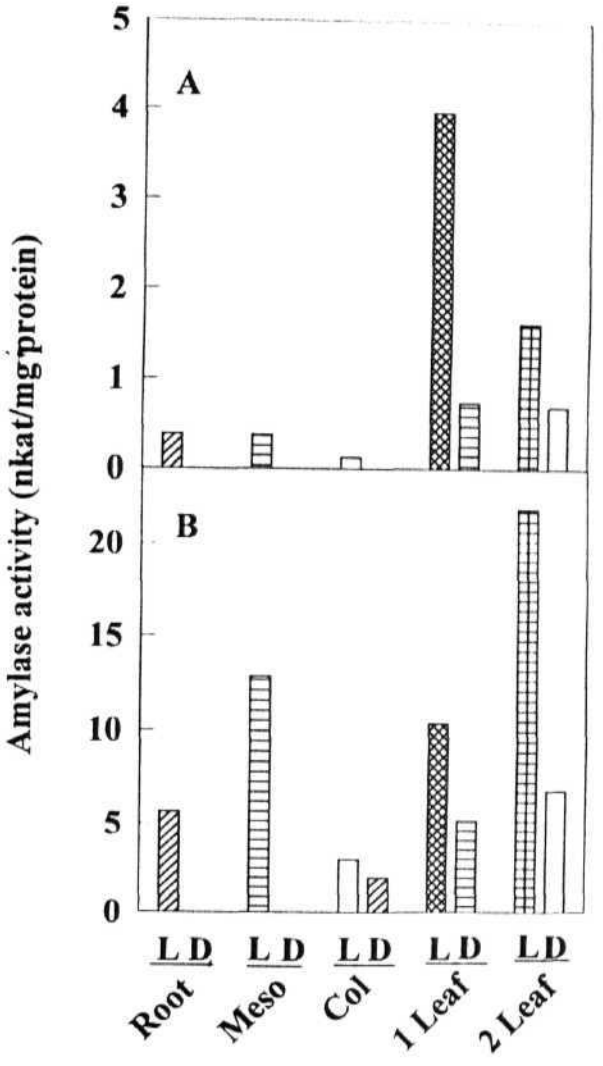


Figure 4.17 Amylase activity in various organs of 7-d-old maize seedlings grown under red light (L) or darkness **(D)**. Seedlings were excised into **seed, root,** mesocotyl **(meso),** coleoptile **(col),** first leaf **(1 Leaf)** and second leaf **(2 Leaf).** Crude extracts of the above organs were assayed for a-amylase (A) or β-amylase (B) activity.

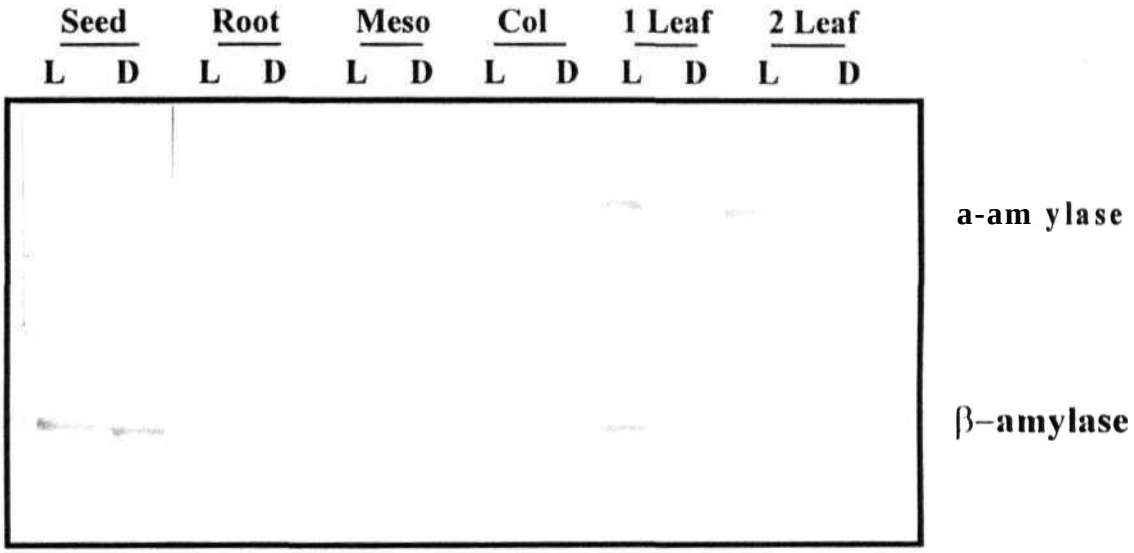


Figure 4.18 Detection of amylases in various organs of red light (L) and dark (D) grown maize seedlings. Seedlings were excised into **seed, root,** mesocotyl **(meso),** coleoptile (**col**), first leaf **(1 Leaf)** and second leaf **(2 Leaf).** Crude extracts of the above organs were subjected to SDS-PAGE and immunoblotting using anti a- and β-amylase antibodies.

4.6.3.1 α-Amylase

α-Amylase activity was below the detectability limit in dark-grown leaves by enzyme assay (Fig 4.19). However, on ND-PAGE, α-amylase-3 and α-amylase-4 could be detected in dark-grown leaves and the activity of both these isozymes increased towards the tip of the leaf. On the other hand, α-Amylase-1 and α-amylase-2 could not be detected in the dark-grown leaves (Fig 4.20). In contrast to dark-grown leaf, in red light-grown seedlings, though α-amylase activity was barely detectable in the first three segments of the leaf, in the next 4 segments of the 7 cm long leaf, the a-amylase activity increased gradually and was maximum at the tip of the leaf (Fig 4.19).

The ND-PAGE of RL-grown leaf showed all 4 isozymes of a-amylase. a-amylase-4, which is the most abundant isozyme in the leaf and all other amylases showed a distribution pattern similar to that obtained by assay, with increasing activity towards the leaf tip. α-Amylase-3 and -4 were detected from the first segment onwards. a-Amylase-1 and -2 appeared in the light-grown seedlings and showed a gradient of increasing activity towards the tip (Fig 4.20). Interestingly, in both dark and light-grown leaves, activity could not be detected in some cases by enzyme assay, but the same could be detected by activity staining. It is likely that since the *in vitro* assay was carried out only for 30 min, low levels of enzyme could not be detected. The same could however be detected by contact printing of the ND-polyacrylamide gel after prolonged incubation for 8 h.

To analyze if the a-amylase distribution pattern in light-grown leaf resulted from the inductive influence of light, when 6-d-old dark-grown seedlings were transferred to red light for 24 h. A 24 h RL exposure induced a-amylase activity almost to the level of continuously red light-grown seedlings (Fig 4.19). The ND-PAGE revealed that transfer from RL to dark led to the induction of α-amylase-4 activity, but this period of exposure to light was not sufficient for the appearance of α-amylase-1 and -2 isozymes (Fig 4.20).

Invariably both by ND-PAGE and activity assay, a-amylase activity in leaf showed an increasing level towards the tip of the leaf. Western blotting confirmed the above pattern of increasing protein towards the tip of the leaf (Fig 4.21). The Western blots were quantitated by densitometric scanning (Fig 4.22). In all the three cases, the profiles obtained ND-PAGE, enzyme assay and Western blotting, as confirmed by

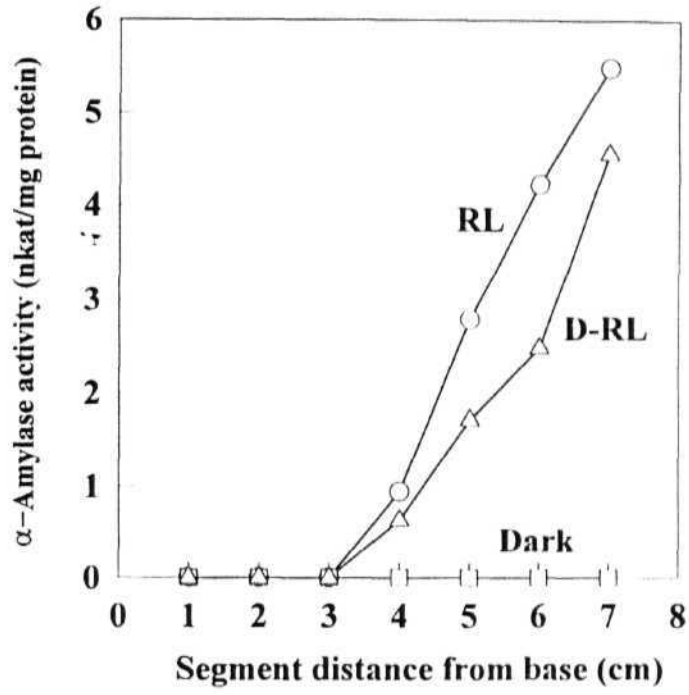


Figure 4.19 The relative photostimulation of a-amylase activity in different sections (1 cm) of 7-d-old maize primary leaf. The segments are numbered from the base (1) to the tip of the leaf. Seedlings were either grown in continuous red light (RL), darkness **(Dark)** or 6 d in darkness followed by 1 d in red light (D-RL). a-Amylase activity was expressed on the basis of nkat mg protein.

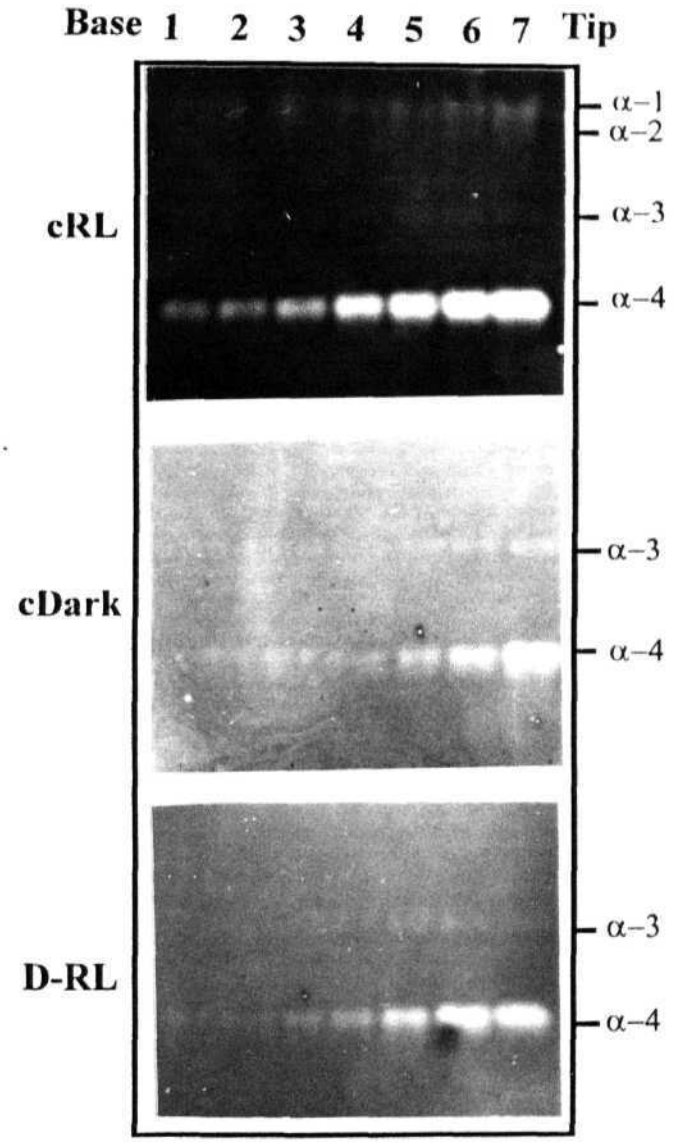


Figure **4.20** Photostimulation of a-amylase activity in 1 cm long sections of the first leaf of 7-d-old seedlings grown in red light **(cRL),** dark **(cDark)** and 6-d-old dark-grown seedlings transferred to light for 24 h (D-RL). The segments arc numbered from the base (1) to the tip of the leaf. The α-amylase bands were visualized by contact printing on a β-limit dextrin agar gel after non-denaturing PAGE. The symbol a- indicates the positions of a-amylase isoforms. The a-amylase isoforms are numbered in an order based on their increasing mobility.

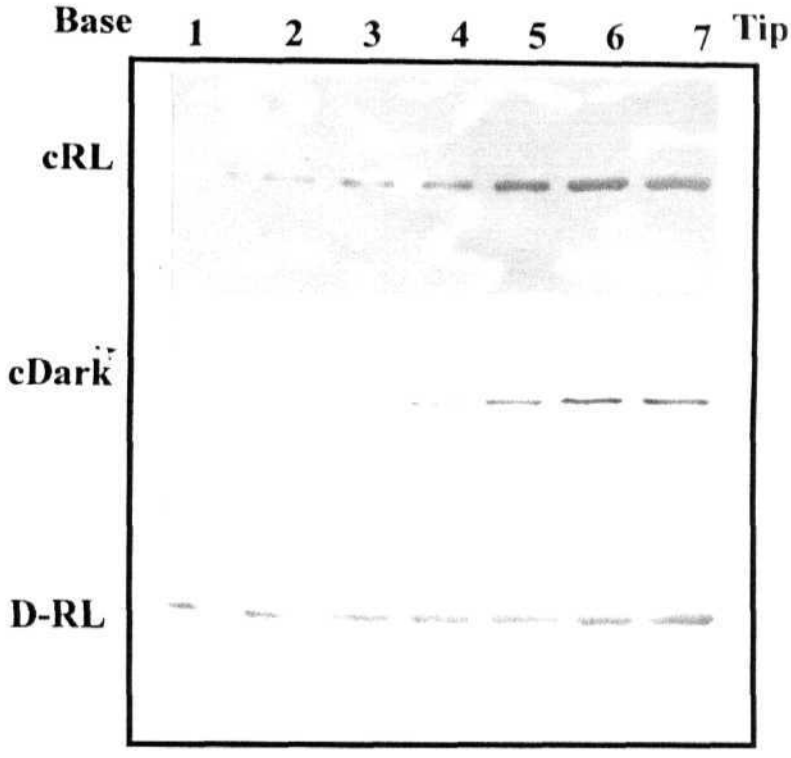


Figure 4.21 Photostimulation of α-amylase level in 1 cm long sections of the first leaf of 7-d-old seedlings grown in light **(cRL),** continuous dark **(cDark)** and 6-d-old dark-grown seedlings transferred to light for 24 h (D-RL). The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti α-amylase antibodies.

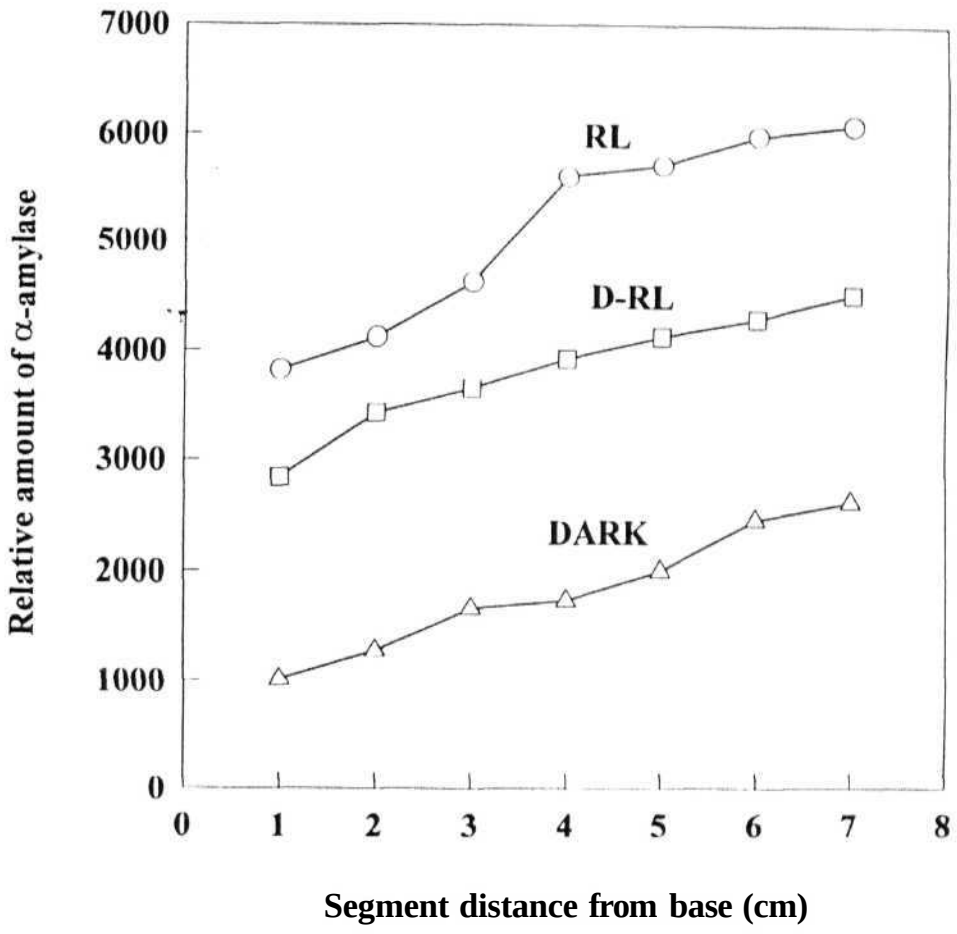


Figure 4.22 Distribution of a-amylase protein level in 1 cm long sections of leaf in 7-d-old seedlings grown in red light (RL), dark **(Dark)** and 6-d-old dark-grown seedlings transferred to red light for 24 h **(D-RL)**. The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti a-amylase antibodies. The level of a-amylase was quantitated by densitometric scanning of the blot displayed in the fig. 4.21.

scanning data, were nearly similar, showing increasing α-amylase level towards the leaf tip.

4.6.3.2 β-Amylase

In dark-grown leaves P-amylase distribution pattern was similar to that of α-amylase with increasing activity towards the tip of the leaf (Fig 4.23). However in contrast to α-amylase, in light-grown seedlings, the distribution of p-amylase followed a different pattern. Estimation of P-amylase activity in 7-d-old leaf segments showed low level of P-amylase activity at the base followed by maximal activity (7-fold induction) in a region 1-2 cm away from the base, and a gradual decline towards the tip of the leaf (Fig 4.23). Red light exposure of etiolated seedlings for 24 h stimulated P-amylase activity in the second segment and shifted the distribution pattern, making it similar to that of continuously red light-grown seedlings. Thus, light exposure not only stimulated β-amylase activity, but it also shifted the stimulation towards the base.

