REVIEW OF LITERATURE
CHAPTER 2

REVIEW OF LITERATURE

In land plants, the leaf is the site for trapping light energy via photosynthesis. To be fully effective as a photosynthetic organ, leaf has to trap light, with the inevitable consequence that it is also exposed to infrared wavelengths that causes heating, and to ultraviolet radiation that causes genetic damage (Dale, 1992). The development of the leaf lamina is an important process in relation to the interception of light and the accomplishment of photosynthetic competence (Leech and Baker, 1983). Though leaf development is genetically determined (Freeling, 1992; Freeling et ed., 1992), the acquisition of photosynthesis and differentiation of leaf cells is triggered by an environmental factor-light. The several facets of leaf development influenced by light are seen most remarkably during de-etiolation of seedlings e.g., light-stimulated unrolling of cereal leaves (Virgin, 1989), and cotyledon or leaf expansion in dicot seedlings (Sangeetha and Sharma, 1988). Additionally, light also influences several metabolic processes in leaf cells via regulation of gene expression, and by controlling activities of enzymes regulating metabolic pathways. In the present review, the role of light in the development of the monocot leaf is summarized with particular emphasis on regulation of starch degrading enzymes.
2.1 **LEAF DEVELOPMENT**

In *angiosperms*, leaves originate at shoot apical meristems, which consist of three distinct cell layers L1, L2 and L3 from which plant organs are derived (Poethig, 1987). These layers have a distinct pattern of cell divisions, e.g., in the L1 layer, which is the outermost, cell division is anticlinal and gives rise to the epidermal tissue of the differentiating organ. The cells in the L2 layer divide mostly in the anticlinal plane, but also in the periclinal plane, and cells of the L3 layer divide both in anticlinal and periclinal planes. In *monocots*, leaves are derived from L1, L2 and L3 layers of apical meristems. In dicots, two tunica layers, L1 and L2, are present, out of which only the L1 layer contributes to epidermis, and the L2 and L3 layers give rise to the internal tissue of the leaf (Poethig, 1987; 1989).

In maize, cells from both L1 and L2 layers of the apical meristem divide to form the leaf primordium (Poethig, 1987). Subsequent growth occurs predominantly from a basal intercalary meristem, where polarized patterns of cell division and expansion produce the elongated structure characteristic of a mature leaf (Sharman, 1942). Clonal analysis has shown that the monocot leaf epidermis is derived from the L1 layer of the apex, whereas the internal leaf structures, vascular tissue, photosynthetic bundle sheath (B) and mesophyll (M) cells are derived primarily from the L2 layer (Poethig, 1987). At the leaf margins, both the epidermis and the internal tissue are often derived from the L1 layer (Langdale *et al.*, 1989). After the formation of leaf primordium further growth of the leaf is largely restricted to a basal meristem, and the leaf possesses a gradient of cell maturation, showing all stages of
development from the most immature at the base to the fully functional and differentiated cells at the tip of the leaf (Sharman, 1942).

2.1.1 Cell differentiation in C₄ plants

In C₄ plants, leaf development is more complex than in C₃ plants due to chloroplastic and cellular dimorphism. C₄ differentiation depends largely on cell position and light-induced signals. In C₄ monocot leaves, the cellular differentiation of 'kranz anatomy' is associated with the development of the basic framework of a vascular system. Vascular development is initiated as the midvein develops from procambium in the shoot apex and differentiates towards the tip of the leaf (acropetally), dividing leaf in half, longitudinally (Sharman, 1942; Esau, 1943). Once procambial meristems become discrete, veins enlarge as a result of periclinal (radial increase) and anticlinal (longitudinal increase) divisions within the procambial strand. As the leaf primordium expands, lateral (large, acropetal) and then intermediate (small, basipetal) veins progressively subdivide the leaf. The overall age-gradient in the vasculature is also seen from the tip (oldest) to the base of the leaf. Bundle sheath and mesophyll cells around the medium vein matures first (acropetally); cells around the lateral veins then develop both acropetally and basipetally. Finally, cells around the intermediate veins mature basipetally. This is the same order in which the cells become morphologically differentiated (Easu, 1943; Sharman, 1942; Russell and Evert, 1985).

The development of bundle sheath cells takes place from cell division of either procambial cells or ground meristematic cells of mesophyll. The
maturation of bundle sheath cells and mesophyll cells is closely linked to vascular development and follows vascular differentiation (Langdale et al., 1987). The bundle sheath cells differentiates coordinately, partly from the outermost layer of the procambium, and partly from the adjacent ground meristem. These divisions generate 'half vein' units which comprise half of the bundle sheath cells around a vein and a single adjacent mesophyll cell (Langdale et al., 1989). Dengler et al., (1985, 1990) however, suggested that bundle sheath cells are derived entirely from procambial meristems, and mesophyll cells from ground meristem.

2.1.2 Regulation of leaf development

The identity of morphogenic factors which determine leaf initiation, and its distinction from reproductive organs is still unknown. A number of mutants have been described which are defective in leaf development, of these Knotted-1 (Kn1) gene of maize is quite interesting. The product of Knl gene is present in the nucleus of the shoot apical meristem cells, but is undetectable in leaf and floral organ primordia of wild-type plants (Freeling et al., 1988; Smith et al., 1992). Mutation at the Knl locus disrupts epidermal-layer development to give finger-like projections on leaf surface. To date, Knl is the first gene whose downregulation may be linked in some way to the earliest known events in leaf initiation. The Knl locus was cloned and its cDNA sequence shows a similarity to a homeo-domain protein present in animals, which acts as a transcriptional regulator controlling animal development (Vollbrecht et al., 1991). The ecotopic expression of the Knl gene product in developing leaf blade interferes with normal leaf development: the regions expressing it differentiate
to the leaf sheath around the veins rather than the normal leaf blade. Similar to the *Kn1* gene product, ROUGH SHEATH-1 and LIGULELESS-3 genes also encode homeo-domain proteins in maize (Smith and Hake, 1992). Similarly, the production of juvenile leaves and adult leaves in maize is controlled by specific gene loci. In maize, juvenile leaves are shorter than adult leaves and possess epicuticular wax which is not present on adult leaves (Poethig, 1990). The mutations controlling juvenile-leaf production in maize act on apical meristems to delay its transformation to the floral stage and promote development of the juvenile leaf (Bassiri et al., 1992). In the maize *Tp 1* mutant, it has been shown that *Tp1* controls the generation and distribution of a diffusible substance which initiates development of juvenile traits (Poethig, 1988).

In addition to genetic regulation, leaf development is strongly influenced by the hormonal status of the leaf. For example, in heterophylllic aquatic species, ABA treatment of the submerged shoot initiates development of aerial leaves, which normally requires exposure to an aerial environment (Mohanram and Rao, 1982). It is proposed that the emergence of the shoot from water results in osmotic stress leading to ABA production and, consequently, aerial leaf development (Goliber, 1989). The young, expanding leaves have higher abscisic acid levels than mature leaves (Raschke and Zeevaart, 1976; Zeevaart, 1980). In woody angiosperms gibberellic acid treatment can induce the formation of juvenile leaves from adult shoots (Rogler and Hackett, 1975).