Isozyme pattern of p-amylase was similar to the pattern observed by enzyme assay in RL, D and transferred leaves (Fig 4.24). Also, the same pattern was obtained by western blotting (Fig 4.25), as confirmed by the densitometric scanning of the blots (Fig 4.26), which shows that the photostimulation observed in the second segment results from the increased level of p-amylase protein in the basal region of the leaf.

4.7. The photostimulation of amylases is mediated by phytochrome

To find out if the photostimulation of amylase is mediated by phytochrome, reversibility of red light exposure by far red exposure was examined. In 4 d old etiolated seedlings, a 10 min exposure to RL led to a stimulation that was equivalent to that of continuous RL-grown seedlings. Whereas, a 10 min FRL pulse did not alter the activity. The inductive effect of the RL pulse was reversed by following it with a FRL exposure, indicating that the stimulation of amylase activity by RL is mediated by phytochrome (Fig 4.27).

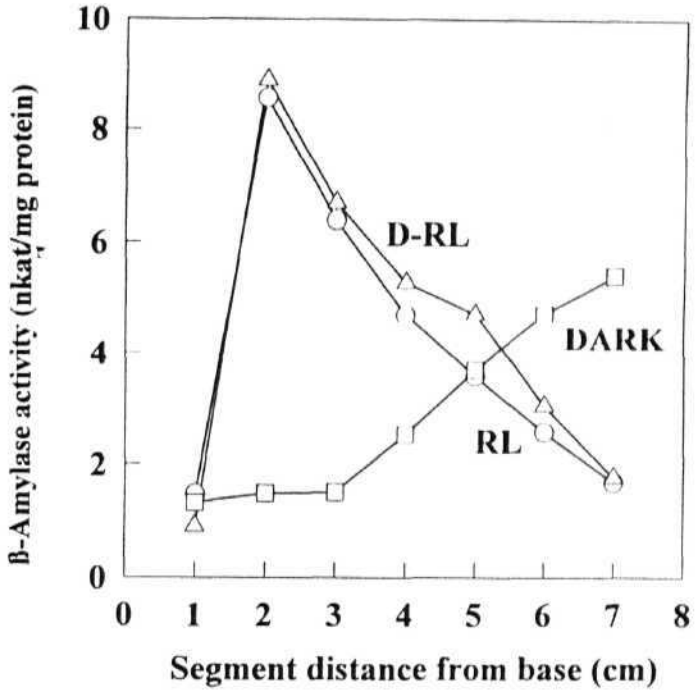


Figure 4.23 The relative photostimulation of β-amylase activity in different sections (1 cm) of 7-d-old maize primary leaf. The segments are numbered from the base (1) to the tip of the leaf. Seedlings were either grown in continuous red light **(RL)** , darkness (I)) or 6-d in darkness followed by 1 d in red light (D-RL). p-Amylase activity was expressed on the basis of mg protein.

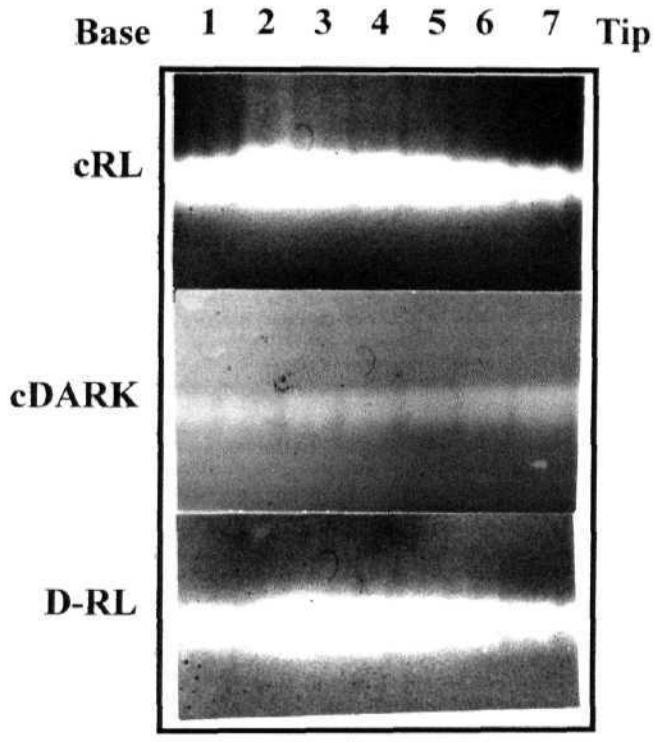


Figure 4.24 Photostimulation of β-amylase activity in 1 cm long sections of the first leaf of 7-d-old seedlings grown in red light **(cRL),** continuous dark **(cDark)** and 6-d-old dark-grown seedlings transferred to light for 24 h (D-RL). The segments are numbered from the base (1) to the tip of the leaf. The [3-amylase bands were visualized by enzyme staining using amylose as the substrate.

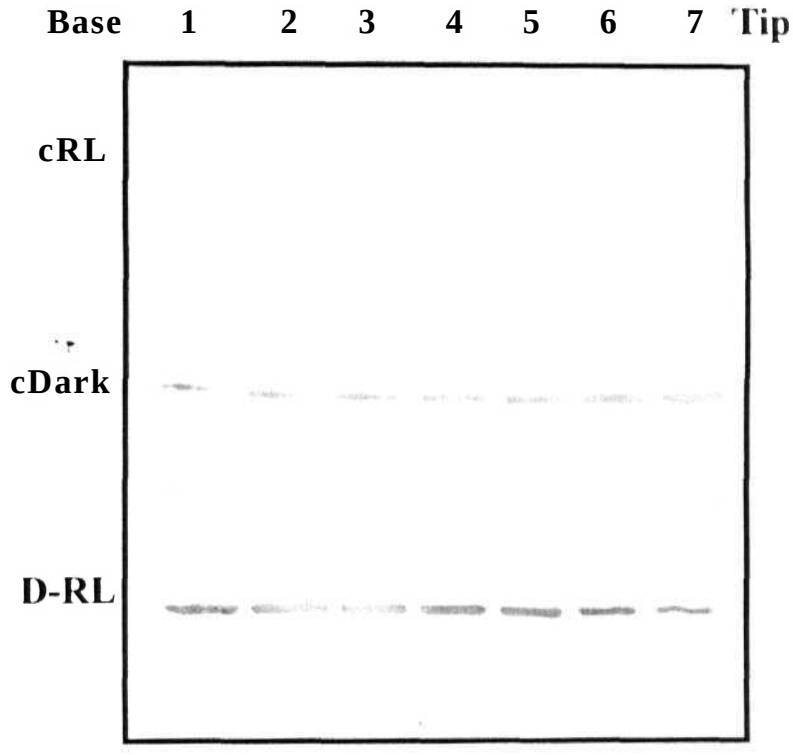


Figure 4.25 Photostimulation of (3-amylase level in 1 cm long sections of the first leaf of 7-d-old seedlings grown in light **(cRL),** continuous dark **(cl)ark)** and 6-d-old dark-grown seedlings transferred to light for 24 h (D-RL). The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and iinmunoblottiiig using anti β-amylase antibodies.

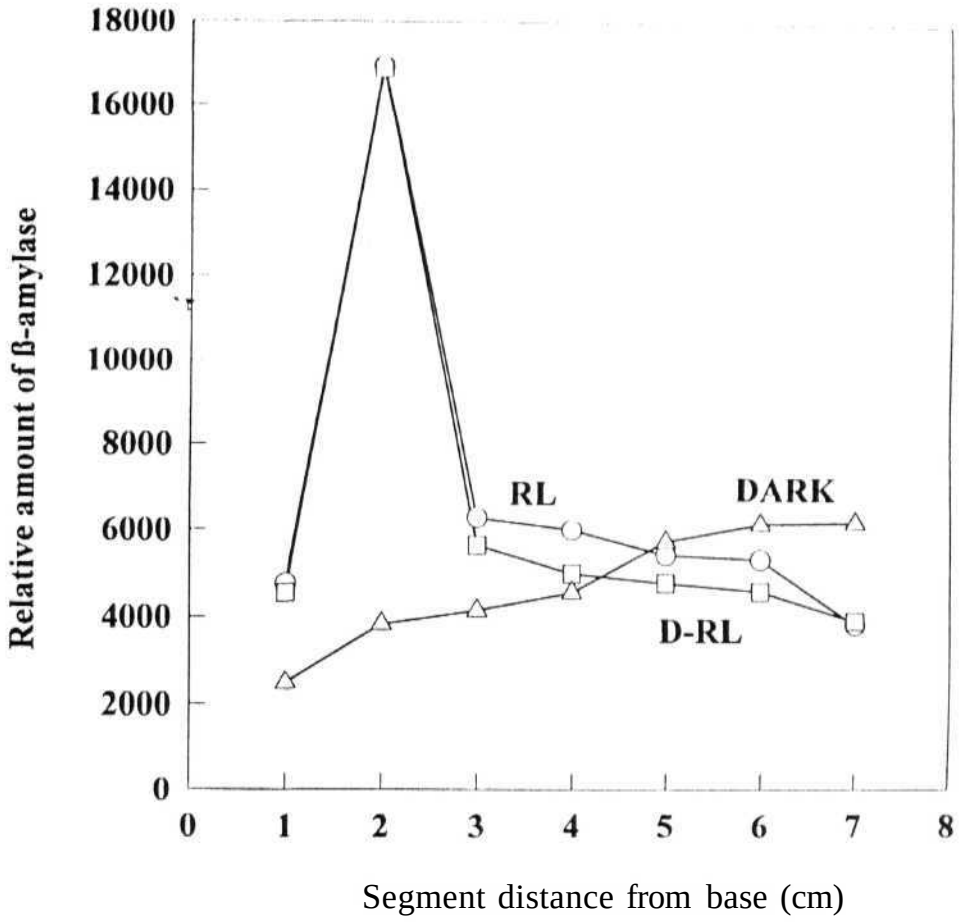


Figure 4.26 Photostimulation of β-amylase level in 1 cm long sections of leaf in 7-d-old seedlings grown in red light **(RL)**, dark **(DARK)** and 6-d-old dark-grown seedlings transferred to red light for 24 h (D-RL). The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti β-amylase antibodies. The level of β-amylase protein was quantitated by densitometric scanning of the blots displayed in the fig 4.25.

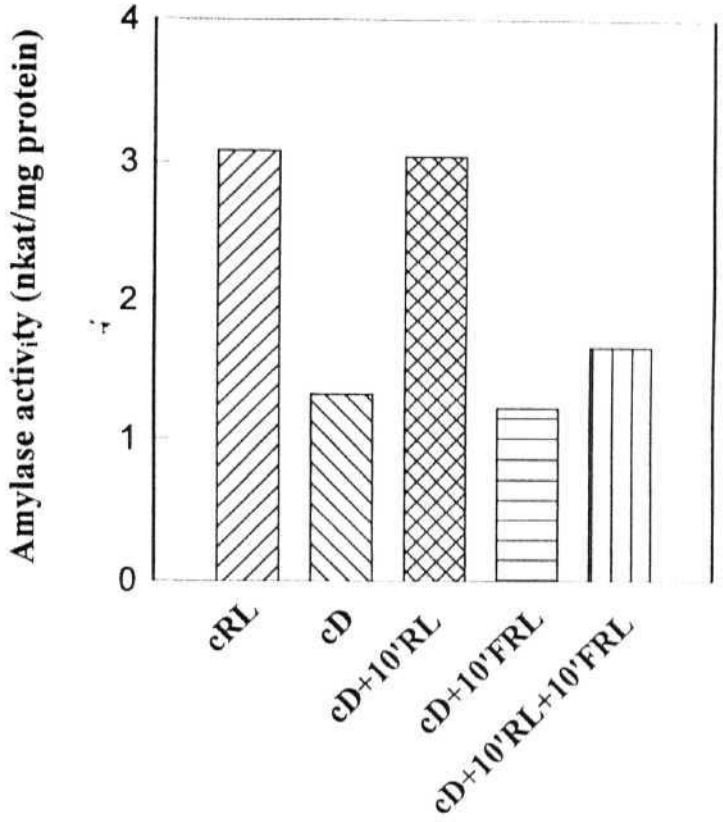


Figure 4.27 Phytochrome control of amylasc photostimulation in maize seedlings. Seedlings were grown in darkness for 3 d, after which they were exposed to 10 min red light, 10 min far red light or 10 min red light followed by 10 min far red light pulses. The seedlings were returned to darkness for a further period of 24 h. Control seedlings were grown in continuous darkness or red light. Amylase activity was assayed and expressed as nkat/ mg protein.

4.8. Intra- and Inter-cellular localization of α-Amylases

Since transitory starch is generated in the chloroplasts during photosynthesis, **which** is degraded during the dark period and transported out of the chloroplasts, it is reasonable to assume that leaf α-amylases are localized in the chloroplasts. Therefore, experiments were done to locate amylases in maize chloroplasts. The intactness of the chloroplasts isolated by sucrose density gradient was checked by ferricyanide reduction test. Ferricyanide was not reduced by the intact chloroplasts, but the reduction could be observed in broken chloroplasts after osmotic shock (data not shown). In order to check for contamination by cytosolic enzymes, PEP Carboxylase was assayed in the isolated Chloroplast preparation after sonication to lyse the chloroplasts. In the chloroplasts preparation, PEPC activity could not be detected, whereas in the cytosolic fraction, PEPC activity was detected (data not shown).

In comparison to cytosol, chloroplasts contain very low amylase activity. Table 2 shows that about 70 % of the amylase activity is localized in the cytosol. The amylase present in the chloroplasts could degrade both amylose and β-limit dextrin with equal efficiency. The α-amylase activity was also checked in isolated etioplasts and etioplasts failed to show any a-amylase activity.

4.9 Localization of amylases in mesophyll and bundle sheath cells

Maize being a C-4 plant consists of two kinds of chloroplasts, the mesophyll and the bundle sheath chloroplasts. In order to characterize the amylase present in these chloroplasts, and localize amylases in various intra- and inter-cellular compartments, mesophyll and bundle sheath protoplasts were isolated. Chloroplasts were separated from the protoplasts and amylases were assayed in the chloroplastic as well as cytosolic fractions. Bundle sheath strands were also isolated by mechanical method and bundle sheath protoplasts were obtained from these strands by enzymatic digestion. Further fractionation of the bundle sheath protoplasts yielded bundle sheath chloroplasts and cytosolic fraction.

Assay of amylases in isolated cell fractions showed that β-amylase is absent in the bundle sheath protoplasts, but it is present only in the mesophyll cells. On a relative scale, In mesophyll cells, 33% of the total amylolytic activity was found to be a-

amylase, **and** the remaining was P-amylase (Table 2). Thus P-amylase is the major form of amylase in the mesophyll cells. β-Amylase was absent in the chloroplasts of both mesophyll and bundle sheath cells. In mesophyll cells, about 15% of the total α-amylase activity of the cell was localized in the chloroplasts. In bundle sheath cells, about 40% of **the** a-amylase activity is chloroplastic and the rest is cytosolic in localization (Table 2).

4.10 Properties of chloroplastic a-amylase

The physico-chemical properties of a-amylase present in chloroplasts was also analyzed. ND-PAGE was used for assigning the a-amylase to chloroplastic and non-chloroplastic groups. Chloroplastic a-amylase treated with 10 mM EDTA lost 70% of its activity, and only 30% was retained. Whereas, when 10 mM EDTA was supplemented with 10 mM $CaCl_2$, 85% of the amylase activity was retained. This showed that calcium is required for the activity of chloroplastic a-amylase and removal of calcium lead to the inactivation of the enzyme (Table 2).

In mesophyll cells, all the four isozymes of a-amylase and a single isozyme of β-amylase were present. In the cytosolic fraction, α-amylase-3 and -4 and P-amylase are present, whereas α-amylase-2 is exclusively chloroplastic in localization. α-Amylase-1 could not be detected both in the chloroplastic and cytosolic fractions after cell fractionation. In the bundle sheath cells, P-amylase was absent. At the same time bundle sheath cells possessed all the four isozymes of a-amylase, out of which a-amylase-2 is chloroplastic and a-amylase-3 and -4 are extra-plastidic in localization (Fig 4.28).

Contact printing of Chloroplast extract after ND-PAGE on a ß-limit dextrin agar gel confirmed that a-amylase-2 is chloroplastic in localization in both mesophyll and bundle sheath cells. Both these chloroplasts do not contain any other isozyme of amylases (Fig **4.29).**

Western blotting of the various cell fractions shows that etioplasts do not contain significant amount of a-amylase protein. α-Amylase-1,2 was detected in both mesophyll and bundle sheath cells. α-Amylase-3,4 was absent in the chloroplasts. It was found in both mesophyll and bundle sheath cells. P-Amylase was localized only in the mesophyll cells, **but** not in the bundle sheath cells or in the plastids (Fig 4.30).

Table 2: Compartnientation of Aniylases

Cell Fraction	Amylase Activity (nKat/mg protein) α	β	% α-Amylase
Mesophyll chloroplasts	0.26	--	15.4
Mesophyll cytosol	0.81	3.28	47.95
Bundle sheath chloroplasts	0.25	--	14.5
Bundle sheath cytosol	0.37	--	22.1
Chloroplast + 10 mM EDTA +10 mM $CaCl_2$	0.21	--	85
Chloroplast +10 mM EDTA	0.08	--	30

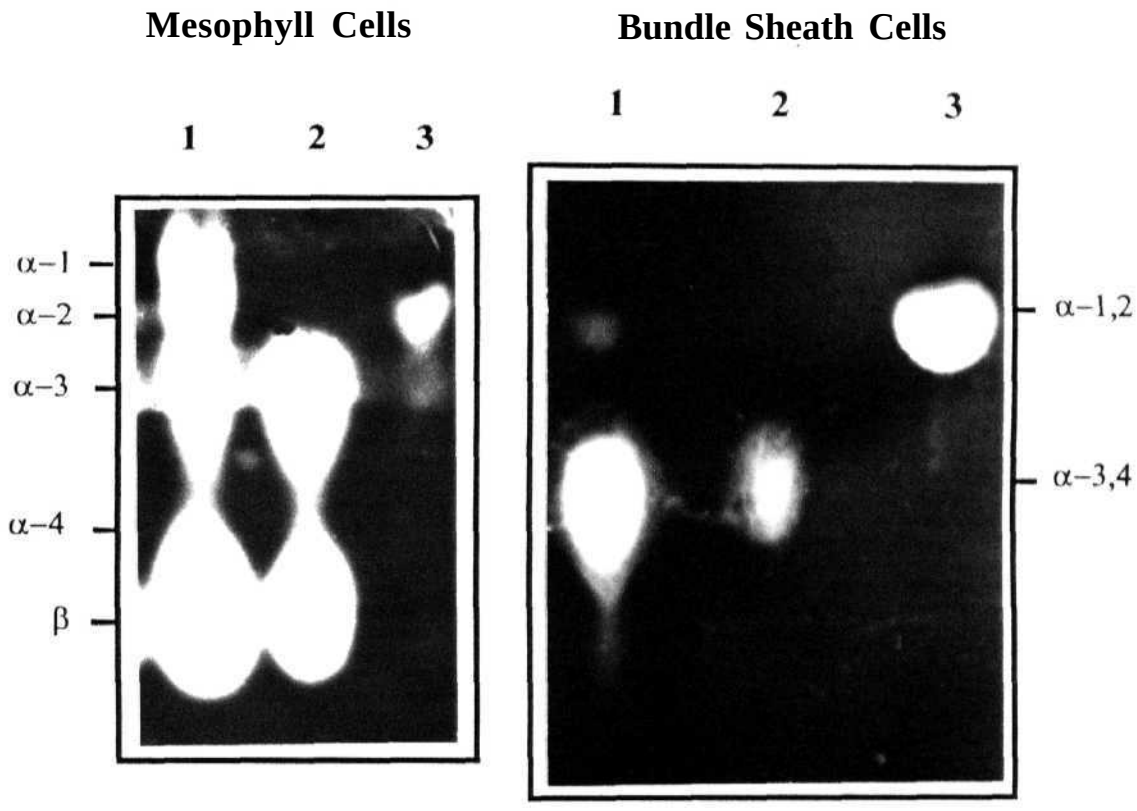


Figure 4.28 Distribution of amylase isoforms in isolated mesophyll and bundle sheath cells. Extracts from chloroplastic and cytosolic fractions of mesophyll and bundle sheath cells were subjected to non-denaturing PAGE and stained for amylase activity using amylose as the substrate. Lane 1, Protoplasts; Lane 2, Cytosolic fraction; 3, Chloroplastic fraction. The symbols a- and β- indicates the positions of a- and β-amylase isoforms respectively.

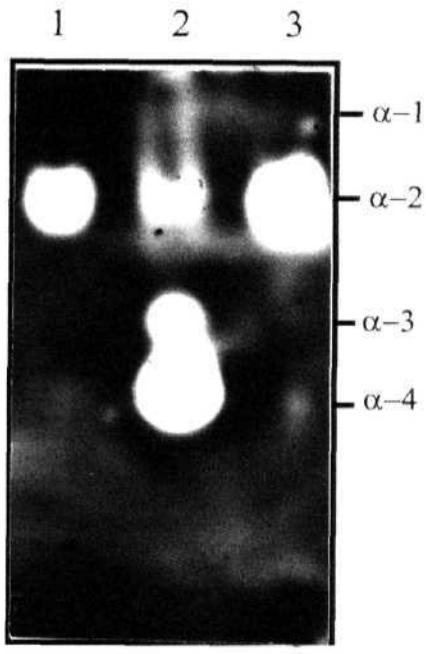


Figure 4.29 Isoforms of chloroplastic a-amylase. Crude leaf extract and Chloroplast extracts from mesophyll and bundle sheath cells were subjected to non-denaturing PAGE. The isoforms were visualized by contact printing the gel on a ß-limit dextrin agar gel. Lane 1, Mesophyll chloroplasts; Lane 2, Leaf extract; Lane 3, Bundle sheath chloroplasts. The symbol α- indicates the positions of a-amylase isoforms.

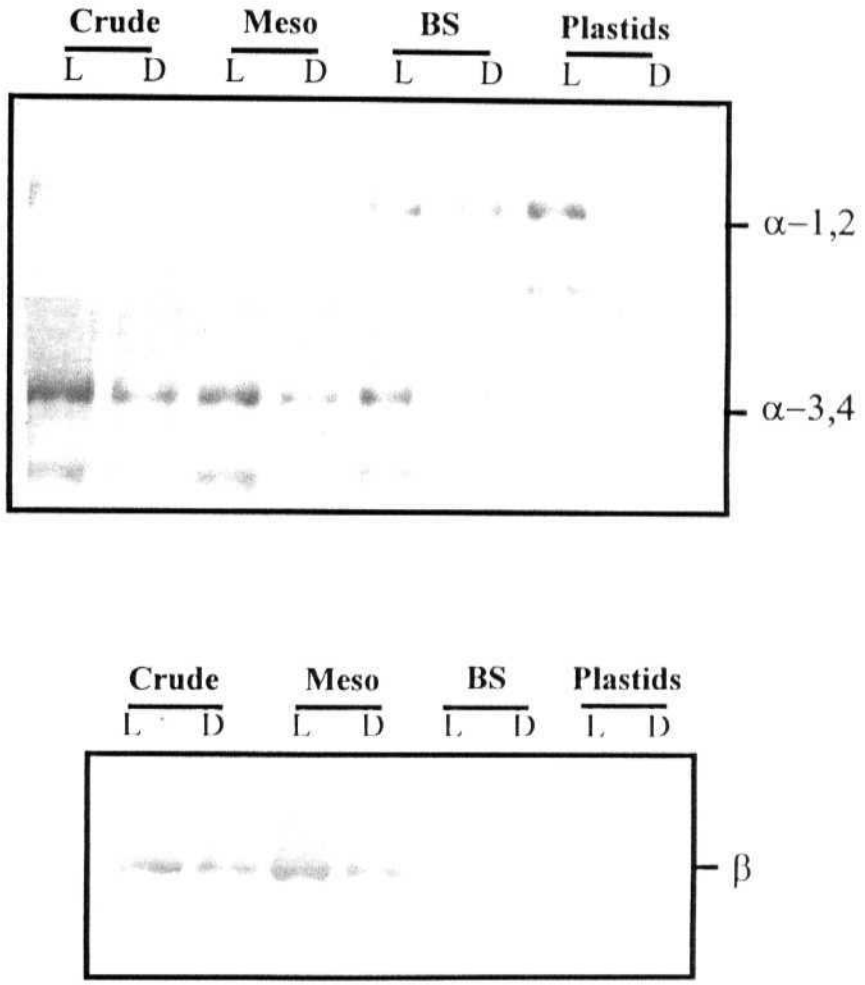


Figure 4.30 Detection of amylases from dii'ferent cell fractions of red light (L) and dark **(D)** grown seedlings by immunoblotting. Extracts from different cell fractions like mesophyll protoplasts (Meso), bundle sheath protoplasts (BS) and plastids were separated by SDS-PAGE and subjected to Western blotting. Blots were probed against α-amylase1/2; α-amylase 3/4 and β-amylase antibodies.

4.11 Purification of chloroplastic amylase

Attempt was made to purify the chloroplastic a-amylase by using CHA-Sepharose chromatography. Since antigenically, chloroplastic a-amylase is similar to seed α-amylase-1,2, attempt was made to purify and compare both α-amylase-1,2 from seed and the chloroplastic a-amylase. While seed a-amylase could be easily purified by CHA-Sepharose chromatography, and the isozymes could be separated by DEAE-Sepharose chromatography into α-amylase-1,2 and α-amylase-3,4, it was difficult to follow up the purification steps in the case of the chloroplastic a-amylase, due to its very low abundance. After concentration of the eluate from CHA-Sepharose column, and blotting against α-amylase-1,2 antibodies of seeds, it was found that chloroplastic a-amylase has a molecular weight of 57 kD, as against 46 kD of seed α-amylase-1,2 (Fig 4.31).

The low amount of chloroplastic a-amylase precluded its purification. However, on Ouchterlony double immunodiffusion, the Chloroplast lysate showed a single precipitin line against α-amylase-1,2 antibodies, and this precipitin line could also be stained for amylase activity. In contrast, precipitin line was not observed in the case of extracts from NF grown leaves, and also from the etioplasts confirming the absence of a-amylase (Fig 4.32).

4.12 Reconstitution of seed and leaf α-Amylase

The fact that the 45 kD band observed by western blotting of leaf extracts probed against a-amylase-3,4 antibodies is due to a-amylase was ascertained by reconstitution of a-amylase activity after denaturation and separation by SDS-PAGE. The reconstitution of a-amylase activity was performed by exchanging SDS in the gel with Triton X-100. Thereafter, the gel was stained for activity using amylose as the substrate. Both seed and leaf a-amylase reconstituted at the same position with a molecular weight of around 45 kD (Fig 4.33). On the other hand, the 57 kD chloroplastic a-amylase could not be reconstituted, probably due to its low amount. Also β-amylase could not be reconstituted by this method. Thus only a-amylase-3,4 of leaf was reconstituted and the position of the reconstituted a-amylase from seed and leaf coincided with each other.

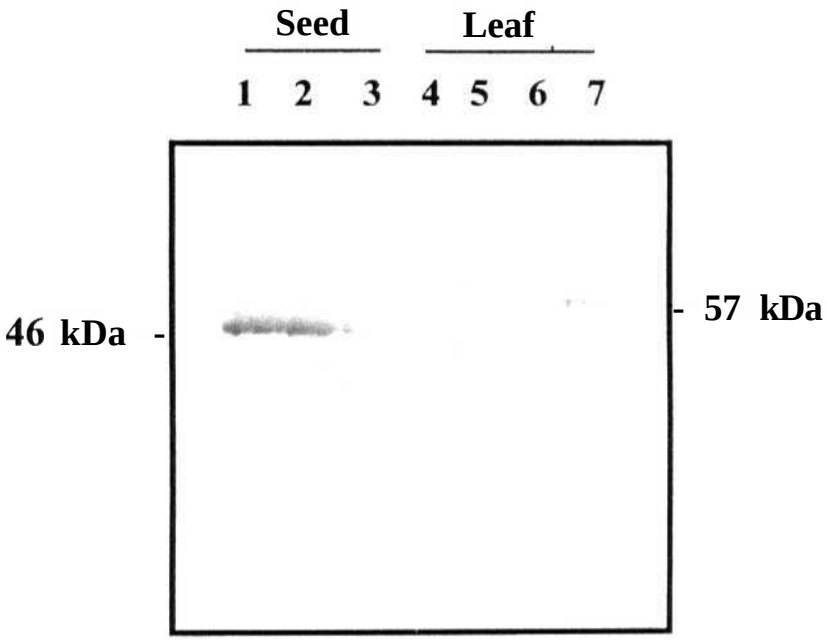


Figure 4.31 Purification of chloroplastic a-amylase. Aliquots from different stages of purification of seed and chloroplastic a-amylase were analyzed by immunoblotting against anti a-amylase 1/2 antibodies. Lane 1, Crude seed extract; Lane 2, Eluate of CHA-Sepharose column; Lane 3, Eluate of DE AE-Sepharose column; Lane 4, Crude leaf extract; Lane 5, Unbound fraction of CHA-Sepharose column; Lane 6, Eluate of CHA-Sepharose column; Lane 7, Eluate of CHA-Sepharose column (concentrated).

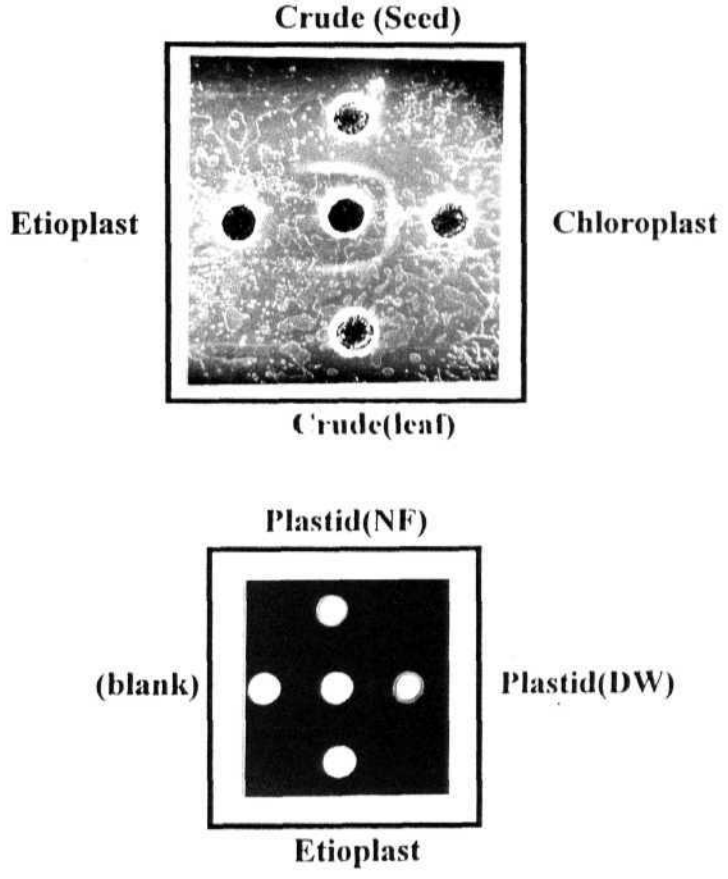


Figure 4.32 Comparison of seed and chloroplastic α-amylase by Ouchterlony double immuno-diffusion. The central wells were loaded with anti α-amylase-1,2 antibodies. The peripheral wells were loaded with seed, leaf and plastid extracts from samples grown in distilled water (DW) or Norflurazon (NF). The bottom photograph represents the activity staining of the precipitin line formed.

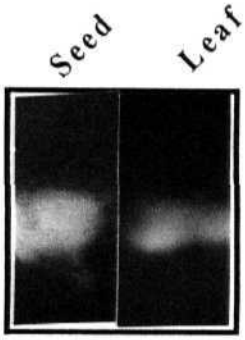


Figure 4.33 Renaturation of denatured a-amylase after SDS-PAGE. Crude extracts from seed and **leaf** were subjected to SDS-PAGE. SDS was exchanged with Triton X-100 and amylase bands were detected by enzyme staining using amylose as the substrate.

4.13 ft-Amylase is localized in the vacuole

This study on intra-cellular fractionation shows that p-amylase is exclusively localized in the extraplastidic compartment of the cell. In view of previous reports of pea and wheat β-amylases being localized in the vacuoles (Ziegler and Beck, 1986), the possibility of a vacuolar localization of p-amylase was examined. Intact protoplasts were isolated from maize leaves, and ruptured to obtain chloroplasts and vacuoles, which were separated. The purity of the vacuolar preparation was checked by assaying acid phosphatase activity as a marker enzyme. About 95% of the total P-amylase activity was found in the isolated vacuoles (Table 4.3). Vacuolar extract was compared with total protoplast extract byNDPAGE followed by enzyme staining and also by western blotting (Fig 4.34). The vacuolar extract showed only P-amylase activity, no α-amylase could be detected in the vacuolar extract.

4.14 Effect of NFon amylase activity

The interrelationship between the photoregulation of amylases and Chloroplast biogenesis was studied in NF-treated seedlings. NF is a herbicide which inhibits carotenoid biosynthesis thereby causing photooxidation of chloroplasts resulting in albino seedlings under white light or continuous red light. Maize seedlings were grown in NF right from the time sowing. While NF treatment completely abolished a-amylase activity, P-amylase activity increased several folds in light-grown seedlings. NF-treated seedlings possessed about 7-fold more P-amylase when compared to the dark control. In dark-grown seedlings, NF did not affect the p-amylase activity.

On analysis of P-amylase distribution in NF-treated seedlings, it was found that the distribution of p-amylase along the leaf remains the same as that of the distilled water grown control seedlings, with the maximum activity in the second segment from the base. Nevertheless, there is super-induction of P-amylase in the second segment, and the enzyme activity is about 5-fold higher than red light-grown control seedlings (Fig 4.35). Analysis of P-amylase isozyme by ND-PAGE and protein by western blotting confirmed that NF leads to an induction of P-amylase activity in the maize leaf, but it does not influence the pattern of P-amylase distribution gradient along the leaf axis (Fig 4.36 and 4.37).

Table 3 : Localization of Amylase in Vacuoles

Cell Fraction	Amylase Activity (nkat/mg protein) α	β	Acid Phosphatase (μM of pnp released)
Crude extract	1.03	4.69	20.5
Protoplasts	0.830	3.51	13.5
Vacuoles	0.0	0.78	3.2

Recovery of β-amylase **in** vacuoles - 22.4%
Recovery of acid phosphatase - 23.7%
Corrected value of (3-amylase in vacuoles - 94.6%

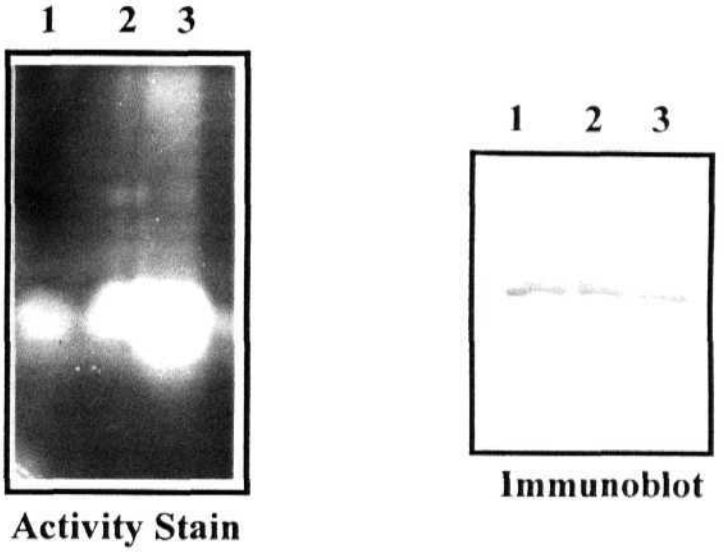


Figure 4.34 Localization of ß-amylase in vacuoles. Non-denaturing PAGE followed by enzyme staining for amylase using amylose as substrate; and SDS-PAGE followed by immunoblotting against anti-B-amylase antiserum. Lane 1, Vacuolar extract; Lane 2, Mesophyll protoplast extract; Lane 3, Crude extract.

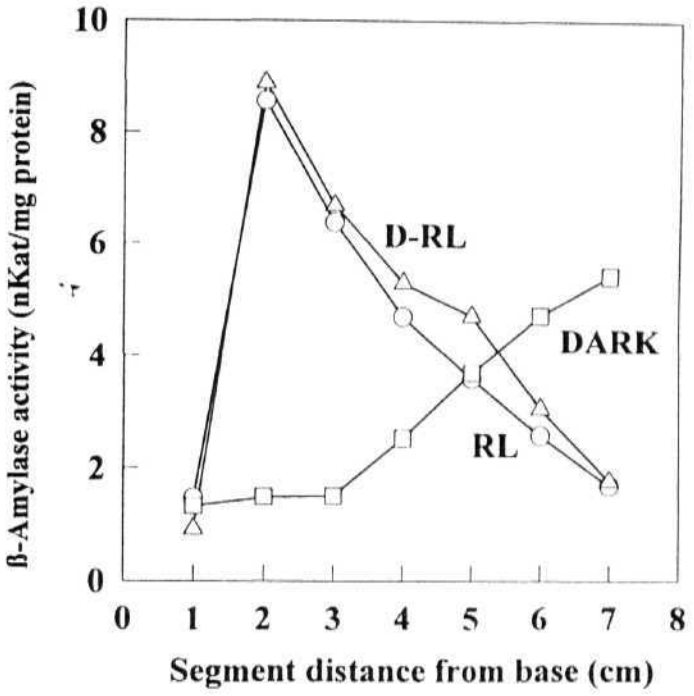


Figure 4.35 The relative photostimulation of β-amylase activity in different sections (1 cm) of 7-d-old maize primary leaf. The segments are numbered from the base (1) to the tip of the leaf. Seedlings were either grown in continuous red light (RL) or darkness **(D)** in distilled water (I)W) or in a solution of norflurazon (NF). β-Amylase activity was expressed as nkat/mg protein.