In several instances hormones have been implicated in regulation of leaf expansion and differentiation. In many dicots the application of cytokinin initiates expansion of cotyledons/leaf (Scolt and Liverman, 1957), which
normally requires exposure to light to trigger leaf expansion (Sangeetha and Sharma, 1988; van Volkenburgh and Cleland, 1979, 1986). In grasses, the unrolling of leaf in seedlings is initiated by light and accompanied by a distinct hormonal change like an increase in gibberellic acid level (Virgin, 1989).

Higher cytokinin contents have been found in immature leaves and apical buds than in mature leaves. Application of cytokinins could promote leaf unfolding and expansion in intact plants (Leech, 1985). It has been reported that the young expanding leaves are the richest source of auxin. The highest concentration of auxin has been found at the leaf base (Allen and Baker, 1980). In basal tissue of the 6th leaf from the bottom in 28-day old maize plants, the IAA level was 2 to 3 times more than that in the apical segments. The auxins at high concentrations inhibited the development of the mesophyll without affecting vein extension (Gifford, 1953). In many dicot species evidence has been obtained that GA$_3$ may function in regulating leaf growth. GA$_3$ application alters leaf shape in many species (Jones, 1987), and leaf growth is altered in GA-deficient mutants of tomato (Koornneef et al., 1980) and pea (Ross et al., 1993). Ethylene inhibits leaf expansion. The inhibition involves a general reduction in leaf dimensions, rather than just a reduction in mesophyll tissue. The application of ethylene inhibits leaf expansion by inhibiting cell division (Leopold and Kriedemann, 1978, Scott and Possingham, 1982).

The development of the leaf is strongly influenced by light, a phenomenon which is most predominantly seen during seedling development. The dark-grown seedlings of dicots possess a hypocotyl hook and unexpanded leaves or plumules. Exposure to light initiates photomorphogenic development
of these seedlings where light activates plant photoreceptors like the UV-B photoreceptor, UV-A\blue photoreceptor and phytochrome, leading to leaf/cotyledon expansion in dicots, and unrolling of leaves in monocots (Shropshire and Mohr, 1983). While the genotype of the plants determines size and shape of leaf, light strongly influences cellular differentiation of leaves, leading to the formation of chloroplasts and induction of several enzymes (Kendrick and Krorenberg, 1986). While it is known that many of light's effects are mediated by activation of plant photoreceptor phytochrome, the components involved in the signal chain are not yet known.

Recently great emphasis has been laid on identification of components which regulate photomorphogenetic development of leaf/cotyledon using *Arabidopsis* as a model and selecting mutants which in dark possess the phenotype of light-grown plants. In *Arabidopsis*, several loci have been identified such as *det1, cop1*, 2, 3, 4, 5 and 9 (Chory, 1992; Chory *et al.*, 1989, 1991; Deng *et al.*, 1991, 1992) which initiate in dark-grown seedlings with a phenotype characteristic of light-grown plants. Of these mutants *cop2*, 3, 4 show the expanded cotyledon which is characteristic of light-grown plants, but, at the same time, have undifferentiated plastids (Hou *et al.*, 1993). Further, the above *cop* mutations do not affect hypocotyl development but only regulate cotyledon development.

It is apparent from these studies that the process underlying leaf development is controlled by several regulatory factors, which are both interdependent as well as independent of each other and lead to cell differentiation. Sun *et al.*, (1993) have shown that the *detl* mutant lacks a
phosphoprotein which specifically binds to the promoter of \textit{cab} 140 gene of \textit{Arabidopsis}. The protein acts as a repressor of the above gene, as mutant seedlings express a high level of \textit{cab} mRNA in darkness and do not respond to brief red light. The gene product of the \textit{cop9} gene encodes a protein with the characters of both G-proteins and Zinc binding proteins (Deng \textit{et al.}, 1992), which may play a role in carrying a signal from photoreceptor to promoter of light-regulated genes.

In addition to the above factors, the cellular position too can determine the cellular differentiation process. The accumulation of photosynthetic gene products in B and M cells is associated with vascular development (Langdale \textit{et al.}, 1987, 1988). The veins limit transport of factors essential for the development of these cell-types. Maize cells that are close to the vein show a C\textsubscript{4} type localization of Rubisco in B cells, whereas cells which are far off show a C\textsubscript{3} type localization of Rubisco, which suggests that veins might produce a signal which is needed for differentiation of B and M cell specific localization of C\textsubscript{4} enzymes. In the monocot leaf a major signal that influences a C\textsubscript{4} type gene expression is light. In dark-grown plants Rubisco is present in both B and M cells in a C\textsubscript{3} pattern, but on exposure to light Rubisco decreases in M cells and increases in B cells.

\textbf{2.1.3 Chloroplast biogenesis}

The development of chloroplast and leaf are two different processes, but chloroplast development is coordinated and regulated by leaf development. Chloroplasts are derived from small undifferentiated proplastids which are
maternally inherited in plants. The **proplastid** is a small organelle, 0.5-1.0 \( \mu \text{m} \) in diameter, without a distinct internal architecture characteristic of the mature chloroplasts and possesses low amounts of nucleic acids and proteins. The **meristematic** cells of plant possess only proplastid which differentiates into chloroplast during leaf development. In differentiating **mesophyll** cells, proplastids transform into chloroplast, a process which is light dependent. The development of a fully functional chloroplast involves regulation of both nuclear and plastidic genes (Mullet, 1988; Mayfield, 1990). However, signals triggering chloroplast development are still not known.

Chloroplast development in angiosperms goes to completion only in the presence of light (Thomson and Whatley, 1980). The development of proplastid to etioplast or chloroplast is regulated by differentiation of leaf cells and availability of light. In absence of light, development follows a different strategy leading to an etioplast stage, characterized by the presence of **prolamellar** bodies (Weier and Brown, 1970) and a small amount of protochlorophyll. The etioplast to chloroplast differentiation requires the phototransformation of the protochlorophyllide to chlorophyllide (Akoyunoglou and Akoyunoglou, 1986). Light-dependent transformation of etioplasts to chloroplasts may proceed without obvious changes in the rate of transcription or the stability of transcripts of plastid genes (Krupinska and Apel, 1989). However, etioplasts do not synthesize a limited set of **chloroplast-encoded** polypeptides, major constituents of thylakoid membranes (Klein and Mullet, 1987).
Monocot leaves offer a special model to study the process of chloroplast biogenesis since, at a given stage of development, it consists of a gradient of cells of differing maturity. Chloroplast development occurs during the conversion of meristematic cells of the leaf base into mature mesophyll cells of the expanded leaf. The plastid and nuclear gene expression is active in the dividing cell of the meristem perpetuating the proplastid population (Mullet, 1988). Ultrastructural examination of different segments of light-grown barley leaf showed different plastidic forms right from the leaf base, proplastids, amyloplasts, amoeboplasts and protochloroplasts in the base region and chloroplasts in the middle and tip region (Fig. 1). Plastid development in light-grown plants involves an obligatory amyloplast stage although eoplasts themselves do not contain significant quantities of starch. After the amoeboplast and protochloroplast stages of development the sizes and numbers of starch grains fall and rise again when photoreduction, photophosphorylation and CO₂ fixation become operational in the chloroplasts (Fig. 1) (Wellburn et al., 1986). In dicots early amyloplast developmental stage has been observed. The reported amoeboid stage that follows the amyloplast phase has not been observed and starch persists until quite late in the development of the chloroplast (Saito et al., 1990). During maturation of leaf cells a marked increase in levels of chloroplast proteins has been observed (Ougham et al., 1987).

In monocot plants, the developmental strategies of chloroplasts depends on positional localization in cells. In the basal region of light-grown leaves, B and M chloroplasts are indistinguishable, do not possess starch, or grana in B-
Figure 1. Electron micrograph showing gradient of chloroplast development along the length of barley leaf (From Wellburn et al., 1986).
chloroplast, and 2-4 grana in **M-chloroplasts**. In the center of the leaves there is a differentiation of chloroplasts, and the M-chloroplasts have 8-10 lamelle but no starch, while **B-chloroplasts** have starch in some cells but rarely have grana (Perchorowicz and Gibbs, 1980).

### 2.1.4 Biochemical gradients in monocot leaves

The monocot leaf provides a spectrum of cells at different developmental stages and has therefore been utilized to study various aspects of leaf development in both C\textsubscript{3} plants, e.g., barley (Viro and Kloppstech, 1980) and C\textsubscript{4} plants, e.g., maize (Aoyagi and Bassham, 1986). The biochemical specialization of different cells is evident from possession of a distinct gradient of chloroplast from base to leaf tip with diminutive proplastid at base and fully functional chloroplast at tip (Baker and Leech, 1977).

Monocot leaf possess a distinct gradient of Rubisco accumulation parallel to chloroplast development with maximal Rubisco protein to chloroplast development with maximal Rubisco Protein being in apical region of leaf. However the accumulation of the gene transcript for **SSU** and **LSU** of Rubisco and the polypeptides in maize leaf do not correlate well in the basal region of the leaf. While **mRNA** for **LSU** and **SSU** can be detected within 1 cm from leaf base, the respective polypeptides accumulate only 2-4 cm from the leaf base (Martineau and Taylor, 1985). In contrast, accumulation of PEP carboxylase **mRNA** and its polypeptides commences 4-6 cm from the leaf base, and that of LHCP, both mRNA, and polypeptide accumulates progressively from the leaf base (Martineau and Taylor, 1985). It is been hypothesized that appearance of
Rubisco polypeptides is in some way linked to differentiation of M and B cells. In barley leaves, the expression of *LSU* of Rubisco and Rubisco activase transcripts follows a gradient with increasing level towards the tip, but *cab* transcript level peaked at 2 cm from the leaf base and its level declined towards the tip (Zielinski *et al.*, 1989). Remarkably, the mitochondrial gene expression and differentiation shows a gradient with a maxima at the leaf base and a decline towards the tip of the leaf (Topping and Leeve, 1990), with maximal activity in the basal 2-3 cm of the leaf. Obviously, the gradient of mitochondria, and chloroplast development run opposite to each other.

The establishment of the distinct gradient of biochemical specialization mentioned above depends on two factors, viz., cell position and illumination; particularly with respect to the development of chloroplasts and associated proteins. Dark-grown barley leaves possess a distinct gradient of plastid development with diminutive proplastids (1.0 \( \mu \text{m} \)) at the base and well-differentiated etioplasts (4.5 \( \mu \text{m} \)) at the tip. Moreover, the tip cells have more plastids per cell than the basal region. The illumination of plants stimulates plastid differentiation in all segments of leaf and within 48 h the plastids are more or less of the same size and different cells possess the same number of plastids per cell (Robertson and Laetsch, 1974). Along with plastid differentiation, illumination also increases expression of nuclear and plastidic genes encoding for plastidic proteins in leaf cells (Nelson and Langdale, 1992). The appearance of these mRNA and polypeptides along the leaf length follows a distinct gradient, e.g, light stimulates PEP carboxylase mRNA from the basal level with an increased level of transcript towards the tip (Nelson *et al.*, 1984, 1989).
The requirement of light is not obligatory for the accumulation of these proteins and a small amount of Rubisco is found in dark-grown maize seedlings, but it is present in both B and M cells (Sheen and Bogorad, 1987; Langdale et al., 1987). Since Rubisco in light-grown leaves is only localized in B cells, it is apparent that in dark-grown maize leaves, the influence of cell position on Rubisco expression is not evident. However, on illumination of maize leaves, there is a suppression of Rubisco in M cells and expression in B cells leading to the appearance of the observed distribution gradient of the plastidic enzymes in B cells alone (Nelson and Langdale, 1992). Moreover, after onset of illumination, Rubisco in M cells is degraded at a faster rate than that in B cells.

Cell position also determines the expression of genes associated with biochemical specialization of Kranz anatomy. It was observed that veins exert an influence on biochemical specialization of nearby cells, and accumulation of C$_4$ proteins is maximal near veins. Further, as cells are distinct from veins, the level of these proteins declines and as a consequence, even parenchyma cells far from the vein express the gene for Rubisco which is supposed to be restricted to B cells (Langdale et al., 1988). It has been suggested that positional control of B cells on expression of C$_4$ enzymes is positive and acts locally within a small radius of each vein (Nelson and Langdale, 1992). The influence of light was related to its intensity and low light favored accumulation of Rubisco in M cells while high light favored accumulation of both Rubisco and PEP carboxylase (Usuda et al., 1985). It is evident that light plays a dual role in the monocot C$_4$ leaf: it stimulates the level of plastidic
enzymes and also suppress the expression of genes (e.g., Rubisco) in incorrect cell types.

Evidently, the expression of genes in the developing leaf occurs after integrating the information about cell types, position and light level. There have been many studies to identify cis-acting elements and trans-acting factors in order to understand how this information is generated and expressed at the molecular/genetic level. While the studies on light regulatory promoter elements are quite advanced and have been extensively reviewed (Gilmartin et al., 1990; Thompson and White, 1991), the information about elements responsible for M or B cell specific expression is very limited. Since many C₄ genes, such as ppc, ppdk, rbcS, can express in dicot C₃ plants like tobacco (Matsuoka and Sanada, 1991), it is evident that the B and M cell's specific expression is dependent on endogenously encoded trans-acting factors (Matsuoka and Numazawa, 1991). It has been suggested that C₄ genes are differently methylated in M and B cells and may help to explain expression pattern of C₄ genes (Ngernprasirtsiri et al., 1989; Langdale and Nelson, 1991). Bansal and Bogorad (1993), using gene bombardment on maize-leaf cells, identified four sequence elements which are involved in differential regulation of **cab-m-1** gene expression in M and B cells: sequence I (-1026 to -989) and II (-949 to -937) are needed to repress the gene in B cells, and sequences I, III (-936 to -897) and IV (-896 to -850) are needed to strongly enhanced expression in M cells. The factors determining suppression and enhancement in B and M cells are yet to be identified.
2.2 STARCH MOBILIZATION

In green leaves, the surplus carbohydrates generated during photosynthesis are stored in the form of starch in chloroplasts. Starch content is higher during the day, during which photosynthesis favors starch accumulation in chloroplasts its level declines during the night, when the sugars are generated by its degradation. Starch is mobilized to other plant parts via pholem. In tissues like seeds/tubers, starch is continually present till onset of a new phase of growth and development and is called 'storage' carbohydrate. The photosynthetically generated starch is called 'transitory' starch to signify diurnal oscillation in its level in active photosynthesizing tissues.