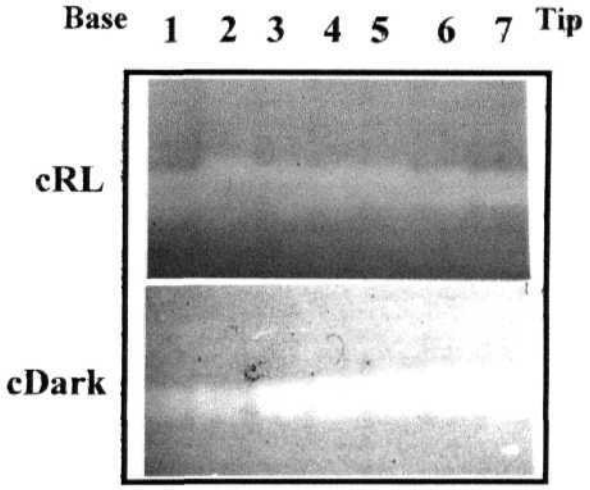


Figure 4.36 Photostimulation of p-amyiase activity in 1 cm Jong sections of the first leaf of 7-d-old NF treated seedlings grown in red light (cRL) and dark **(cDark).** The segments are numbered from the base (1) to the tip of the leaf. The ß-amylase bands were visualized by enzyme staining using amylose as the substrate.

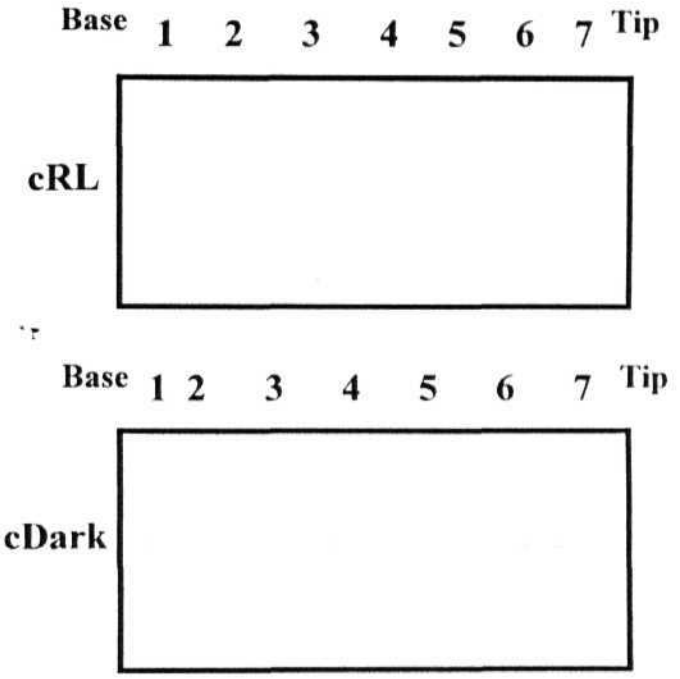


Figure 4.37 Photostimulation of (3-amylase level in 1 cm long sections of the first leaf of 7-d-old NF treated seedlings grown in red light **(cRL)** and dark **(cDark).** The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti ß-amylase antibodies.

In view of previous report of amylases being induced by various kinds of stresses like water stress, light stress and viral infection, etc. The effect of NF was speculated to be induced by light which acts as a stress factor in the absence of any pigments in albino leaves. Therefore, experiments were conducted to find out if p-amylase responds to water stress in the same manner as it responds to NF.

4.15 fl-Amylase is induced by water stress

Four-day-old maize seedlings were subjected to water stress by transferring them to various concentrations of sorbitol and sodium chloride for the next four days. With increasing concentration of both sorbitol and sodium chloride of upto 300 mM, an increase in p-amylase activity was observed in white light-grown seedlings. Further increase in concentration of sorbitol or sodium chloride did not produce any increase in P-amylase activity. The large induction observed in continuous white light-grown seedlings could also be obtained in seedlings transferred to white light from darkness for 24 h. In case of dark-grown seedlings also a slight increase in P-amylase activity could be observed in 300 mM sorbitol and sodium chloride (Fig 4.39). This pattern of β-amylase induction was also confirmed by ND-PAGE, which showed the same pattern as observed by p-amylase assay (Fig 4.40).

When the P-amylase induction by NF, sorbitol and sodium chloride was compared, all the three treatments increased p-amylase activity to almost the same extent in both white light-grown as well as in transferred seedlings. The stress induction was dependent on light as the effect of these treatments was minimum in dark-grown seedlings (Fig 4.41). The isozyme pattern confirmed the above finding. The pattern of p-amylase obtained by activity staining was similar to that observed by amylase assay (Fig 4.42). On immunoblotting it was found that the above stimulation in P-amylase due to stress is the result of increased level of p-amylase protein. The pattern obtained by western blotting closely resembled the pattern obtained by amylase assay (Fig 4.42). Thus the effect of NF on p-amylase activity was interpreted as the effect of light stress. Strong white light probably acts as a stress factor producing an effect similar to that of water stress on P-amylase.

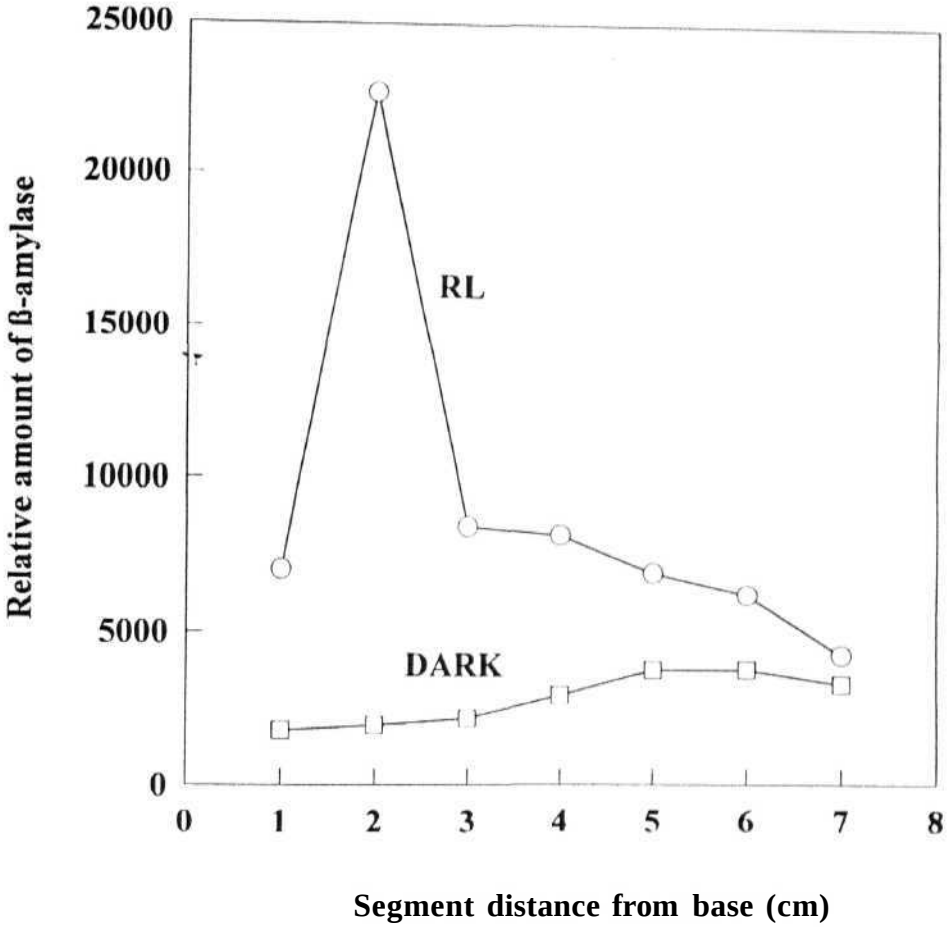


Figure 4.38 Photostimulation of β-amylase level in 1 cm long sections of leaf in 7-d-old Norllurazon treated seedlings grown in red light (UL) and darkness **(Dark).** The segments are numbered from the base (1) to the tip of the leaf. Crude extracts were subjected to SDS-PAGE and immunoblotting using anti ß-amylase antibodies. The level of fl-amylase was quantitated by densitometric scanning of the blots displayed in fig. 4.37.

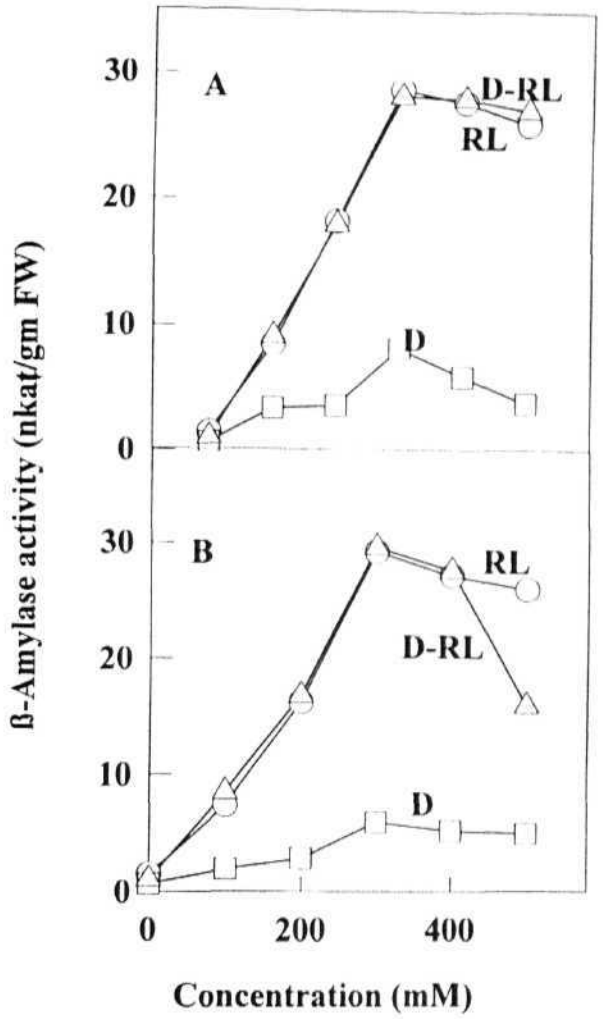


Figure 4.39 Effect of different concentrations of sorbitol and sodium chloride on β-amylase activity. Maize seedlings were grown for 4 d in distilled water and 4 d in different concentrations of sorbitol or sodium chloride. Seedling were grown under white light (WL), darkness (D) or 7 d in darkness and 1 d in white light (D-WL). The primary leaf was assayed for ß-amylase activity and expressed as nkat/gm fresh weight.

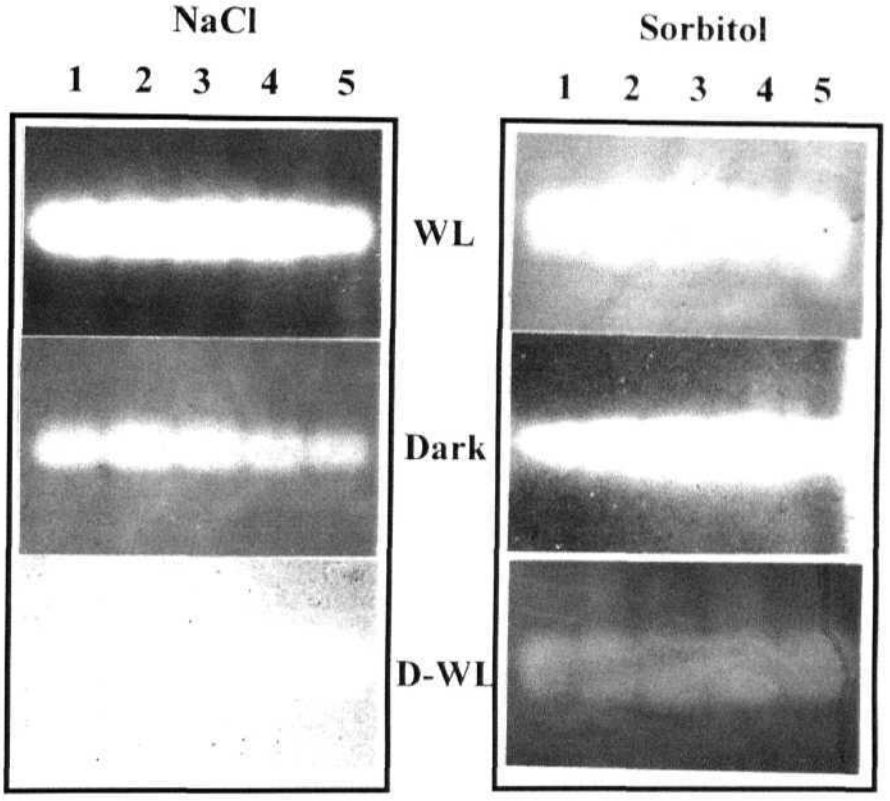


Figure 4.40 Effect of stress on B-amylase activity. Seedlings were grown for 4 d in DW followed by 4 d in different concentrations of sodium chloride or sorbitol in white light **(WL), dark** or 7 d dark + 1 d WL **(D-WL)**.β-Amylase from the first leaves of the seedlings were extracted and subjected to non-denaturing PAGE and enzyme staining using amylose as the substrate. Lane 1, 0.1 M; Lane 2, 0.2 M; Lane 3, 0.3 M; Lane 4, 0.4 M; Lane 5, 0.5 M.

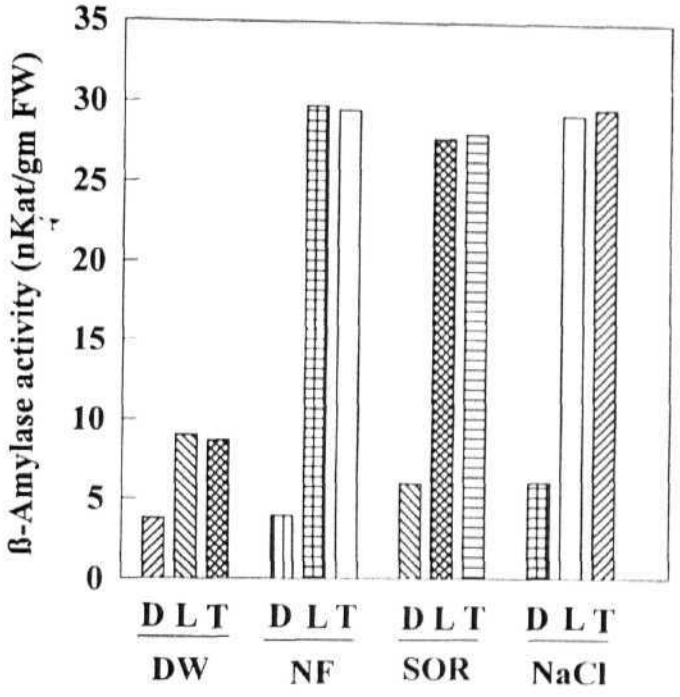


Figure 4.41 Effect of stress on β-amylase activity. Maize seedlings were grown for 4 d in distilled water and 4 d in 300 mM sorbitol **(SOR),** 300 mM sodium chloride **(NaCl)** or for 8 d in distilled water **(DW)** or a solution of norflurazon (NF). Seedling were grown under white light (L), darkness (D) or 7 d in darkness and 1 d in white light (T). The primary leaf was assayed for β-amylase activity and expressed as nkat/gm fresh weight.

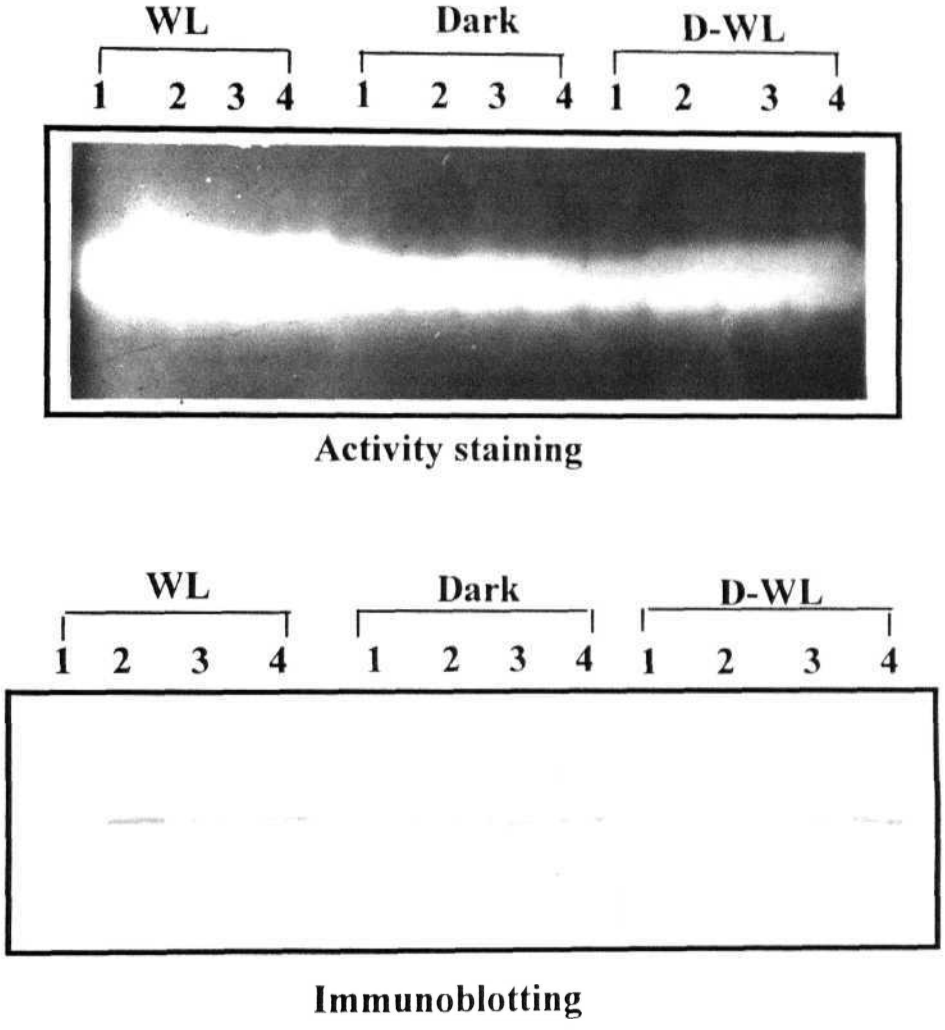


Figure 4.42 Effect of stress on β-amylase activity and protein level. Seedlings were grown in DW or NF for 8 d or for 4 d in DW followed by 4 d in 300 mM sodium chloride or 300 mM sorbitol in white light **(WL), dark** or 7 d dark + 1 d white light (D-WL). First leaves from the seedlings were extracted and subjected to non-denaturing PAGE and enzyme staining using amylose as the substrate or immunoblotted against anti ß-amylase antibodies after SDS-PAGE. Lane 1, Distilled water; Lane 2, Norflurazon; Lane 3, Sorbitol; Lane 4, Sodium chloride.

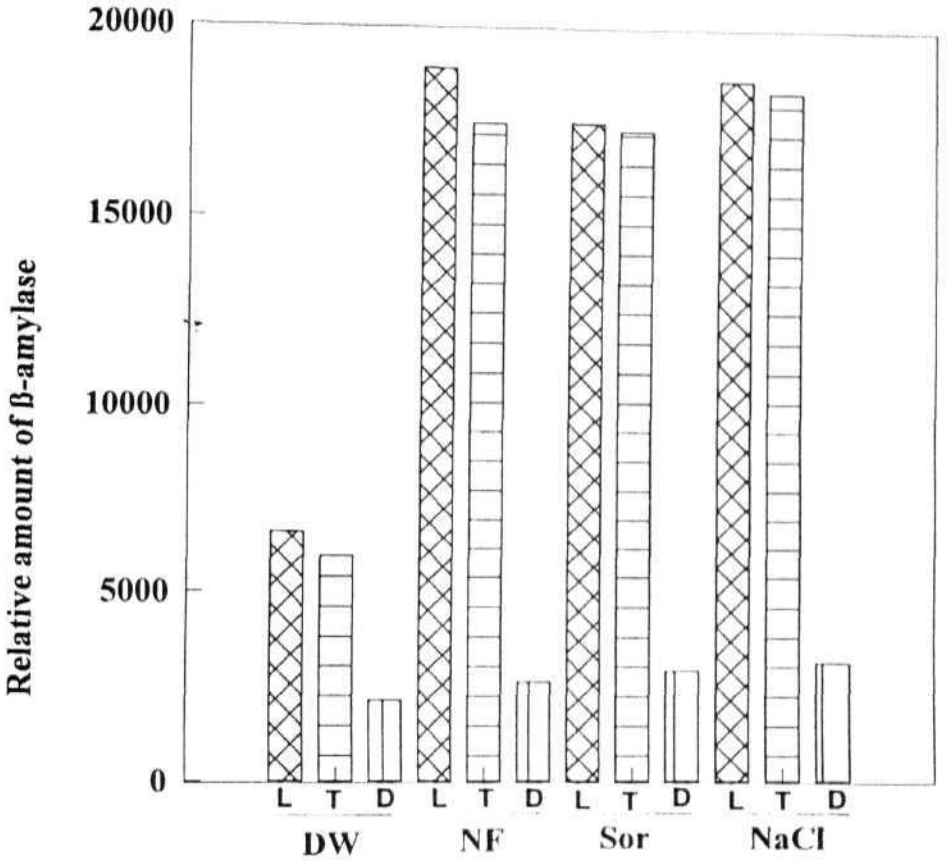


Figure 4.43 Effect of stress on β-amylase protein level. Seedlings were grown in distilled water **(DW)** or Norflurazon (NF) for 8 d or for 4 d in DW followed by 4 d in 300 mM sodium chloride (NaCl) or 300 mM sorbitol **(Sor)** in white light (L), dark (D) or 7 d dark + 1 d white light (T). First leaves from the seedlings were extracted and subjected to immunoblotting against anti β-amylase antibodies after SDS-PAGE. The level of β-amylase was quantitated by densitometric scanning of the blot displayed in fig. 4.42.

4.16 ***Effect of nitrate on nitrate reductase and nitrite reductase activity***

Maize seedlings were grown in various concentrations of nitrate right from the time sowing for 7 d. Assay of NR showed an increase in NR activity when plants were grown in increasing concentrations of nitrate upto 40 mM. Beyond this concentration, there was a sudden decline in NR activity at 50 mM nitrate concentration. Upto 80 mM nitrate, there was a slow decline in activity followed by an increase again at 90 and 100 mM nitrate. Dark-grown seedlings also showed a similar pattern as red light- grown seedlings, but the level of NR was much lower in dark-grown seedlings when compared to light-grown seedlings. At 40 mM nitrate concentration, a 5-fold increase in NR activity in red light was observed when compared to dark control (Fig 4.44).

A similar biphasic response to nitrate was observed in the case of NIR activity as NR activity. A gradual increase in NIR activity was observed with increasing concentrations of nitrate till it reached a maximum at 40 mM nitrate concentration. At 50 mM nitrate there was a sudden decline in NIR activity followed again by a gradual increase upto 100 mM nitrate concentration. A 4-5 fold light induction was observed at all concentrations of nitrate over dark controls (Fig 4.44).

4.17 *Time course* ***of NR*** *and NIR activity*

Time course of NR activity was checked in leaves of 4-10-d-old seedlings grown in 20 mM nitrate both in RL and darkness. In RL-grown leaf NR activity gradually increased upto 6 d after sowing. From 7 d there was a decline in activity upto 10 d. Dark-grown leaf showed a similar pattern to that of light-grown seedlings, but between 4-6 d NR activity in RL-grown leaf was considerably higher. RL led to an induction of about 2.5-fold on the 6 d (Fig 4.45).

Similarly NIR activity too gradually increased from 4 d upto 7 d and then declined upto to the 10 d. However, while NR activity of D and RL-grown leaf were nearly similar from 8 d onwards, NIR activity was higher in the RL-grown seedlings than dark control at all time points. Moreover, NIR activity attained its maximum a day later than NR, at a time when NR activity had already declined considerably. Thus NR peaked a day earlier than NIR during leaf development. While NR peaked on 6 d after sowing, NIR peaked on the 7 d (Fig 4.45).

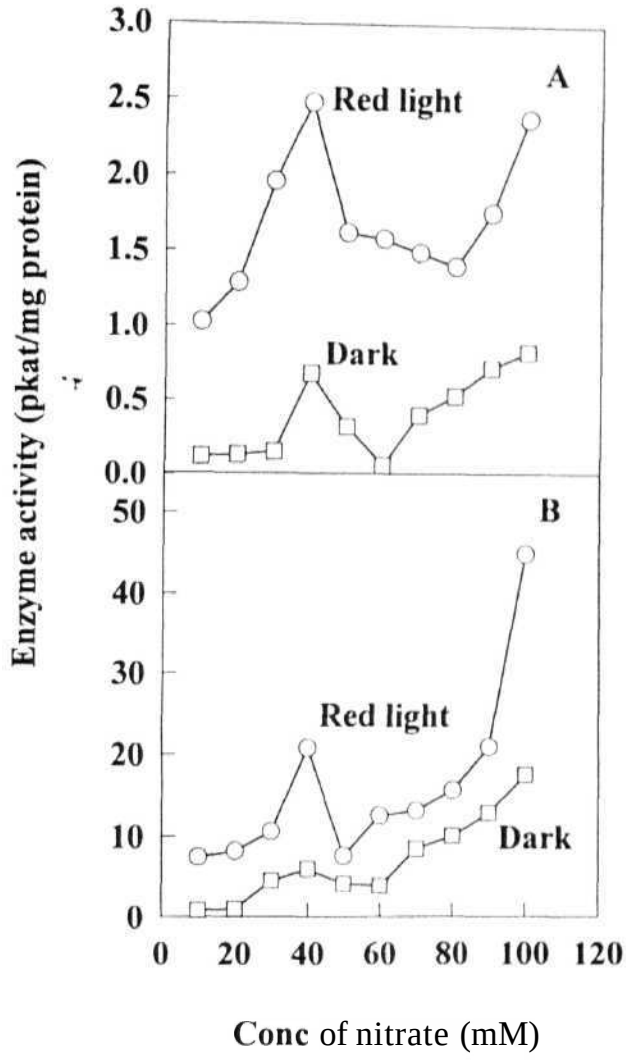


Figure 4.44 Effect of different concentrations of nitrate on nitrate reductase (A) and nitrite reductase (B) activity. Maize seedlings were grown in different concentrations of nitrate solution in red light or darkness for 7 d. The first leaf was assayed for nitrate and nitrite reductase activity and expressed as pkat/mg protein.

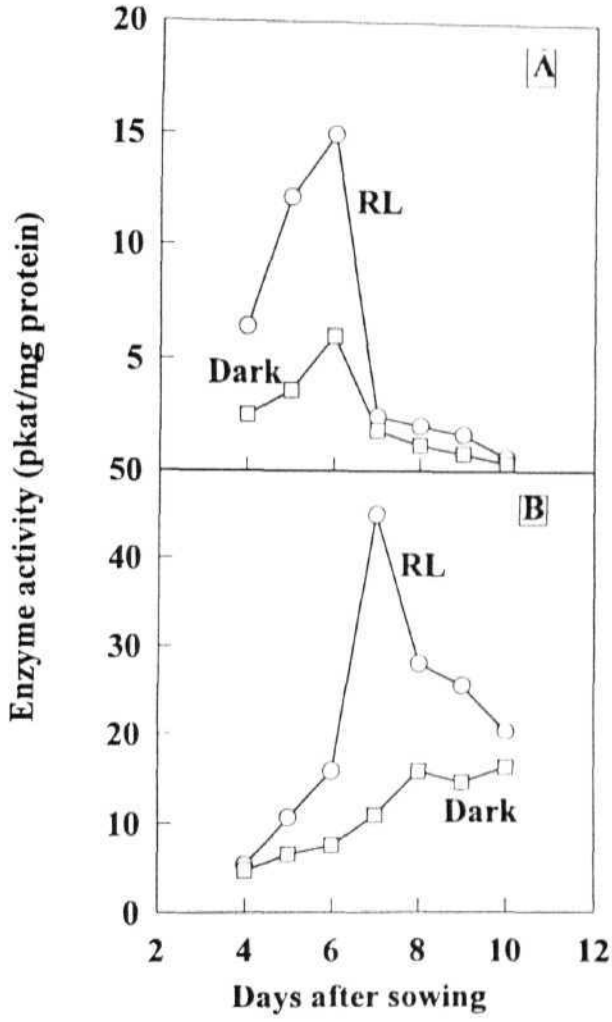


Figure 4.45 Time course of nitrate reductase (A) and nitrite reductase (B) activity in the first leaf of maize seedlings on different days after sowing. Seedlings were grown in distilled water in red light **(RL)** or in darkness **(Dark).** The enzyme activity was assayed and expressed as pkat/ mg protein.

4.18 The effect of light and nitrate on NR and NIR activity

4.18.1 Intact leaves:

The effect of light and nitrate on seedlings was studied by comparing the activity of NR and NIR on dark, RL-grown and transferred seedlings. DW-grown seedlings were supplemented with 20 mM or 60 mM nitrate just before transfer from darkness to RL and *vice versa.*

4.18.1. *J. Transfer from RL to darkness*

In 6-d-old dark-grown seedlings, the NR activity was lower than light-grown seedlings. When 6-d-old light-grown seedlings were transferred to darkness, about 1.2-fold decrease in NR activity was observed. However, NR activity increased by 1.2-fold when supplemented with 20 mM nitrate before transfer, and in the presence of 60 mM nitrate, 1.6-fold increase in NR activity was observed. Thus, when 6-d-old seedlings were transferred to darkness, a decline in the NR activity was observed which could be offset by supplementing with nitrate (Fig 4.46).

In case of NIR also a similar pattern was observed. There was a 1.9-fold decline in NIR activity when RL-grown seedlings were transferred to darkness. But when transferred seedlings were supplemented with 20 mM nitrate, there was a 1.1-fold induction in NIR activity. Supplementing with 60 mM nitrate lead to an induction of about 1.7-fold. Thus the effect of nitrate on NIR was similar to that of NR (Fig 4.46).

4.18.1.2. *Transfer from darkness to red light*

When NR activity was assayed in seedlings transferred from darkness to RL, it was found that transfer to RL led to an increase of about 1.7-fold in NR activity. In presence of 20 mM nitrate 3.2-fold induction was observed over dark control and in the presence of 60 mM nitrate 4.8-fold induction was observed. Thus RL by itself leads to a 40% induction which was further enhanced in the presence of increasing concentrations of nitrate (Fig.4.46).

In the case of NIR, when seedlings were transferred to RL, there was an increase of 1.6-fold in NIR activity. When transfer to RL was done along with 20 mM nitrate,

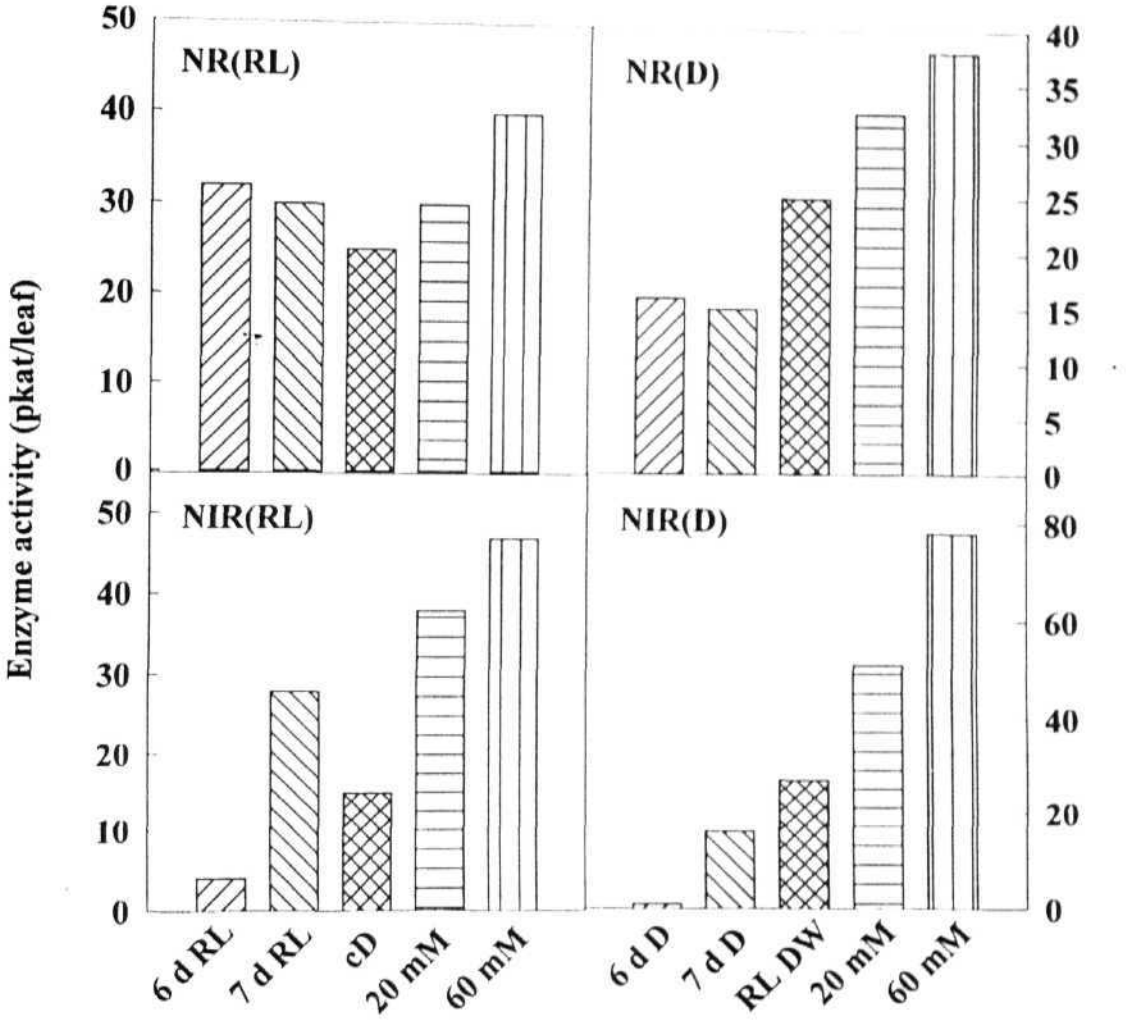


Figure 4.46 Effect of light and nitrate on nitrate reductase (NR) and nitrite reductase **(N1R)** activity in intact primary leaves of maize. Maize seedlings grown in distilled water under red light (RL) or darkness (D) for 6 d were transferred to darkness or light respectively for 24 h after supplementing with either 20 mM or 60 mM nitrate solution. Enzyme activity was assayed and expressed as pkat/ leaf.

there was about 2.9-fold induction in NIR activity. Addition of 60 mM nitrate leads to further increase in the induction of NIR by 4.5-fold. Thus nitrate and light lead to the induction of both NR and NIR in seedlings transferred to RL. Nitrate lead to an increase over and above the induction by RL alone (Fig 4.46).

4.18.2. **Detached** leaves

Most studies have examined NR regulation in detached leaves, and relatively little is known about NR regulation in leaves on the plant. In these studies, leaves after detachment were transferred to RL or darkness in the presence or absence of nitrate. In this study, comparison of intact and detached leaves were done to check whether detachment of leaves leads to any alteration in the results obtained in intact leaves. It was observed that in general detachment lead to a decline in both NR and NIR activity but the pattern observed in the case of intact leaves was similar to the pattern in detached leaves. Thus the response of detached leaves to RL and nitrate treatment was similar to the intact leaves, but the magnitude was different (Fig 4.47).

4.18.2.1. Transfer from RL to darkness

Transfer of RL-grown seedlings to darkness lead to a decline in the level of NR by 1.34-fold. Supplementing with 20 mM nitrate not only blocked the decline but also lead to an increase of 1.5-fold in NR activity which further increased to 2-fold in the presence of 60 mM nitrate (Fig 4.47).

Similar to NR, transfer to darkness also lead to a decline of about 1.5-fold in NIR activity. Supplementing with 20 mM nitrate lead to a 2-fold increase over control leaves. However, increasing the concentration of nitrate to 60 mM nitrate caused a reduction in induction. Apparently, in this case 20 mM nitrate saturated the NR induction, beyond which there was a decline in the NIR activity (fig 4.47).