The studies on starch degradation in seeds/tubers have revealed that breakdown of starch is mediated mainly by a small number of enzymes, viz., starch debranching enzyme, phosphorylase and amylases, which act on starch to generate sugars. The process of starch biosynthesis and its degradation have been extensively reviewed (Preiss, 1984; Beck and Ziegler, 1989). In the present review, an overview of presence, localization, regulation and molecular properties of amylases and phosphorylase in green leaves is presented, and is compared with properties of enzymes present in storage tissues.

AMYLASES

Amylases are principal starch degrading enzymes and are present in both plants and animals. In plants, amylases can be distinguished into two
principal forms on the basis of their action on starch molecules, which are long linear polymers of glucose with a high degree of branching. The amylase hydrolyzing internal glycosidic bonds of starch leading to fragmentation of molecule is called as \( \alpha \)-amylase or endoamylase, while the one selectively removing reducing-disaccharide maltose from the non-reducing end of the starch is called as \( \beta \)-amylase. The properties of both enzymes from green leaves are reviewed below.

### 2.2.1 \( \alpha \)-amylase

\( \alpha \)-amylase (1,4-\( \alpha \)-D-glucan glucanohydrolase, E.C.3.2.1.1) catalyzes the endohydrolysis of 1,4-\( \alpha \)-D-glucoside linkages of starch and related poly- and oligo-saccharides. On incubation with starch, \( \alpha \)-amylase action is characteristically seen as a decrease in the viscosity or liquification of starch.

#### 2.2.1.1 Physicochemical properties

\( \alpha \)-amylase is a metalloprotein (Fischer and Stein, 1960), possessing one atom of \( \text{Ca}^{2+} \) per molecule of enzyme (Bush et al., 1989). The structure of the \( \text{Ca}^{2+} \) binding domain is nearly similar in all \( \alpha \)-amylases. The presence of \( \text{Ca} \) is essential for \( \alpha \)-amylase activity, as removal of bound \( \text{Ca}^{2+} \) by incubation with either EDTA or EGTA results in a complete loss of its enzymatic activity (Thoma et al., 1971; Koshiba and Minamikawa, 1983). The activity of \( \alpha \)-amylase can be restored by removing the chelator and providing \( \text{Ca}^{2+} \) (Huang and Kao, 1992). The role of \( \text{Ca}^{2+} \) in maintaining amylase stability is now well recognized and therefore \( \text{Ca}^{2+} \) is included in the incubation medium along with
α-amylase to stabilize its activity. It has also been shown that in presence of calcium (10 mM) α-amylase can be subjected to heating at 70°C for 5 min to 5 h, without any significant loss of catalytic activity (Stein and Fischer, 1958; Koshiba and Minamikawa, 1983; Beers and Duke, 1990) and this property has been used to distinguish α-amylase from other starch hydrolyzing enzymes. However, it is now recognized that α-amylases may exist in two sub-groups, Amyl and Amy2 and one of it possesses lower affinity for Ca$^{2+}$ and is largely insensitive to chelation by EDTA (Bush et al., 1989). For instance, Jacobsen et al., (1986) found that EDTA inactivates Amy2 of barley seed but not Amyl. Likewise, endoamylase from spinach chloroplast too has no apparent requirement for Ca$^{2+}$, lacks heat stability, and is sensitive to sulfhydryl oxidizing agents (Okita et al., 1979).

α-amylase is stable in pH ranges 5.5 - 8 (Beers and Duke 1990), but denatures rapidly at acidic pH, eg., at 3.6 (Thoma et al., 1971). α-amylases in many plant species are devoid of cysteine and therefore are not susceptible to damage by SH reagents (Preiss and Levi 1980).

2.2.1.2 Seed α-amylase

The molecular properties and regulation of α-amylase in plant systems are best characterized in barley, summarized below, where GA-induced α-amylase synthesis has been investigated in great detail to decipher molecular mechanisms of hormone action. In essence, in barley, α-amylase is encoded by 2 multigene families, located on chromosome 1 and chromosome 6 respectively (Knox et al., 1987). The gene family located on chromosome 6 consists of four
genes which encode polypeptides for $\alpha$-amylase whose isoelectric point is about 5 and are called low $pI$ amylases (Muthukrishnan et al., 1983). The gene family located on chromosome 1 is more complex and consists of at least 7 genes, which encode for polypeptides of $\alpha$-amylase bearing higher isoelectric point of about 6 and are therefore called high $pI$ amylases (Khursheed and Rogers, 1988). Both groups of amylases are characteristically different in terms of their regulation by hormones, and requirement of $\text{Ca}^{2+}$ for stability and secretion. It is pertinent to note that $\alpha$-amylase is a secretary protein and after synthesis it is translocated to the cell exterior via the endoplasmic reticulum-golgi body pathway (Jones and Jacobsen, 1991). The role of gibberellins in inducing $\alpha$-amylase synthesis and secretion in barley seed is well known and has been extensively reviewed (Beck and Ziegler, 1989; Fincher, 1989).

2.2.1.3 Leaf $\alpha$-amylases

There have been relatively few studies concerning $\alpha$-amylases in leaf. In comparison to seed amylases, only a little information is available regarding the physicochemical properties and regulation of leaf amylases. Basically, $\alpha$-amylase isolated from leaf/cotyledons possesses similar physicochemical properties as seed amylase; however knowledge about them is fragmentary. The presence of $\alpha$-amylase in green leaves has been detected in many plant species (Gates and Simpson, 1968) such as spinach (Okita and Preiss, 1980), pea (Ziegler, 1988), barley (Jacobsen et al., 1986), Arabidopsis (Lin et al., 1988), maize (Echeverria and Boyer, 1986) to name but a few. However, in view of its low activity in leaves, its purification and molecular characterization have been
done only in pea (Ziegler, 1988), spinach (Okita and Preiss, 1980) and *Arabidopsis* (Lin *et al.*, 1988).

The properties of pea leaves $\alpha$-amylase is summarized below. The molecular weight of purified pea $\alpha$-amylase is 45 kD, which is also close to the molecular weight of seed amylases, which range between 40-50 kD. The enzyme activity depends on the presence of $\text{Ca}^{2+}$ ion in incubation media, as inclusion of 10 mM EDTA results in a rapid loss of activity pointing out the $\text{Ca}^{2+}$ metalloprotein nature of the leaf enzyme. As is the case with cereal seed $\alpha$-amylases, excess calcium stabilizes purified $\alpha$-amylase against denaturation, effect of heat and low pH. There is no inhibition of amylolytic activity of the above amylase by $\alpha$-, $\beta$- or $\gamma$-cyclodextrins but heavy metal ions, particularly mercury, strongly inhibits $\alpha$-amylase activity. Similarly, maltose also inhibits $\alpha$-amylase activity. On other hand, the activity of the enzyme is unaffected by the presence of sulfhydryl reagents like DTT, and sulfhydryl oxidizing or alkyllating reagents lead to only a moderate loss of activity. The substrate specificity and activity patterns of pea $\alpha$-amylase on various substrates is akin to seed $\alpha$-amylase. The pH maxima of amylase is at 6.0 in Na-acetate buffer. It is clearly evident that physicochemical properties of $\alpha$-amylase purified from pea leaves are very similar to seed amylase and it possesses almost all the characteristic properties of $\alpha$-amylases. The molecular properties of $\alpha$-amylase isolated from shoots and cotyledons of pea (Swain and Dekker, 1966; Beers and Duke, 1990) are identical to pea leaf amylase (Ziegler, 1988), as the peptide maps and molecular weights of these amylases are the same.
It is now known that within the oc-amylase enzyme family, two kinds of amylase can be recognized: type B which are EDTA sensitive but insensitive to -SH reagents, and type A which are insensitive to EDTA but sensitive to -SH reagents (Jacobsen et al., 1986). It has been reported that while pea \( \alpha \)-amylase belongs to type B, in barley (Jacobsen et al., 1986) and spinach leaves (Okita and Preiss, 1980), \( \alpha \)-\text{amylases} are of the A type. In spinach leaves, though \( \alpha \)-\text{amylases} have almost all the typical properties of oc-amylases, such as substrate specificity and a typical action pattern on amylose. But unlike the typical \( \alpha \)-amylase, it is resistant to EDTA or EGTA, it is completely inactivated on heating at 65°C even in the presence of \( \text{Ca}^{2+} \), and -SH modifying reagents also reduces its activity (Okita and Preiss, 1980). Similarly, oc-amylases from Arabidopsis leaves lose its activity on heating at 60°C even in the presence of 20 mM Ca (Lin et al., 1988). In Arabidopsis leaf, while \( \alpha \)-amylase was detected on the basis of its capacity to hydrolyze \( \beta \)-limit dextrin, analysis of its products revealed generation of only maltose (Lin et al., 1988). It is evident that depending on the species, leaf amylases may basically constitute 2 groups which can be distinguished as type A and B.

In green leaves, in view of generation and localization of starch in the chloroplast, it is expected that the oc-amylase of the leaf is a plastidic enzyme. However, the localization of oc-amylase in plastids has still not been unequivocally demonstrated: e.g., while the presence of an oc-amylase in chloroplasts has been reported for sugar cane (Bourne et al., 1970), pea (Ziegler, 1988), other reports are equivocal about its presence (Beers and Duke, 1990). In barley, no oc-amylase could be detected in chloroplast (Jacobsen et al.,
1986). Most reports favor the view that a part of α-amylase activity of green leaves may be plastidic in nature, and this plastidic enzyme may possess properties of either type-A or type-B α-amylases. In spinach leaves, Okita and Preiss (1980) detected both chloroplastic and extrachloroplastic α-amylases which had characteristics of type-B amylases, and about 5% of α-amylase was in chloroplasts. In pea leaves, while a major amount of α-amylase is present in the extracellular compartment, chloroplast possesses about 5% of total α-amylase activity (Ziegler, 1988). The extracellular α-amylase is predominantly present in apoplasts in the cell walls of pea leaves and shoots, but plastidic α-amylase has been envisaged as a contamination (Beers and Duke, 1990). In Vicia faba leaves, about 40% of α-amylase activity is likely to be associated with chloroplasts while the rest is localized outside plastids (Ghiena et al., 1993). The presence of α-amylase in chloroplasts in many species is still doubtful due to lack of convincing experiments.

The location of extraplastidic α-amylase is presumed to be cytosol. However, Ziegler (1988), on isolating protoplasts from pea leaves observed that in comparison to leaf, protoplast contained much lower α-amylase activity, suggesting that a significant amount of α-amylase may be located in extracellular compartments. The infiltration experiments conducted on pea stem indicate that about 87% of α-amylase activity is located in the apoplast in cell walls (Beers and Duke, 1990).

The regulation of α-amylase activity by GA in barley seeds, now a classic observation, signifies hormone-mediated gene expression of α-amylase in aleurone layers. In comparison, the role of hormones in induction of α-amylase
in green leaves is not evident. In the same barley leaves, water stress rather than GA stimulated accumulation of \( \alpha \)-amylase in leaves (Jacobsen et al., 1986). The above accumulation results via stress-mediated induction of \( \alpha \)-amylase mRNA which lead to de novo synthesis of \( \alpha \)-amylase protein, which belongs to only type-A \( \alpha \)-amylase. It is interesting to note that while barley seeds have low pI and high pI amylases, in leaves only low pI amylase is present, and it is not translocated to the chloroplast but rather is present in the extraplastidic compartment. The question whether the signal peptide of \( \alpha \)-amylase, which directs its secretion in aleurone layer cells, is redundant in leaf cells still needs to be examined. In pea leaves, the level of \( \alpha \)-amylase, which is principally located in the apoplast, is in some way determined by chloroplast density and function (Saeed and Duke, 1990b). In fact, the loss of functional chloroplast by Norflurazon treatment increased the \( \alpha \)-amylase level in the apoplast by about 80-fold (Saeed and Duke, 1990a). It has been postulated that chloroplast produces a negative signal for regulation of \( \alpha \)-amy cases activity in pea, and in some organs, e.g., senescing leaves, increase in \( \alpha \)-amylase activity may result due to loss of chloroplasts (Saeed and Duke, 1990a,b). Since \( \alpha \)-amylase purified from different organ of pea such as cotyledon/stem/leaves appears to be the same (Ziegler, 1988; Beers and Duke, 1990), the putative signal directing secretion of \( \alpha \)-amylase to the apoplast is also not known.

While hormonal involvement in regulation of \( \alpha \)-amylase activity in green leaves is not seen, hormones have been implicated in the regulation of \( \alpha \)-amylase activity in hypogeal cotyledons of pea seedling, which serves as a storage organ, where the axis induces de novo synthesis of \( \alpha \)-amylase in pea
cotyledons (Hirasawa, 1989; Hirasawa and Yamamoto, 1991). In contrast to barley where water stress promotes accumulation of α-amylase, in deep-water rice submergence of shoot promotes an increase in α-amylase activity at the internodes (Smith et al., 1987), which can also be induced by \( \text{GA}_3 \) or ethylene treatments to excised internodes. In bean stem, the modulation of α-amylase level correlates with cellular disassembly (Davis, 1984). In mung bean cotyledons, α-amylase development is blocked by an endogenous inhibitor whose action can be blocked by auxins, or imbibition of the cotyledon in water (Morohashi et al., 1989). In tobacco leaves too, activity of two apoplastic α-amylases greatly increases on infection with tobacco mosaic virus (Heitz et al., 1991) and the α-amylase induction is characteristically similar to other pathogenesis-related proteins.

One important factor which may influence the \( \alpha \)-amylase activity in shoot/green tissue is light. Light may have a two-fold effect on amylase activity, first, by promoting photosynthetic generation of starch, and second, by stimulating chloroplast biogenesis, a principal site of α-amylase localization. In spinach chloroplast, amylolytic activity oscillates in a diurnal fashion in light/dark phase, the activity being twice as high in the dark phase. It has been presumed that diurnal oscillation of stromal \( \text{pH} \) may modulate the amylolytic activity of chloroplasts. In leaves of developing maize seedlings, very little influence of light has been observed on the activity of \( \alpha \)-amylase (Subbarao, 1992). However, a detailed analysis of the α-amylase level along the axis shows that in maize leaves α-amylase activity is photostimulated particularly at the base of the leaf (Datta, 1992) and is present in both B and
M cells (Echeverria and Boyer, 1986). In contrast, in maize coleoptile and radicle on transfer to light an induction of isozyme band is observed, which has been interpreted as photoinduction of an amylase isozyme which could be a \( \beta \)-amylase (Segundo et al., 1990). However, the interrelationship between chloroplast biogenesis and \( \alpha \)-amylase induction is yet to be investigated.