4.18.2.2. Transfer from darkness to RL

There was an increase by 1-fold in NR activity when detached leaves were transferred from darkness to RL. The presence of 20 mM nitrate stimulated the induction to 1.6-fold which increased to 2-fold in the presence of 60 mM nitrate (Fig 4.47).

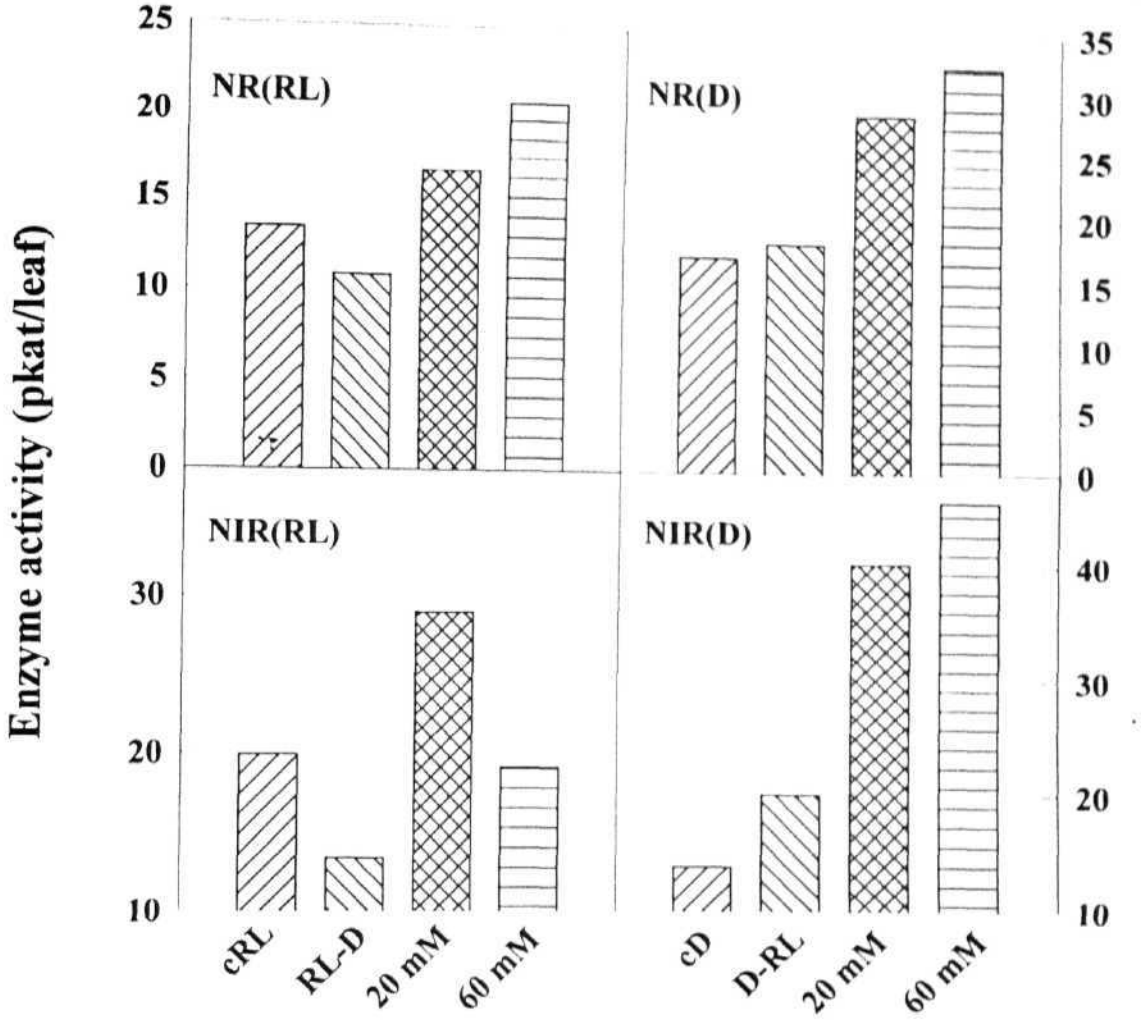


Figure 4.47 Effect of light and nitrate on nitrate reductase (NR) and nitrite reductase **(NIR)** activity in detached primary leaves of maize. Maize seedlings grown in distilled water under red light (RL) or darkness (D) for 6 d were excised at the mesocotyl node and transferred to darkness or light respectively for 24 h after supplementing with either 20 mM or 60 mM nitrate solution. Enzyme activity was assayed and expressed as pkat/ leaf.

In case of NIR, transfer from darkness to RL lead to an increase in activity by 1.5-fold. The addition of 20 mM nitrate resulted in a 3-fold induction, however, a further increase in nitrate concentration to 60 mM lead to only a marginal increase in NIR activity to 3.3-fold (Fig 4.47).

4.19 Distribution of NR and NIR along the length of first leaf

Maize leaves were cut into 1 cm long segments from the base to the tip and the distribution of NR and NIR in these segments was analyzed. The analysis of the distribution of NR and NIR in 5. 7, and 9-d-old seedlings showed that NR activity was highest at the base of the leaf and it gradually declined towards the leaf tip (Fig 4.48). The above pattern was observed for NR irrespective of the age of the seedlings. However, on similar analysis, NIR showed a diametrically opposite pattern. The highest activity of NIR was found at the tip of the leaf and the activity gradually declined towards the leaf base (Fig 4.48).

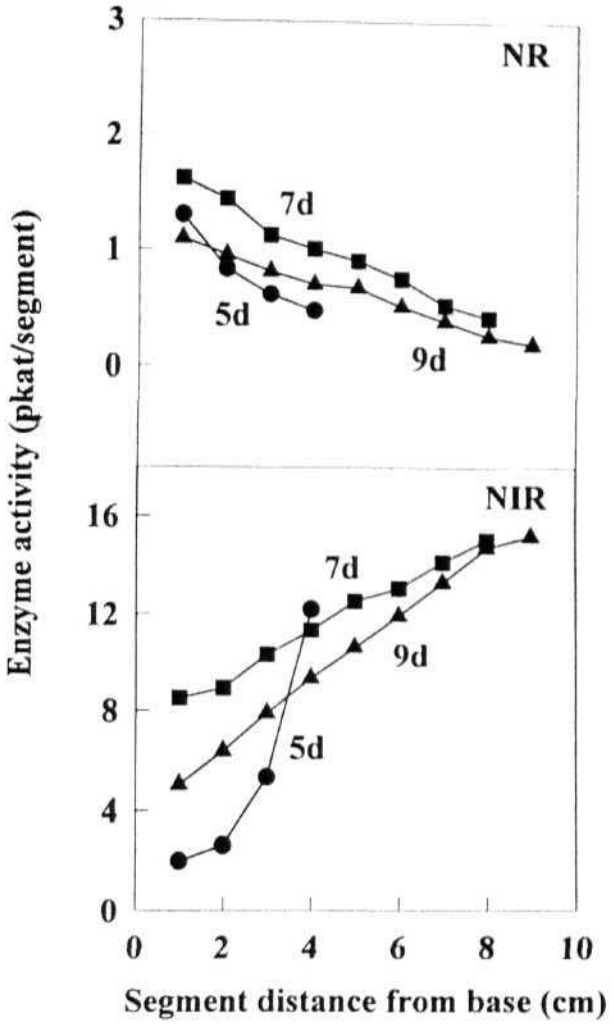


Figure 4.48 Gradient of nitrate reductase (NR) and nitrite reductase **(NIR)** activity in maize leaf segments. Maize seedlings were grown in distilled water under red light. The first leaf was excised 5 d, 7 **d** and 9 **d** after sowing at the mesocotyl node and cut into 1 cm long segments from the base to the tip. The enzyme activity was assayed in each segment and expressed as pkat/segment. The segments are numbered from the base (1) to the **tip.**

CHAPTER 5

DISCUSSION

Plant organs are formed by highly variable patterns of cell division, yet, the final arrangement of cell types within each organ remains the same. The differentiation of newly formed cells in plants depends on the interpretation of positional information. For example, in C-4 plants, bundle sheath and mesophyll cells interpret positional information with respect to each vein to correctly express bundle sheath cell specific genes. Light plays a crucial role in the generation and interpretation of this positional information (Nelson and Langdale, 1989). In monocots leaves, a basal meristem generates files of cells that remain aligned from the base to the tip of the leaf (Poethig, 1984). In a young leaf, the cells at the tip are most photosynthetically developed, whereas cells at more basal location are at progressively younger stages of differentiation (Nelson and Langdale, 1992). In the present study, the interrelationship between leaf development and photoregulation of some chloroplastic and cytosolic enzymes such as amylases, nitrate reductase and nitrite reductase was studied in maize leaves.

5.1. Distribution of amylases in maize seedlings.

Maize seedlings possess both α- and β-amylases in all organs of the seedlings, but at different levels. While α-amylase was the predominantly active enzyme in seeds, in leaf and other vegetative organs, β-amylase activity was higher. Comparatively, in 7-d-old light-grown maize seedlings, about 90 % of the total amylolytic activity was present in the seeds, and only about 10 % was present in the rest of the seedling. Among the vegetative organs, maximal amylolytic activity was present in the leaf, while other organs had little activity. In contrast to seeds, in leaf amylolytic activity predominantly consisted of β-amylase. The above expression pattern of amylases correlates well with the localization and synthesis of its substrate starch, which is predominantly stored in seed and is also synthesized in leaf during photosynthesis.

5.2 Amylase isozymes in maize leaf

The pattern of amylase isozymes in seed and leaf was examined by ND-PAGE followed by enzyme staining. The substrate specificity of the isozymes against starch and β-limit dextrin revealed that the maize leaf possesses four α-amylase and one β-amylase isozyme. The isozyme pattern in the leaf was broadly similar to that of seed, where five amylase isozymes were observed (Subba Rao, 1992). Moreover, on Western blotting seed and leaf extracts after ND-PAGE, and probing with a- and β-amylase antibodies raised against seed amylases, bands corresponding to all 4 isozymes of a-amylase and a single β-amylase isozyme were detected in both seed and leaf.

Although ND-PAGE and Western blotting revealed only five amylase isozymes, the presence of additional isozymes of amylases cannot be ruled out. While leaf α–amylase-4 on ND-PAGE showed a single band, on immnoblots, several closely migrating bands were observed, indicating that the observed band of a-amylase on ND-PAGE may be a conglomerate of multiple isozymes with little difference in mobility. Since on activity staining, diffusion of the substrate covers a greater area, resulting in a broad band, which basically results from the activity of closely migrating isozymes. In fact, the immunoblot of ND-PAGE after prolonged incubation showed several additional bands, which could either be minor a-amylase isozymes, other proteins with low affinity to a-amylase antibodies. The similarity in isozyme profiles and antigenicity of amylases between seed and leaf indicate that in both organs, these enzymes are encoded by a similar set of genes.

5.3 Maize amylases are antigenically similar to other cereals

The antibodies obtained in this study recognized amylases from other related cereal species. In an earlier study, it was shown that maize antibodies recognize a-amylase and β-amylase from pearl millet leaf (Vally and Sharma, 1995). In this study, it was found using Western blot that a-amylase and β-amylase from maize are antigenically similar to the respective amylases present in rice, wheat and oat. In addition, the molecular weights of these amylases are also identical to that of maize. It is therefore evident that within cereal amylases, the protein is antigenically fairly conserved, atleast against polyclonal antibodies.

5.4 fi-Amylase is the major form of amylasein maize leaves

β-Amylase was the major amylolytic enzyme in maize leaf. Out of the total amylase activity in the 7-d-old light-grown leaf, 70 % was contributed by p-amylase. and the remaining 30 % by α-amylase. In higher plants, while α-amylase is the major enzyme in seeds, in leaves p-amylase is the major form of amylase in most species. In *Vicia faba* (Ghiena at al, 1993), *Pennisetum americanum* (Vally 1994) and barley (Drier et al, 1995) leaves, p-amylase appears to be the predominant form of amylase. In mustard cotyledons, no a-amylase activity could be detected, and β-amylase is the sole amylase enzyme (Subbaramaiah and Sharma, 1989).

In most species while a-amylase consists of several isozymes, studies revealed mostly a single or two isozymes of p-amylase. In barley, two isoforms of p-amylase were reported, one predominantly expressed in the seeds and the other in the leaves (Shewry et al 1988). Similarly, two isoforms of p-amylase have been reported in wheat and rye, one specific to the endosperm and the other to the leaf and the root (Daussant and Laurière, 1990; Daussant et al 1991). In sweet potato (Yoshida et al 1991), mustard (Subbaramaiah and Sharma 1988) and *Arabidopsis* (Lin et al 1988), a single isozyme of P-amylase was reported. In this study, only a single p-amylase isozyme was observed on ND-PAGE, which could perhaps consist of two closely migrating isozymes. Two closely migrating bands of leaf P-amylase in maize were detected by Western blotting and immunoprecipitation using antibodies raised against seed p-amylase (Subba Rao, 1992).

5.5 Photostimulation of amylases is mediated by phytochrome

Amylase activity was higher in all organs of light-grown seedlings; except in seeds, where light had no influence on amylase activity. In seedlings grown in continuous RL, photostimulation of amylase activity could he observed right from the beginning of leaf expansion. That phytochrome mediates the above photostimulation of amylase was confirmed by R/FR reversibility of the response (Fig 4.27). A pulse of RL-induced amylase activity almost to the level of continuously RL-grown seedlings (Fig 4.27), while a pulse of FRL had no effect on amylase activity. Moreover, the inductive effect of RL could be effectively nullified by a FRL pulse.

Though phytochrome regulates both α–amylase and β–amylase activities, their regulators are independent of each other. The time course of α–amylase accumulation shows that a-amylase activity appears late, but continue to increase upto 10 d, whereas β–amylase activity appears earlier and peaks by the 7 d.

Information on the photoregulation of amylases is sparse and has been reported only in a few cases such as in chick pea cotyledons, where a-amylase activity is induced by RL (Casado et al 1991). Photoregulation of amylases has also been reported in mustard cotyledons (Sharma and Schopfer 1987; Subbaramaiah and Sharma 1989), maize leaves (Datta, 1991, Subbarao, 1992) and pearl millet leaves (Vally 1994). In all these cases the photoreceptor involved appears to be phytochrome. Though in mustard cotelydon blue light regulates β-amylase activity, it likely acts through phytochrome (Manga and Sharma 1988).

In maize, amylase activity in seed is not is not regulated by phytochrome. The synthesis and secretion of amylase from the aleurone and scutellum are strictly regulated by an endogenous development program, which can be stimulated by the presence of calcium in the medium (Subba Rao, 1992). However, in leaf, amylase activity is stimulated by phytochrome. Since seed and leaf amylases appear to be isozymically and antigenically similar, it would be of interest to examine if promoters of these proteins have dual regulation and operate differentially in seed and leaf, or the seed and leaf amylases are entirely different amylases restricted to their respective organs.

5.6 Photostimulation of amylases results from higher level of amylase proteins

Amylases are known to be synthesized *de novo* during seed germination excepting P-amylase which in barley is activated by the proteolysis of the stored form. Very few studies have examined if the variations in amylase activity in vegetative organs such as the leaf is due to *de novo* synthesis or the result of activation of the previously inactive protein. The evidences obtained in this study favor the view that the photostimulation of amylase activity is associated with increase in protein levels. In leaf, the photostimulation of a-and p-amylase activity is broadly correlated to a corresponding increase in the protein level. For example, the analysis of p-amylase activity distribution along the length of a leaf shows highest level of activity near the leaf base. The same pattern is also observed both on non-denaturing PAGE and Western blotting of p-amylase protein after SDS-PAGE. These results strongly support the notion that light stimulates P-amylase activity by increasing the level of p-amylase protein. Similar to β-amylase, α-amylase protein level was also photostimulated by light.

However, the results obtained in this study do not distinguish if the increase in the protein level was due to the *de novo* synthesis of the protein or decrease in the rate of turnover. Nevertheless, the results obtained rule out the possibility that the photostimulation of amylase activity is due to the activation of a preexisting protein. In a similar study on photoregulation of amylase in pearl millet leaves (Vally, 1994), it was observed that light stimulated amylase activity through *de novo* synthesis of the protein. It is also quite likely that a similar process may occur in maize leaves too.

5.7 Intra- and Inter-cellular localization of amylases

Most reports indicate that while a- and β-amylases are predominantly extra-chloroplastic in localization (Ziegler, 1988). It is often debated whether amylases are chloroplastic enzymes. In most instances, the chloroplastic localization of amylases has been questioned, as Chloroplast isolation leads to a cross-contamination of cytosolic enzymes. In this study, a possible localization of α-amylase in plastids was examined by different methods, like isolating chloroplasts after mechanical shearing of maize leaves, which gives a mixture of intact and broken chloroplasts, which were separated on a

sucrose density gradient. In a second and more gentle method of isolation of chloroplasts, mesophyll and bundle sheath protoplasts were isolated by enzymatic digestion, followed by isolation of chloroplasts. In either case, the intactness of the chloroplasts was checked by the ferricyanide reduction test. At the same time, the amount of α-amylase present in the cytosol was also examined.

The intra-cellular fractionation studies showed that α-Amylase activity was localized in both mesophyll and bundle sheath cytosol and chloroplastic fractions. In bundle sheath cells, about 60 % of the total a-amylase activity was localized in the cytosol and 40% in chloroplasts. In contrast, bundle sheath cells did not show any β-amylase activity. In mesophyll, about 25% of the a-amylase activity was localized in the chloroplasts. Isolated etioplasts failed to show a-amylase activity, and also a-Amylase protein could not be detected by western blotting in etioplasts.

It is therefore evident that maize chloroplasts possess only a-amylase activity, and β-amylase activity is absent in the chloroplasts. Since etioplasts do not show a-amylase activity, evidently a-amylase activity in chloroplasts appears during light-mediated Chloroplast biogenesis. The presence of a-amylase activity in chloroplasts is in conformity with its likely role in mobilization of photosynthetic starch. However, maize being a C4 plant, most of the photosynthesized starch is generated in the bundle sheath chloroplasts. It has been reported that maize chloroplasts contain the full complement of starch synthesizing enzymes such as ADP glucose pyrophosphorylase and starch synthase (Spilatro and Preiss, 1987). The subcellular localization studies have further shown that bundle sheath cells are rich in the activity of starch synthesizing enzymes. Since a-amylase is also localized in the bundle sheath chloroplasts, which is the predominant site for starch generation during photosynthesis, it may play an important role in the mobilization of starch. However, the presence of a-amylase in the mesophyll chloroplasts is intriguing, as under normal conditions, mesophyll chloroplasts of C-4 plants such as maize does not accumulate starch.