### 2.2.2 \( \beta \)-Amylase

\( \beta \)-amylase (1,4-D-glucanmaltotohydrolase, E.C. 3.2.1.2), was named after the product \( \beta \)-maltose, by Kuhn (1925) and Ohlsson (1930). It is present in a few species of higher plants and microbes, but is absent in animals (Thoma et al., 1971). It hydrolyzes amylose from its non-reducing end, releasing \( \beta \)-maltose, and leading to a complete hydrolysis of amylose. Since, it can not bypass \( \alpha \)-1,6-glucoside linkages; thereby, the hydrolysis of amylpectin results in \( \beta \)-limit dextrin. \( \beta \)-amylase is unable to hydrolyze native starch grains from pea and glucans smaller than maltotetraose (Lizotte et al., 1990).

#### 2.2.2.1 Physicochemical properties

\( \beta \)-amylase has a broad pH optima of 4.0-5.5, and requires free sulfhydryl groups for activity. It is believed that the active center of \( \beta \)-amylase contains a \( \alpha \)-SH group, because on incubation with SH reagents like \( p \)-chloromercurybenzoate, \( N \)-ethylmaleimide, \( \beta \)-amylase activity is inhibited (Thoma et al., 1971). However, it has also been suggested that there is no direct involvement of \( \alpha \)-SH groups in the enzyme reaction (Mikami and Morita, 1983). \( p \)-amylase has no disulfide bridges and 2 \( \alpha \)-SH groups were deeply buried
inside the enzyme. Heavy metals as well as other thiol binding reagents inhibit \( \beta \)-amylase activity (Thoma et al., 1971).

### 2.2.2.2 Seed \( \beta \)-amylase

As with \( \alpha \)-amylase, the molecular properties of \( \beta \)-amylase summarized below, have been best studied from the storage organs particularly from barley seeds. \( \beta \)-amylase in barley is encoded by 2 genes, one located on chromosome 4 (Jonassen et al., 1981) and a second locus on the short arm on chromosome 4 (Kreis et al., 1988). The MW of barley \( \beta \)-amylase, predicted from its cDNA sequence from barley is 63 kD, which is also close to MW of purified \( \beta \)-amylase protein. However, in the presence of reducing agents 4 forms can be purified from seeds having MW 59.7 kD, 58 kD, 56 kD and 54 kD with corresponding isoelectric points 5.2, 5.3, 5.5 and 5.7 respectively. These multiple forms of \( \beta \)-amylase result from C-terminal processing of a single gene product of \( \beta \)-amylase by proteases (Lundgard and Sevensson, 1987). Unlike \( \alpha \)-amylase, which is induced de novo during barley seed germination, p-amylase in barley endosperm is synthesized during seed development along with storage proteins, and is stored in a latent form which is activated during germination by post-translational modification (Lundgard and Sevensson, 1987). The majority of \( \beta \)-amylase in barley endosperm exists in a bound form which is considered to be latent or inactive. The proteolysis or reducing agents releases the enzyme from bound form and also increases its activity (Sandergren and Klang, 1950). The notion that bound p-amylase is latent and free p-amylase is active has been questioned recently as it has been shown that reduced activity of bound \( \beta \)-amylase is due to steric hindrances (Sopanen and Lauriere, 1989).
2.2.2.3 Leaf β-amylases

In barley, the developing endosperm possesses 2 β-amylase isoforms. One appears early in endosperm development and is followed by the other which is the major enzyme of barley endosperm. Both enzymes are encoded by different population of mRNA whose levels correspond to development of two respective β-amylase proteins (Shewry et al., 1988). It has been found that the β-amylase encoded by the locus on chromosome 4 is the major seed form of β-amylase while other enzyme encoded by chromosome 2 constitutes the protein expressed in the leaves and shoots (Shewry et al., 1988; MacGregor and Dushickn, 1989). Similarly, two distinct β-amylases have also been reported in wheat and rye, one which was specific to starchy endosperm, and another present in root, leaves or green tissue (Daussant and Lauriere, 1990; Daussant et al., 1991). It is found that while one β-amylase has an endosperm-specific promoter and is expressed only during seed development, the other β-amylase is ubiquitously expressed and therefore is also present in other organs of plants.

A perusal of cDNA sequence of the ubiquitous and endosperm-specific β-amylase sequence of rye reveals that ubiquitous β-amylase is only 77% homologous with endosperm β-amylase (Sadowski et al., 1993; Rorat et al., 1991). It is also distinct from dicot β-amylases and on comparison with Arabidopsis leaf β-amylase, it shows only 61% homology (Monroe and Preiss, 1990) and shows 62% homology with sweet potato tubers β-amylase.
In contrast to cereals however, sweet potato β-amylase gene is encoded by only one gene (Yoshida et al., 1991, 1992). Recently, Ishiguro et al., (1993) detected a nuclear factor which binds 5' upstream sequence of β-amylase gene in sweet potato and is a member of bZIP family.

The cDNA clones of the ubiquitous β-amylase of rye have been obtained and its properties have been compared with endosperm-specific β-amylase (Sadowski et al., 1993). It codes for 503 amino acids and has a deduced MW of 56,700 daltons. It differs characteristically from endosperm specific β-amylase of cereals as it lacks gly-rich repeat sequences in the C-terminal of β-amylase. In contrast to endosperm specific β-amylase which undergoes as C-terminal specific proteolytic processing, ubiquitous β-amylase (lacks C-terminal repeat region) does not undergo this processing (Daussant et al., 1991).

In green tissue, p-amylase has been purified from Vicia faba leaves (Chapman et al., 1972), mustard cotyledons (Subbaramaiah and Sharma, 1987a, 1987b, 1988), Arabidopsis (Lin et al., 1988), pea epityocyl (Lizotte et al., 1990) etc. The leaf p-amylase basically possesses molecular properties similar to the p-amylase of storage tissue, but differs in a few aspects. The pH optima of mustard p-amylase is 5.8-6.2 which is more towards neutral pH than that of potato or barley which range between pH 4 to 5 (Subbaramaiah and Sharma, 1990; Lin et al., 1988; Greenwood and Milne, 1968). Also mustard p-amylase is not susceptible to inhibition by cyclodextrins which are competitive inhibitors of p-amylase (Thoma and Koshland, 1960), while pea p-amylase is slightly inhibited (Lizotte et al., 1990). The specific activities of leaf p-amylase is also lower than that of seed p-amylase (Lin et al., 1988).
β-amylases from leaf constitute a heterogenous group with molecular weights in range of 50 - 65 kD (Subbaramaiah and Sharma, 1988; Lizotte et al., 1990), with Vicia faba p-amylase being an exception with a MW of 27 kD: in contrast to other p-amylases which exist as monomer, it is present as a tetramer in leaves. p-amylase from mustard cotyledon (Subbaramaiah, 1987), and pea shoot (Lizotte et al., 1990), do not bind to concanavalin A and are not glycoproteins. p-amylase from mustard (Subbaramaiah and Sharma, 1987b), and pea (Lizotte et al., 1990), also have isoelectric points more towards the acidic side, than that of p-amylase storage tissues. Both mustard and pea p-amylase do not require SH-groups for catalytic activity of enzyme.

Analogous to α-amylase, in the majority of plant species, p-amylase in leaves is located in extraplastidic fractions. In a few cases, the presence of β-amylase has been detected in chloroplasts, e.g., in Arabidopsis (Lin et al., 1988), pea (Kakefuda, et al., 1986; Levi and Preiss, 1978), but a cytoplasmic contamination contributing to presence of p-amylase in chloroplast can not be ruled out (Beers and Duke, 1990). Moreover others have failed to detect p-amylase activity in pea chloroplasts (Ziegler, 1988). In mustard cotyledons, p-amylase activity is localized exclusively in cytosolic fraction; no p-amylase activity has been detected in chloroplast (Manga and Sharma, 1990). While chloroplastic localization of p-amylase is uncertain, vacuoles isolated from protoplasts of pea, wheat and Arabidopsis (Monore and Preiss, 1990), showed high activity of p-amylase (Ziegler and Beck, 1986). However, in spinach and Chenopodium, p-amylase is cytosolic in nature (Ziegler and Beck, 1986). The distribution of p-amylase activity in different tissue has not been analyzed in
detail. In sweet potato roots p-amylase was present throughout the root (Hagenimana et al., 1992), but in broad bean leaves, p-amylase is localized specifically in the epidermal cell layers (Chapman et al., 1972).

In general, p-amylase in most species is found in extraplastidic compartments, but in view of its localization in vacuoles of pea, Arabidopsis and wheat, the possible vacuolar localization in other species needs to be examined. The signal directing p-amylase to the vacuole is, however, not known. Though p-amylase of Arabidopsis is located in vacuoles, its sequence is also very similar to N-terminal of soybean and sweet potato (Monroe and Preiss, 1990).

The information on regulation of p-amylase activity in leaf is scanty and most studies have been concerned only with its detection and localization. Further, most available information on p-amylase pertains only to cereal seeds (Pan et al., 1988). In mustard cotyledons involvement of GA has been ruled out in photoregulated increase in amylase activity in leaves. The photoregulated P-amylase activity results from phytochrome-mediated enhancement of p-amylase synthesis (Sharma and Schopfer, 1982, 1987). Unlike in cereal seeds, no inactive form of p-amylase, could be immunochemically detected in mustard cotyledons, and increase in activity of the enzyme strictly correlated with the increase in its protein level (Subbaramaiah and Sharma, 1989).

The photoregulation of p-amylase seems to be a characteristic feature of monocot seedlings too, as it has also been detected in maize seedlings (Subbarao, 1992). In barley leaves, though water stress enhanced α-amylase
activity, there was no induction of 6-amylase activity (Jacobsen et al., 1986). In pea seedlings, chloroplast destruction which specifically elevated $\alpha$-amyrase activity, showed only a slight stimulation of 6-amylase (Saeed and Duke, 1990b). The most significant elevation of 6-amylase activity has been noticed in Arabidopsis mutants having altered starch metabolism. Activity up to 40-fold higher than in wild types has been noticed in mutants, only when it was grown under 12 h photoperiod, but not under continuous light (Casper et al., 1985). Interestingly, both starchless and starch-overproducing mutants possessed the elevated 6-amylase activity. Casper et al., (1989) suggest that sucrose acts as an inducer of 6-amylase activity in the Arabidopsis mutant. The role of sucrose on 6-amylase induction is evident in sweet potato petiole cuttings, where sucrose induces an accumulation of 6-amylase by de novo synthesis of enzymes (Nakamura et al., 1991). In addition to sugar, a slight induction of 6-amylase has also been noticed with respect to light (Nakamura et al., 1991).

The functional role of extraplastidic 6-amylase in shoots is not yet known. It is likely that 6-amylase located in the extraplastidic fraction may have a defense related function (Ohto et al., 1992), as chitosan and galactosan fragments can induce 6-amylase synthesis in leaf petiole cuttings of sweet potato (Ryan and Farmer, 1991). Moreover, expression of 6-amylase gene can also be induced by ABA and induction by all of above promoters can be suppressed by GA (Ohto et al., 1992). However, more information is needed to establish a clear role of extraplastidic 6-amylase.
2.2.3 Phosphorylase

Phosphorylases (1,4-\(\alpha\)-D-Glucan:orthophosphate D-glucosyltransferase, E.C. 2.4.1.1.) are transglucosylases that catalyze the reversible transfer of glucose residues between (1 \(\rightarrow\) 4) \(\alpha\)-D glucan and inorganic phosphate. Phosphorylase activity can be detected in all starch-containing plant tissues. Phosphorylase can act both as glucan degrading and glucan-synthesizing enzyme (Beck, 1985; Tsai and Nelson, 1968, 1969), depending on relative concentrations of glucose-1-phosphate and iP. But primarily, it is a degradative enzyme (Steup, 1988, 1990). It degrades linear chains of (1 \(\rightarrow\) 4) \(\alpha\)-linked glucose residues in the presence of inorganic phosphate, with the formation of glucose-1-phosphate. Like \(\alpha\)-amylase, phosphorylase is also present in animals and plants, and the most characterized phosphorylase is the one purified from rabbit muscle. In plants most information about molecular properties of phosphorylase, summarized below, has been obtained from potato tuber phosphorylase.

2.2.3.1 Tuber phosphorylase

Potato phosphorylase (type I) is a large molecule containing 916 amino acids with a MW of about 103916 daltons, and in vivo, it exists as a dimer (Nakano et al., 1989; Lee, 1960a). Phosphorylase also contains one molecule of pyridoxal phosphate per monomer as a cofactor (Lee, 1960b). Potato phosphorylase can be distinguished from rabbit muscle phosphorylase by lack of allosteric regulation; and its activity is also unaffected by AMP (Lee, 1960a). It also has no phosphate and is not phosphorylated by phosphorylase kinase.
from rabbit muscle (Lee, 1960a). It acts on maltose, dextrin, amylose, amylopectin, but poorly on glycogen (Smith, 1977). Potato tuber also contains another type of phosphorylase called type II which has a MW of 90,000 daltons. Unlike type I phosphorylase, it has high activity for glycogen. The type II phosphorylase is a minor phosphorylase of potato tubers and its level is 100-fold lower than type I phosphorylase (Gerbrandy and Verleur, 1971; Shimamura et al., 1982).

The cDNA of potato phosphorylase type I is cloned, it encodes for 966 amino acids, of which 50 amino acids constitute signal peptides directing its translocation to chloroplast/amyloplasts (Nakano et al., 1989). It is pertinent to note that 51% and 40% of the amino acid sequence of the above phosphorylase is identical with rabbit and E.coli phsphorylase, respectively. However, plant phosphorylase is unique in having an amino acid sequence consisting of 78 amino acids mostly hydrophobic, near the central portion of protein sequence (Nakano et al., 1989).