In pearl millet, a C-4 plant similar to maize, both mesophyll and bundle sheath cells possess a-amylase activity. However, in pearl millet, bundle sheath cytosol does not possess a-amylase activity, and the entire a-amylase activity is localized in the

bundle sheath chloroplasts (Vally et al.. 1995). In contrast in maize a-amylase activity is localized in both cytosol and chloroplasts of bundle sheath cells.

5.8 Chloroplasts possess novel a-amylase

The physico-chemical properties of chloroplastic a-amylase were similar to typical α–amylases. The chloroplastic a-amylase degraded amylose and β-limit dextrin with equal efficiency, and was resistant to heating at 70°C for 10 min in the presence of calcium. It was sensitive to EDTA, and lost almost 85 % of its activity by EDTA treatment. The activity could however, be protected to a large extent by supplementing calcium along with EDTA (Table 2).

On activity staining of ND-PAGE, chloroplastic isozyme corresponded to the a-amylase-2 isozyme of seed and leaf in mobility (Fig 4.28, 4.29). Ouchterlony double immuno-diffusion. and Western blotting revealed that, chloroplastic a-amylase cross-react with α-amylase-1,2 antibodies (Fig 4.30, 4.32). Besides, the precipitin lines formed by the cross-reaction of seed a-amylase-1,2 and chloroplastic a-amylase with a-amylase-1,2 antibodies fused completely, showing immunological identity (Fig 4.32). On the other hand, the chloroplastic a-amylase failed to cross-react with α-amylase-3,4 and β-amylase antibodies (Fig 4.30). Thus from the migration pattern on non-denaturing PAGE and from immunological studies, apparently the chloroplastic a-amylase is antigenically similar to a-amylase-1,2 of seed.

α-Amylases are encoded by a small multigene family consisting of several members, comprising of two groups, the high pI and the low pI group (Jacobsen and Higgins, 1982). Information regarding the molecular properties and expression pattern is available for amylases expressed during cereal seed germination, which are secreted into the endosperm from the encircling aleurone layer and the scutellum (Subba Rao, 1992). By contrast, in vegetative organs like leaves, a-amylases appear to be localized within the cell and are not secreted, although in a few cases, apoplastic location of a~amylases has also been reported (Saeed and Duke, 1990a). Since a-amylase is a secretory protein in seeds, it may be reasonable to assume that a-amylases present in vegetative tissues are either encoded by a different gene family or atleast do not possess the N-terminal signal for secretion into the apoplast.

The results obtained in this study however indicate that the a-amylase present in the chloroplasts is atleast antigenically similar to that present in the endosperm **This is** evident from the complete fusion of precipitin lines obtained as a result of the cross reaction between endosperm and leaf a-amylase against anti α-amylase-1,2 antibodies on double-diffusion gels. Similarly, Western blotting showed antigenic similarity between seed and leaf a-amylase as both cross-react with α–amylase specific antibodies. However, the molecular weight of the partially purified chloroplastic a-amylase was 57 kD, as against the antigenically similar seed α-amylase-1,2 that has a molecular weight of 46 kD. Moreover, the chloroplasts a-amylase isozyme is similar to α-amylase-2 of leaf and seed. It is however difficult to reconcile how one particular amylase isozyme is secreted to the medium whereas an antigenically similar counterpart is retained within the cell in another organ and directed to an organelle.

No information is available regarding the genes encoding the seed and leaf a-amylases proteins in maize. Since seed α-amylase-1,2 is a secretory protein, being secreted during germination of the seed, and chloroplastic α-amylase-1,2 should be directed to the Chloroplast after translation in the cytoplasm, the nature and reason for their antigenic similarity, and also similar pattern of migration on a non-denaturing gel can only be speculated.

One explanation could be that in this study polyclonal antibodies were used which may have recognized a broad range of a-amylases which may have been copurified during the purification of seed amylases; and are difficult to resolve on SDS-PAGE and Western blots due to their similarity in molecular weights. Therefore, though the seed and vegetative tissue a-amylases are different, immunological analysis using polyclonal antibodies were ineffective in resolving the differences between them. The antigenic similarity between seed and chloroplasts a-amylases is a clear indication of such a possibility.

The seed and leaf α-amylase-1,2 could be encoded by the same gene, and translated into two kinds of proteins. Though no evidence is available that the a-amylase present in the endosperm and Chloroplast are encoded by the same gene, the strong antigenic similarity between seed and leaf α-amylase-1,2 indicates such a possibility. In several cases in eukaryotes it has been reported that a single gene by a variety of

mechanisms such as multiple transcription initiation sites, variable mRNA splicing or multiple translation initiation sites can generate two proteins which are translocated to two different sites (Danpure, 1995). For example in yeast, fumarase gene produces two transcripts, the larger transcript product is located in the mitochondria and the smaller transcript is located in the cytosol (Wu and Tzagoloff, 1987). Similarly, yeast invertase encoded by a longer transcript is secreted and that encoded by a shorter transcript remains in the cytosol.

5.9 *Major portion of fi-amylase is localized in vacuoles*

In maize leaves, P-amylase appears to be a vacuolar protein, as 95% of the β-amylase activity present in leaf protoplasts is located in the vacuoles (Table 3). β-Amylase was localized exclusively in the mesophyll cells, and isolated bundle sheath protoplasts did not show any β-amylase activity. This finding is similar to that of pea and wheat as reported by Zieglcr and Beck (1986). These authors found that about half the p-amylase activity in pea is localized in the vacuoles, whereas in wheat, almost the entire activity of P-amylase is localized in the vacuoles. Since in maize also p-amylase is a vacuolar protein, it would be interesting to examine if p-amylase has a vacuolar targeting sequence. Vacuolar proteins are synthesized in the endoplasmic reticulum and progress through the Golgi apparatus and clathrin coated vesicles before accumulation in vacuoles. Targeting of the soluble proteins to the vacuoles is mediated by determinants that reside in the polypeptides. Both N-terminal and C-terminal targeting determinants have been identified (Kirsch et al 1994). However, no information is available regarding the presence of such a determinant in the P-amylase protein sequence. The localization of P-amylase in vacuole in some way indicates the possibility that photostimulated β-amylase after synthesis in the cytosol gets translocated to the vacuoles. In developing monocot leaves, the process of vacuole formation, expansion and maturation is maximal in the region proximal to the leaf base. The expression of a vacuolar protein, tonoplast intrinsic protein, γ-TIP gene expression is the highest in vascular bundles of the cells that are elongating and differentiating. The expression of this gene is shut down at the older regions, and high level of expression is coincident with vacuolation and cell

differentiation (Ludevid et al 1992). Its expression is also increases due to water stress (Guerrero et al 1990).

In case of β-amylase, it is likely that in light-grown leaves, photostimulation of activity must be linked to vacuolar differentiation, or light mediates its translocation into vacuoles. The analysis of β-amylase in dark-grown leaves showed an increase in activity and protein level towards the tip of the primary leaf. Light exposure stimulated the activity and also switched the gradient of (Vamylase distribution with maximal activity near base of the leaf. Similarly, in pearl millet leaves too, maximal synthesis of β-amylase was observed at the leaf base (Vally 1994). Since β-amylase is a vacuolar protein and is stimulated by light, and light-grown leaves show a distribution similar to that of γ–TIP, a relationship may exist vacuolar differentiation and β-amylase induction.

5.10 Spatial distribution of a- and β-amylase in first leaf

The maize leaf has been used as a favorite model for the study of several aspects of leaf development (Freeling, 1992). These studies have pointed out that leaf development in maize consists of three phases, the meristem founder cell, primordial and post-primordial phases (Sylvester et al 1992). During the post primordial phase of development, the leaves mature basipetally, with more mature cells being at the leaf tips and the youngest cells at the meristems. The leaf veins arise independently and differentiate basipetally into stem. In addition, the leaf can be distinguished into two regions, the sheath and the blade, with distinctive features and a clear demarcation of tissues. Several studies have used this cell maturation gradient to examine the relationship between the gene expression of nuclear and plastidic genes in the developing maize leaf (Nelson and Langdale, 1992).

The analysis of a- and β-amylase activity in dark-grown maize leaves revealed a similarity in their distribution with their level being low at the base and gradually increasing towards the leaf tip. Such a pattern is generally seen in the case of nuclear encoded plastidic proteins and pigments. In contrast, light-grown leaves showed a different pattern. In light-grown leaves, while the level of α-amylase was photostimulated mainly towards the leaf tip, (3-amylase level was mainly stimulated at the region close to the base of the leaf. The above photostimulation was clearly

associated with the increase in the level of P-amylase protein as seen by western blotting, which showed higher level of P-amylase protein in red light-grown leaves. Thus, light had contrasting effects on distribution of a- and P-amylase proteins along the length of the leaf. While light stimulated the level of α-amylase, it did not alter the distribution pattern of a-amylase along the leaf axis. However, light did alter the p-amylase distribution pattern, and the maximal level of protein shifted from the tip of the leaf to the base. It can be speculated that p-amylase gradient in light-grown leaves may be linked to its vacuolar localization as expansion of vacuoles is maximal near the leaf base (Ludevid et al 1992).

The molecular mechanism through which light generates this differential pattern of a- and p-amylase distribution is not known. In principle, a given pattern of enzyme distribution in leaf would be generated by regulation of its synthesis and degradation along the leaf length. The distinct variation in the distribution of β-amylase in dark and light-grown maize leaf may result by several mechanisms. The argument that this distribution pattern results from the determination of photoresponses of cells by a differential cell maturity gradient is strongly supported by transfer experiments. In maize, the transfer of dark-grown seedlings to red light shifts the gradient of P-amylase from the dark-grown distribution pattern to light-grown distribution pattern. The observed β-amylase distribution pattern light-grown leaves signifies dual effect of light. One, light enhances the level of p-amylase protein at the basal region of the leaf. Second, in segments close to the leaf tip, light promotes the rapid turnover of P-amylase protein, as P-amylase activity in segments near the leaf tip is lower than the dark control.

The distribution pattern of amylases in maize leaf is similar to that of a- and p-amylase gradient in pearl millet leaves (Vally 1994). The p-amylase distribution pattern observed in maize is also similar to the distribution pattern of cytosolic phosphorylase in pearl millet, which increases towards the tip in dark-grown leaves, whereas in light-grown leaves, the level is highest next to the leaf base (Vally et al., 1995). In barley leaf too, p-amylase activity is higher at the leaf base (Jacobsen et al 1986). Thus, the observed p-amylase distribution in light-grown leaf of maize is probably a general characteristic of cereal leaves.

Similar to β-amylase, α-amylase distribution also ensues from the differential photostimulation of its synthesis along the length of the leaf. For a-amylase too, this differential photostimulation is determined by the existing gradient of cell maturity and other associated components in maize leaf such as Chloroplast development. Since α-amylase synthesis is dependent on Chloroplast biogenesis, and as the chloroplasts are progressively more mature towards the tip of the leaves, the amount of a-amylase protein also increases towards the tip. A direct link between a-amylase activity and Chloroplast development is supported by the fact that in dark-grown leaves, a-amylase activity is very low, and also isolated etioplasts fail to show a-amylase activity.

5.11 Norflurazon abolishes both cytosolic and chloroplastic α-amylase activity

One way to prove that a-amylase is located in the chloroplasts is to check if the herbicide Norflurazon, which destroys chloroplasts, also abolishes a-amylase activity. a-Amylase activity was completely abolished in NF-treated seedlings. Treatment with NF which totally destroys chloroplasts supports this possibility. Interestingly, NF also destroyed the cytosolic isoform of a-amylase indicating that the a-amylase activity in the cytosolic compartment also depends on the Chloroplast biogenesis. ND-PAGE followed by contact printing on ft-limit dextrin containing gel failed to show both chloroplastic and cytosolic a-amylase activities in NF-treated leaf. That loss of a-amylase activity was accompanied by the loss of its protein was evident by the observation that no a-amylase protein could be detected by western blotting of NF-treated leaves using α-amylase-1,2 and α-amylase-3,4 antibodies. Thus, photooxidation of chloroplasts abolished chloroplastic as well as cytosolic α-amylases.

The loss of a-amylase activity in the chloroplasts of NF-treated leaves are in consonance with the chloroplastic localization of a-amylase. It is well established that the level of most plastidic proteins is drastically reduced in NF-treated leaves due to the photooxidation of chloroplasts. May field and Taylor (1984) demonstrated that in NF-treated seedlings, mRNA for LHCP fails to accumulate in cytosol. Similarly, a class of maize mutants, in which the accumulation of carotenoid pigments is blocked, leading to the development of albino seedlings similar to NF-treated seedlings, is deficient in both

nuclear and plastid encoded Chloroplast proteins (Jabben and Dietzer 1979). These results have been taken as an evidence for the existence of a signal originating from the Chloroplast and required for the optimal transcription and mRNA accumulation of nuclear genes encoding proteins destined for chloroplasts. Photooxidation of chloroplasts destroys this signal. This phenomenon has been observed in several plants such as maize (Mayfield and Taylor 1984) barley (Batschauer et al 1986), tobacco (Simpson et al 1980), mustard (Oelmuller and Mohr 1980) and tomato (Scolnik et al 1986).

In comparison to plastid proteins, the effect of NF on the level of enzymes in other sub-cellular compartments is equivocal. The loss of cytosolic α-amylase in NF-treated leaves indicate a clear interrelationship between the presence of functional chloroplasts and cytosolic α-amylase activity. The loss of cytosolic α-amylase by NF-treatment could happen in several ways, such as the degradation of cytosolic a-amylase, or the inhibition of transcription of a-amylase gene by a Chloroplast derived signal. It can also be assumed that both the cytosolic and plastidic isoforms of a-amylase are regulated by a similar promoter, and the activity of this promoter is dependent on functional chloroplasts. It is therefore likely that the loss of functional chloroplasts by NF-treatment down-regulates both cytosolic and plastidic isoforms of a-amylase.

Very few instances of the disappearance of cytosolic enzymes by NF treatment are available in literature. Börner et al (1986) showed that NR, a cytosolic enzyme, is reduced in barley and maize albino mutants and in mustard seedlings treated with NF. NR level was found to be dependent on the presence of intact chloroplasts and not on photosynthesis (Rajasekhar and Mohr, 1986). The interpretation for the above phenomena is that the expression of nuclear genes coding for plastidic proteins as well as extraplastidic enzyme levels related to functions in the plastids are controlled by a positively acting signal originating from the plastid (Oelmuller and Mohr, 1980). An alternative explanation is that some factor accumulates in the cytoplasm after photooxidative destruction of the plastids, which prevents the transcription of nuclear genes and accumulation of extraplastidic enzymes with functions related to intact chloroplasts (Oelmuller 1989).

The molecular mode through which plastidic signal may work is being examined by analysis of mutants. Susek et al (1993) isolated *gun* mutants of *Arabidopsis* which

have defective nuclear-plastidic gene interaction. Hess et al (1994) analyzed the transcription of 10 nuclear genes in the *albostrians* mutant of barely, which lacks plastid ribosomes. A strong reduction in the accumulation of transcripts of genes encoding Chloroplast enzymes involved in the Calvin cycle, LHCP and NR were observed. The run-on transcription assay in isolated nuclei showed that the plastid signal acts at the level of transcription. The main argument in favor of the plastidic origin of the signal is that white and green seedlings have the same genotype and differ from each other only in the type of the plastids.

5.12 Amylases are induced by various kinds of stress

The functions of amylases present in the extra plastidic compartments is not known, as these compartments do not possess polymeric carbohydrates. One potential function of amylase could be that of defense against putative pathogens or act as endogenous markers of stress expressed by plants. Relatively few studies have examined these properties, but there are isolated reports favoring the view that amylases are induced by various stresses. For example in barley leaves subjected to water stress, α-amylase was induced in the extraplastidic compartment. The above increase was due to *de novo* synthesis (Jacobsen et al 1986). Similarly, tobacco mosaic virus infection elevated apoplastic α-amylase in tobacco leaves along with other hydrolyses which are known to be pathogenesis related proteins. The two isozymes of α-amylase induced by infection are apoplastic in localization (Heitz et al 1991).

In the present study, P-amylase was strongly induced by treatment with NF, in cWL (Fig 4.41). Since the NF-treated leaves are albino with no protection against the damaging effects of light, p-amylase activity may be induced as light acts as a stress factor. To elucidate if the stimulation of P-amylase is due to light-stress, maize seedlings were subjected to water stress by growing them in different concentrations of NaCl and sorbitol. In light-grown seedlings, increase in the concentration of NaCl and sorbitol from 0-300 mM led to increasing induction of P-amylase activity, while no effect was observed on α-amylase. The experiments using NaCl and sorbitol showed that the extent of increase in p-amylase activity under water stress conditions is similar to that of the NF-induced increase in p-amylase activity. Thus the vacuolar P-amylase is induced by

various stresses in maize leaves, and the response to NF can be concluded to be due to light acting as stress factor in the absence of pigments in albino seedlings. By Western blotting, it was confirmed that the above stress induction in p-amylase was due to the increase in the level of P-amylase protein.

Induction of several other enzymes by NF has also been reported. For example, chalcone synthase (CHS) and phenylalanine ammonia lyase (PAL) is induced in mustard by NF treatment (Reiß et al 1983). CHS mRNA levels were also induced in the albino *albostrians* mutant of barley (Hess et al 1994) and also in NF bleached leaves of barley (Ehmann and Schafer 1988).

Similarly, when pea seedlings were treated with NF, and grown in cWL, a 76 to 85-fold increase in α-amylase activity was observed. This α-amylase was apoplastic in localization, either the concentration of NF, or the fluence rates were lowered, the induction in a-amylase activity was also reduced.

Saeed and Duke (1990 b) observed an inverse relationship between Chloroplast function and the level of apoplastic a-amylase and vacuolar P-amylase activity. Senescing leaves of pea showed 32-fold (protein basis) and 3.8-fold (FW basis) higher α-amylase activity when compared to fully mature leaves. Also, petals with low chlorophyll and starch content showed 6.5-fold higher p-amylase activity when compared to leaves. Thus p-amylase activity is inversely related to chlorophyll and starch concentration and also to Chloroplast density and function. Therefore it was proposed that chloroplasts produce a negative signal for the regulation of apoplastic *a*-amylase in pea (Saeed and Duke 1990 a). In barley, p-amylase activity NF bleached light-grown leaves showed a 3 to 5-fold higher activity compared to green leaves. Also, P-amylase was induced by high light intensity, increase in growth temperature and water stress. The induction by NF was interpreted as a stress response to light in the absence of pigments (Drier et al 1995).

5.13 Role of cytosolic amylases

It is not known if the cytosolic amylases have any significant role in the starch metabolism of plants. Since photosynthesis results in the formation of transitory starch in the chloroplasts, which are degraded and transported into the cytoplasm, the chloroplastic amylases could be responsible for the degradation of this transitory starch along with

chloroplastic phosphorylases. However, in most of the vegetative tissues, α-amylases are cytosolic enzymes and their role in starch degradation is not clear. However, cytosolic α-amylases, like NR, behave as if they were chloroplastic proteins and disappear in the absence of intact chloroplasts (Börner et al, 1986, Rajasekhar and Möhr, 1986).

In cereal seeds, amylases are strongly induced by hormones (Fincher 1989), but in the case of leaves hormones do not play such a role (Jacobsen et al 1986). In leaves, amylases have been reported to be regulated by viral infections (Heitz 1991), water stress (Jacobsen et al 1986), light stress (Drier et al 1995) etc. β-Amylases are also regulated metabolically. β-Amylase in sweet potato leaf is induced by sucrose, glucose and fructose. P-Amylase is also induced by mechanical wounding (Ohto et al 1992). In both sweet potato and transgenic tobacco containing β-amylase:GUS fusion gene, the expression of β-amylase is under complex regulatory mechanism which involves phosphorylation and dephosphorylation of proteins (Takeda et al 1994). Calcium mediated signaling was also shown to be involved in the sucrose induced expression of β-amylase gene in both sweet potato and tobacco (Ohto et al 1995). Thus, in spite of p-amylase expression being highly regulated, its role has not been clearly established. The vacuolar localization and stress-mediated induction of p-amylase protein indicate that β-amylase may either be a marker for the stress experienced by plants, or plays some role in alleviation of stress.

5.14 Both NR and NIR activities are induced by nitrate

The effect of various concentrations of nitrate in induction of NR and NIR activity on seedlings grown in darkness and WL was studied, it was found that nitrate response curve is biphasic in case of both NR and NIR. The NR and NIR activity peaked at 40 mM nitrate concentration, followed by a decline. With further increase in nitrate concentration, there was an increase in NR and NIR activity upto 100 mM nitrate. This induction in activity was further enhanced in the light-grown seedlings when compared to dark-grown seedlings (Fig 4.44).

A similar study using detached leaves of wheat did not show such a response. The activity of NR increased steadily from 10 to 100 mM KNO_3 concentration (Vijayraghavan et al 1979). In most of the higher plants studied, NR is nitrate inducible.

NR activity, protein and mRNA have been reported to be induced by nitrate; and the effect of nitrate is at the level of transcription (Hoff et al 1992). RNA analysis and transcription assay with isolated nuclei show that nitrate induction of NR mRNA is due to the *de novo* synthesis of the transcript (Melzer et al 1989, Callaci and Smarrelli, 1991).

Since both NR and NIR show biphasic profile to increasing substrate concentration, it indicates a mechanism which may be common to both enzymes causing this phenomenon. Most likely, this biphasic induction profile of NR and NIR result from a biphasic uptake of nitrate by leaf cells. Such a biphasic uptake may signify the operation of two carriers at the membrane level operating at high affinity and low affinity of nitrate (Glass et al., 1992, Hole et al., 1990). So, the first phase of induction may be caused by the high affinity carrier, and the second phase of induction by the low affinity carrier. Together these two carriers would modulate the endogenous nitrate concentration which gives rise to the observed profile of NR and NIR induction. However, other alternative explanations based on a dual affinity cytosolic inducer protein are equally plausible and cannot be excluded.

5.15 NR and NIR activities are not strictly corregulated in intact leaves.

NR and NIR induction in maize leaf show that the activity of both enzymes is strictly regulated by development on a temporal scale. The overall profile of enzyme induction in both dark and light-grown leaves is similar. The exposure to light elevates the level if NR and NIR by acting on the present pattern of temporal induction and differentiation. Apparently, while NR/NIR are dependent on some substrate for induction, their developmental programs are not strictly linked. This is evident from the observation that the maximal activity of NR and NIR are observed on different days. While NR activity peaked on the 6 d after sowing, NIR activity peaked on the 7 d (Fig 4.45). Moreover, the profile of decline is also different.

It is evident from the present study that while substrate induced corregulation is evident in NR and NIR, the developmental programming of their gene expression is not that strictly corregulated, leading to difference in the peaking of maximal activity in NR and NIR.

Since NR activity peaks earlier compared to NIR activity, and it may be associated with the age of the seedlings. For example, in detached corn leaves, treatment with nitrate led to a higher induction in younger seedlings compared to older seedlings (Kenis et al, 1992). Though the transcript levels in younger and older tissue are similar, the efficiency of translation in older tissue is much lower, which accounts for the decrease in NR activity and protein found in older leaves (Kenis et al 1992). Similarly in soybean, the youngest leaves had more NR activity, which dropped off with age. The NR activity was reported to reach the maximum level 2-4 days after new leaf formation (Li and Gresshoff, 1990).

5.16 Gradients of NR and NIR activity along maize leaf are opposite to one another

The analysis of the distribution of NR and NIR along the length of the maize leaf showed diametrically opposite patterns. While the activity of NR was maximum at the base of the maize primary leaf and gradually declined towards the tip of the leaf, the opposite was true in the case of NIR (Fig 4.48). Since in the maize leaf, the basal cells are the youngest, and the cell age increases towards the tip, the above finding agrees well with previous studies showing that younger tissues have higher NR activities (Kenis et al 1992, Li and Gresshoff, 1990).

It is evident that the distribution of NIR is similar to a typical chloroplastic enzyme, with a gradient of increasing activity towards the tip of the leaf. In a young leaf, the chloroplasts are at various stages of development, the ones at the tip being the most matured and photosynthetically active. The gradient of NIR in maize leaves is similar to the gradient of several other chloroplastic enzymes such as LHCP, RUBISCO, etc. Thus, the expression of NIR is linked to Chloroplast biogenesis and it is regulated in a manner similar to other photosynthetic genes such as the SSU of RUBISCO (Vincentz et al 1993).

The observed diametrically opposite gradients of NR and NIR in maize leaf support the notion that these two enzymes are regulated by different developmental programming. NR is apparently regulated by cell age, and NIR by Chloroplast development. However, NR activity is also dependent on chloroplasts and the loss of

chloroplasts causes the loss of NR activity. However, gradual decline of NR activity is somewhat similar to mitochondrial differentiation in wheat leaves (Topping and Leaver, 1990) which is maximal at the leaf base and lowest at the leaf tip. The occurrence of maximal activity of NR at the leaf base also raises question about the possible source of reductant energy which is normally met from surplus of photosynthesis. Near the leaf base, the chloroplasts are least differentiated and photosynthetically active, therefore NR cannot be dependent on chloroplasts for their energy source.

5.17 Effect of light and nitrate on induction of NR and NIR activity

The effect of light and nitrate was checked in 6-d-old maize leaves transferred from darkness to light and vice versa in the presence of two concentrations of nitrate. Comparisons were also made between the effect of nitrate and light in intact and detached seedlings.

In leaves of seedlings grown in DW, a reduced amount of NR and NIR were observed even in the absence of nitrate. Light can independently modulate NR and NIR activity which is evident from the observation that even in seedlings grown in the absence of nitrate, the activities of both the enzymes are high in light when compared to dark. It is therefore evident that NR and NIR genes are under leaky regulation and some amounts of the enzymes are formed even in the absence of the inducer nitrate, which is also subject to photostimulation. On transfer of 6-d-old light-grown leaves to darkness, NR activity was reduced, which could be offset by supplementing with nitrate. The same pattern was observed in the case of NIR activity. The protective effect of nitrate was more evident in detached leaves, which could be due to the fact that the detached leaves were floated in nitrate solution leading to the accumulation of high amount of nitrate in these leaves. In contrast, intact leaves obtain their nitrate supply through the root. However, detachment of the leaves caused a decline of NR activity, which was only partially offset by incubation with nitrate solution.

An inhibitory effect of leaf detachment on NR activity is evident when induction of NR in intact and detached leaves are compared after transfer of seedlings from darkness to light. The fold induction was better in intact leaves than detached leaves, though detached leaves had a better supply of nitrate as they were floated on nitrate

solution. On the other hand, the detachment of etiolated leaves did not lead to a similar decline in NR activity, showing that the light-inducible component of NR was subject to decline, but the NR present in etiolated leaves was not subject to a similar decline on detachment. A part of the decline in NR activity could also be due to the fact that NR activity peaks on the 6 d, and then declines, and the application of nitrate at this stage helps to offset this decline.

Exposure to light in the presence of nitrate strongly photostimulated NR, whereas in case of the detached leaves, the quantum of photostimulation was lower. However, in detached leaves, light alone did not stimulate NR activity, unlike in intact leaves, where light alone without nitrate caused a 50 % stimulation in NR activity. It is therefore evident that the detachment of the leaves caused a loss of responsiveness of the leaf to light mediated NR induction.

The results obtained NIR in intact leaves are in conformity with NR in general trends, where enzyme inductions are more effective in intact leaves than detached leaves. Like NR, the best stimulations in NIR activity is found when dark-grown leaves are detached and exposed together to red light and nitrate. Though intact leaves show a significant induction of NIR activity its magnitude is low. The low responsiveness of detached leaves to nitrate induction may be related to decreased metabolism. In intact leaves, it has greater access to resources of the leaf, and therefore shows higher stimulation of NR/NIR activity. In contrast, detached leaves are under stress and detachment causes the initiation of senescence. This would cause a drain on the energy resources of the detached leaves leading to a decline in activity.

Phosphorylation of NR is known to play a greater role in modulation of its activity. In addition, light and nitrate causes induction of transcription of NR and NIR genes. Given the complexity of NR regulation, it is likely that more than one mechanism may operate during detachment causing a decline in its activity. Similarly, its photostimulation in the presence of nitrate may result from multiple control points, the nature of which are not known and need to be investigated.

The molecular nature of the signaling intermediate in nitrate and red light mediated induction of NR in maize leaves has been recently examined. One of the studies showed that the light stimulation through phytochrome of NR activity in etiolated

maize leaves could be mimicked in darkness by the **addition** of phorbol **myristate acetate (PMA). The** stimulation in NR activity in dark by PMA was equivalent to stimulation by RL irradiation. Since Protein kinase C is known to be stimulated by phorbol esters, it was suggested that a protein kinase C type enzyme is involved in the light mediated induction of NR (Chandok and Sopory, 1992). Raghuram and Sopory (1995) further showed **that PMA** mimicks RL effect and induces NR transcripts in a fashion similar to RL exposure in **a** nitrate dependent fashion. Further, stimulation of phosphoinositides turnover, serotonin and 5 hydroxy tryptamine also mimick RL effect and increase NR transcripts. These results indicate that RL-mediated induction of NR mediated by phosphoinositide cycle and Protein kinase C type enzyme in maize leaves.

CHAPTER 6

Summary

Earlier studies in this laboratory have shown that the endosperm of germinating maize seeds show four isozymes of α-amylase and a single isozyme of β-amylase on ND-PAGE. The a-amylase isozymes were designated as α-amylase-1,-2,-3 and -4 according to their relative position on the gel. The band at the top of the gel was named as α-amylase-1 followed by α-amylase-2, -3 and -4 in an increasing order of their mobility on the gel. β-Amylase migrated as a broad band on the gel and had greater mobility than α-amylase-4. The amylase isozyme pattern of leaf was similar to that observed for endosperm displaying four a-amylase and one β-amylase isozymes.

Amylases were purified from endosperms of 6-d old maize seedlings following the procedure of Subba Rao (1992). The endosperm extract after concentrating by ammonium Sulfate precipitation, was subjected to affinity chromatography on amylose column. Only α-amylases bound to the amylose column and β-amylase and some amount of a-amylase-3,4 was recovered in the unbound fraction. The bound a-amylases were eluted using white dextrin solution and the eluate consisted of all the four isozymes of α-amylases purified to homogeneity. The isozymes of a-amylases were then separated was by ion-exchange chromatography on a DEAE-Sepharose column, and eluting the bound a-amylases using a gradient of 0-1 M sodium chloride. This step separated a-amylases into two groups, α-amylase-1,2 and a-amylase-3,4. On subjecting the unbound fraction of the amylose column to glycogen precipitation, β-amylase and a-amylase-3,4 were purified to homogeneity. The separation of p-amylase from a-amylase-3,4 was achieved by DEAE-Sepharose chromatography.

The homogeneity of the purified amylases was confirmed by SDS-PAGE followed by silver staining, and their molecular weights were determined. a-Amylase-1,2 migrated as a single band with a molecular weight of 46 kD, whereas, α-amylase-3 and -4 were resolved into two separate, closely migrating bands with

molecular weights of 45 and 47 kD respectively. β-Amylase had a molecular weight of 56.8 kD.

The purified amylases were injected into rabbits to raise three sets of polyclonal antibodies against α-amylase-1,2; α-amylase-3,4 and β-amylase. The specificity of these three sets of antibodies against their respective antigens present in leaf was checked using several techniques such as Ouchterlony double immuno-diffusion, immunoprecipitation and Western blotting. Immunoblotting of the amylases separated by non-denaturing PAGE revealed a pattern of a- and P-amylase isozymes similar to that observed by activity staining of ND-PAGE. Both anti a- and anti β-amylase antibodies also cross-reacted against the respective amylases from other monocots such as rice, wheat, pearl-millet and oat.

Spatial and temporal expression and distribution of a- and β-amylases were studied in the first leaf of maize seedlings using amylase assay, enzyme staining of non-denaturing gels and Western blotting. On the temporal scale, the accumulation of both amylases depends on the age of the leaf and also on the availability of light. While α-amylase activity was maximal on the 10 d after sowing, p-amylase activity was highest on the 7 d, followed by a decline thereafter. In comparison to seed, where a-amylase is the predominant enzyme, in leaf, P-amylase is more prominent. β-Amylase activity constituted about 80 % of the total amylase activity in light-grown leaf. Red/far-red light reversibility experiments confirmed that the photoinduction of amylases is mediated by phytochrome. The magnitude of photoregulation of amylases varied in different organs, and among all the organs, the leaf was the most responsive to light.

The effect of cell maturity on the expression pattern of amylases was studied by examining amylase activity in light- and dark-grown maize seedlings along the length of the leaf. In the dark-grown leaf, both a- and P-amylase activity and protein level increase from the base to the tip. Exposure to light drastically altered this expression pattern for P-amylase. Though light stimulates a-amylase activity and protein level, its distribution pattern is not altered by light and its activity and protein level increases progressively from the base to the tip in the light-grown leaf. In contrast, p-amylase activity and protein level increase from a low level at the leaf base, to an

induction of about 7-8 fold in the region close to the leaf base (1-2 cm from the leaf base), followed by a decline towards the leaf tip.

In order to find out if the observed gradient of P-amylase in light-grown leaf is innate to the leaf, dark-grown seedlings were transferred to light for 24 hours, and the P-amylase pattern was examined along the leaf length. Exposure to red-light altered the gradient pattern of p-amylase to a pattern typical of continuously light-grown leaves. Light increased the level of α- and P-amylase and the Western blotting of the leaf extracts revealed that a- and p- amylase protein patterns are similar to their respective activity patterns.

The intercellular and intracellular distribution of amylases in leaf was examined by isolating mesophyll and bundle sheath cells. The entire activity of β-Amylase was localized in the mesophyll cells, where 95% of the activity could be detected in the vacuoles. P-amylase activity could not be detected in the bundle sheath cells.

While mesophyll cells had 48% of the total α-amylase activity, bundle sheath cells showed about 22% of the total a-amylase activity. In light-grown leaves, a chloroplast-specific a-amylase was detected, which was absent in etioplasts isolated from dark-grown leaves. About 15% of the total a-amylase is present in the mesophyll chloroplasts and the same amount in the bundle sheath chloroplasts. Chloroplastic a-amylase migrated with a Rf value similar to that of seed α-amylase-1,2. On Western blotting, chloroplastic a-amylase cross reacted with seed a-amylase-1,2 antibodies but not with α-amylase-3,4 antibodies. Mesophyll and bundle sheath cytosol also contain a-amylase activity which migrate with Rf value similar to seed a-amylase-3,4 and on Western blotting, cross react with seed a-amylase-3,4 antibodies, but not with α-amylase-1,2 antibodies.

Attempt was made to purify the chloroplastic a-amylase using affinity chromatography on a cycloheptaamylose-Sepharose column. However, enough amount of the protein could not be recovered to make physico-chemical characterization of the plastidic a-amylase, because of the very low amount of protein in the leaves. Nevertheless, a partially purified chloroplastic a-amylase was

detected on blots probed against anti α-amylase-1,2 antibodies of maize seeds. It showed a molecular weight of 57 kD as against 46 kD of seed α-amylase-1,2.

The effect of Chloroplast biogenesis on the activity of a- and β-amylases were studied by growing maize seedlings in the herbicide Norflurazon right from the time of sowing. Norflurazon (NF) treatment causes photooxidative loss of chloroplasts in light and also the loss of most of the plastidic proteins, giving rise to albino seedlings.

The photooxidation of chloroplasts by NF treatment leads to a complete loss of both chloroplastic and cytosolic α-amylase activity, whereas it induced p-amylase activity 6-fold. Photooxidation of chloroplasts did not alter the distribution pattern of P-amylase in the leaf, which was similar to that of light-grown control leaves. The highest activity as well as protein level of p-amylase was in the region close to the leaf base (1-2 cm) in NF-treated seedlings, followed by a decline towards the tip of the leaf. The NF mediated induction of P-amylase appears to be a stress induced response. Similar induction of P-amylase was also observed in the seedlings subjected to water stress by sorbitol and sodium chloride treatment.

The influence of cell maturity on the induction and distribution of nitrate reductase, a cytosolic enzyme and nitrite reductase, a chloroplastic enzyme was also examined. The effect of various concentrations of nitrate on the activity of NR and NIR showed a biphasic response which probably resulted from the biphasic uptake of nitrate in seedlings. The time course of accumulation of NR and NIR in maize leaf followed a pattern that was loosely coordinated, with NR activity peaking on the 6 d after sowing and NIR activity peaking on the 7 d. The transfer of seedlings from darkness to light showed that nitrate is essential for sustaining both NR and NIR activity, which is further stimulated by light. The magnitude of induction in intact and detached leaves were different, the profiles of NR and NIR induction were basically similar. A study of NR and NIR distribution in maize leaf showed diametrically opposite profiles of distribution, with NR activity being maximal at the base of the leaf, and NIR activity being maximal at the tip, indicating that the temporal and spatial regulation of NR and NIR are not tightly corregulated, as seen for induction by light and nitrate.

CHAPTER *7*

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