2.2.3.2 Leaf phosphorylase

Leaf phosphorylase has been purified from a number of plant species such as maize (Mateyka and Schnarrenberger, 1984, 1988), spinach and potato (Steup and Schachtele, 1981). In potato leaf, both type I and type II phosphorylase are detectable and are located in cytosol and plastid respectively (Conrads et al., 1986). In leaf, platidic phosphorylase exists as a dimer, has a subunit of 104 kD, and immunologically cross-reacts to type I tuber phosphorylase. The minor, type II phosphorylase of tuber cross-reacts with
cytosolic phosphorylase of potato leaves and has a molecular weight of 94 kD. The leaf plastidic and cytosolic phosphorylase represent two antigenically distinct enzymes as they do not cross-react with each other (Conrads et al., 1986).

The properties of cytoplasmic and plastidic phosphorylase purified from spinach leaves have been compared and found similar in many respects such as activity towards inorganic phosphate during phosphorolysis, and pH optima of glucan synthesizing and degrading activities (Steup and Schachtele, 1981; Shimamura et al., 1982). However, their activity towards glucans varies considerably whereas chloroplastic phosphorylase has affinity for linear low molecular weight maltodextrins and very low affinity for branched polyglucans glycogen, cytoplasmic phosphorylase possesses high affinity for branched polyglucans and has low affinity for maltodextrins (Steup et al., 1980, Steup and Schachtele, 1981). Unlike plastidic phosphorylase, the cytosolic phosphorylase is effectively inhibited by maltotetrose on cyclodextrins, both acting as competitive inhibitors (Steup and Schachtele, 1981; Shimamura et al., 1982). The two phosphorylases are not interconvertible proteins; possess different primary structure as is evident from the difference in immunological properties and peptide patterns (Conrads et al., 1986).

The regulation of phosphorylase activity in plant leaves has not been investigated in detail. In pea cotyledons both cytosolic and plastidic phosphorylases have been detected and plastidic phosphorylase has been detected in both proplastids and amyloplasts. The amount of either enzyme depends on the developmental state of the organ. During seed development,
cytosolic enzyme is a minor enzyme but plastidic phosphorylase is a major enzyme. However, during germination, the level of plastidic enzyme remains constant but the level of cytosolic enzyme increases significantly (Berkel et al., 1991). The plastidic phosphorylase possesses a distinct N-terminal transit peptide which is processed during its translocation to chloroplast, as is evident from the fact the MW of precursor and mature polypeptide is 116 kD and 105 kD respectively. In pea, chloroplasts possesses two types of plastidic phosphorylases, one of which is present in etioplasts and the other appears during photo-induced in chloroplast biogenesis (Berkel et ed., 1991). Plastidic phosphorylase in pea cotyledon is localized in the stromal space of the chloroplast (Berkel et al., 1991). In potato tuber, the location of plastidic phosphorylase depends on tuber age. In the young tuber, it is present in the stroma of amyloplasts, and in mature tubers, it is localized outside the amyloplasts near the membrane of the amyloplasts (Brisson et al., 1989).

In maize leaves, phosphorylase enzyme is present in two distinct locations, i.e., mesophyll and bundle sheath cells, the cytosolic one being confined to mesophyll, and plastidic one is located in bundle sheath chloroplasts (Mateyka and Schnarrenberger, 1984; 1988). Both enzymes have a pH optima around pH 7 and their affinity for substrate is similar to spinach phosphorylases and akin to cytosolic and plastidic phosphorylase, respectively (Mateyka and Schnarrenberger, 1988). However, there is no information about the regulation of phosphorylase in both monocot and dicot leaves during leaf development. In the only study done, it was observed that, in spinach leaves, the activity of cytosolic phosphorylase increased after 35 days of sowing by 4-8
fold, which declined back to initial level by 45th day from sowing. On the other hand, the level of plastidic phosphorylase remained nearly constant (Hammond and Preiss, 1983).

**Functional role of Starch degrading enzymes**

The multiplicity of starch degrading enzymes viz \( \alpha\)-amylase, \( \beta\)-amylase and starch phosphorylase have engendered the question regarding relative importance and role of these enzymes in the process of starch degradation in plant tissues. A clear role for \( \alpha\)-amylase in degradation of starch is evident in cereal seeds germination, where *de novo* synthesized \( \alpha\)-amylase after secretion to endosperm is responsible for the degradation of starch stored in endosperm (Beck and Ziegler, 1989). The degradation of starch in endosperm is initiated by \( \alpha\)-amylase although other enzymes also participates in this response. The role of \( \beta\)-amylase in cereal seeds germination is less clear and has been speculated that due to presence of an excess amount of \( \beta\)-amylase, it may have a nutritive role as a storage protein (Shewry *et al.*, 1988). In potato tuber phosphorylase plays a major role in starch mobilization (Steup, 1988; Steup, 1990).

The close association of \( \beta\)-amylase and starch grain in cereal seeds indicates its logical role in starch degradation during seed germination. Even dry cereal seeds consist of a low level of free \( \beta\)-amylase which may participate in the initial degradation of starch during seed imbibition (Pan *et al.*, 1988). Although \( \beta\)-amylase can not degrade intact starch grains, it can act on gelatinized starch after boiling (Lizotte *et al.*, 1990). However, \( \beta\)-amylase has
been demonstrated to attack native starch granules that have not been partly degraded by other enzymes or solubilized by boiling (Nakamura et al., 1991; Sun and Henson, 1991).

Enzyme extracted from resting barley seeds is able to convert the entire starch content of grain into maltose in less than half an hour at 20°C. Therefore, it appears that the enzyme present in grain is in large excess with regards to its enzymatic function. β-amylase activity in cereal seeds is 500-fold more than needed. A 100-fold reduction would still have enough β-amylase for the mobilization of endosperm starch (Sopanen and Lauriere, 1989).

The functional role of these enzymes is less clear in photosynthetic tissues such as leaves. Since a major portion of amylolytic enzymes both α-amylase and β-amylase is localized in extraplastidic compartment, which does not contain a suitable carbohydrate polymer, there have been doubts about their importance in mobilization of photosynthetic starch (Dunn, 1974). In green leaves, β-amylase is reported to be present predominantly in cytosol (Manga and Sharma, 1990), in few cases in the vacuole (Ziegler and Beck, 1986) and in chloroplasts (Kakefuda et al., 1986; Okita et al., 1979). Due to the location of this enzyme outside the chloroplasts, its function is unclear (Monroe and Preiss, 1990).

Moreover, in most species a clear demonstration of localization of α-amylase in chloroplasts has not been shown (Beers and Duke, 1988). In case of β-amylase, it is now firmly established that above enzyme in green leaves is extraplastidic in nature (Manga and Sharma, 1990) and the reports
describing its localization in chloroplastic may result from contamination of cytosolic $\beta$-amyrase. Moreover precursor to $\beta$-amyrase lack a N-terminal transit peptide which is needed to decrease its import to chloroplasts (Kreis et al., 1988, Mikami and Morita, 1983). Similar to amylases in vivo function of cytosolic phosphorylase is also unclear (Yang and Steup, 1990). Not with standing these reports, it is now assumed that $\alpha$-amyrase by virtue of its capacity to hydrolyze native starch granule, is the primary enzyme responsible for initiating starch degradation in chloroplasts, on which other starch degrading enzyme act. Therefore, it has been proposed that phosphorylase rather than amylase present in chloroplast is important for mobilization for photosynthetic starch.