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Light-Induced Chloroplast α-Amylase in Pearl Millet (*Pennisetum americanum*)[1]

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In pearl millet (*Pennisetum americanum*) seedlings light induces the appearance of a leaf α-amylase isozyme. The leaf α-amylase isozyme was present in enriched amounts in isolated chloroplasts but it could not be detected in isolated etioplasts. The chloroplast α-amylase was present in both mesophyll and bundle-sheath chloroplasts. Preliminary characterization indicated that molecular properties of chloroplast α-amylase were like those of a typical α-amylase. The plastidic α-amylase had a molecular mass of 46 kD, pH optimum of 6.2, required Ca^{2+} for activity and thermostability, but lost activity in the presence of ethylenediaminetetracetate. Plastidic α-amylase activity after sodium dodecyl sulfate-polyacrylamide gel electrophoresis could be renatured in situ by Triton X-100. Western blot analysis demonstrated that this protein was antigenically similar to a maize seed α-amylase. In vivo [^{35}S]methionine labeling of bundle-sheath strands isolated from light-grown leaves followed by immunoprecipitation revealed that bundle-sheath strands synthesized plastidic α-amylase de novo.

In green leaves surplus carbohydrates generated during photosynthesis are stored in the form of starch in chloroplasts. The level of chloroplastic starch undergoes a daily oscillation, where its level becomes higher during the day when photosynthesis favors its accumulation, and its level declines during the night because of its mobilization. Although the metabolic pathway of transitory starch mobilization in leaves has not yet been fully elucidated, studies of storage organs such as seeds and tubers have revealed that starch degradation is mediated by a small group of enzymes, α- and β-amylases, phosphorylases, and starch-debranching enzymes (Beck and Ziegler, 1989). It is assumed that the above starch-degrading enzymes may also participate in the mobilization of transitory starch in chloroplasts.

Since starch is exclusively localized in chloroplasts, it is reasonable to expect that starch-degrading enzymes too are localized in chloroplasts. However, studies of intracellular localization of starch-degrading enzymes in green leaves are somewhat contrary to the above expectation. These studies have indicated that most of the activity of starch-degrading enzymes such as α-amylase and β-amylase in leaves is rather confined to the extraplastidic compartment (Okita et al., 1979; Okita and Preiss, 1980; Lin et al., 1988; Ziegler, 1988; Li et al., 1992; Ghiena et al., 1993). Among the amylases, β-amylase activity seems to be completely absent from chloroplasts of most plants (Beck and Ziegler, 1989) such as mustard (Manga and Sharma, 1990) and pea (Saeed and Duke, 1990). In fact, in pea and wheat leaves it appears to be localized in the vacuolar compartment (Ziegler and Beck, 1986; Beers and Duke, 1988). Likewise, the major part of α-amylase activity in pea (Ziegler, 1988; Beers and Duke, 1990) is localized in the extrachloroplastic compartment, and less than 5% of α-amylase activity seems to be associated with chloroplasts (Ziegler, 1988). The presence of a chloroplastic α-amylase has been found in spinach (Okita et al., 1979), *Arabidopsis* (Lin et al., 1988), sugar beet (Li et al., 1992), and *Vicia faba* (Ghiena et al., 1993), but evidence in favor of plastidic α-amylase in many plants is equivocal and the likelihood of cytosolic α-amylase contaminating chloroplast preparations is difficult to exclude (Beers and Duke, 1988, 1990; Ghiena et al., 1993).

It is thought that either the amylolytic pathway or phosphorolytic pathway or a combination of the two may mobilize transitory starch in chloroplasts (Okita et al., 1979; Steup and Latzko, 1979; Beck and Ziegler, 1989). Since in several species the presence of α-amylase in chloroplasts has not been unequivocally established, the probability of operation of the amylolytic pathway in starch degradation is ambiguous. Although the existence of a plastidic phosphorylase in many species has been reliably established (Steup, 1988), the absence of α-amylase in plastids is intriguing. It has been reported that starch-degrading enzymes other than α-amylase and α-glucosidase do not seem to be capable of degrading native starch grain, at least in vitro (Steup et al., 1983; Sun and Henson, 1991). Therefore, the presence of an α-amylase (or α-glucosidase) activity in the chloroplast may be needed at least to initiate the degradation of starch. Whereas α-glucosidase activity has been detected in the chloroplasts (Beers et al., 1990), evidence of a plastidic α-amylase is ambiguous in many species.

The present study was undertaken to determine the presence or absence of a chloroplastic α-amylase in leaves of pearl millet (*Pennisetum americanum*) seedlings. In an earlier study we observed that the appearance of an α-amylase isozyme in greening pearl millet leaves was dependent on light-induced chloroplast biogenesis (Vally and Sharma, 1991). Here we report that this α-amylase is exclusively localized in the chloroplast and is synthesized de novo during greening of the leaf.

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MATERIALS AND METHODS

Plant Materials and General Methods

Pearl millet (*Pennisetum americanum*) var WCG.75 seeds were obtained from AP State Seed Corp. (Hyderabad, India). The seeds were sown on moist germination papers and the seedlings were grown at 25°C in distilled water under continuous red light (0.67 W m^{-2}) or darkness. Chloroplasts or etioplasts were isolated from leaves of 10-d-old red light-grown or 8-d-old dark-grown seedlings, respectively. The procedures for isolation of chloroplasts and etioplasts from leaves (Palmer, 1986), isolation of mesophyll protoplasts (Day et al., 1981), and mechanical isolation of bundle-sheath strands (Chollet and Ogren, 1973) were nearly the same as described earlier (Vally and Sharma, 1991). The intactness of chloroplasts was confirmed by the ferricyanide reduction test (Lilley et al., 1975) and also by phase contrast microscopy. The chloroplasts were disrupted by osmotic shock or sonication in a lysis medium containing 50 mM Na-acetate buffer, pH 4.6. The inclusion of 10 mM $CaCl_2$ in the lysis mixture significantly increased the recovery of α-amylase activity in the homogenate by stabilizing α-amylase activity. α-Amylase activity was assayed using β-limit dextrin as a substrate, and β-amylase activity was assayed using amylose as a substrate as described earlier (Vally and Sharma, 1991). The pH optimum of plastidic amylase was determined in 50 mM Na-acetate buffer (pH 4.2–6.6). The thermostability of the enzyme in the presence (10 mM $CaCl_2$) and absence of calcium and the effect of EDTA (10 mM) were determined in 50 mM Na-acetate buffer, pH 4.6, by the protocols described by Okita et al. (1979).

Maize α-amylases were purified to homogeneity from endosperm of 6-d-old seedlings by ammonium sulfate precipitation, starch affinity chromatography, and DEAE cellulose chromatography into two α-amylase peaks possessing physicochemically distinct α-amylases. On the bases of increasing mobility of isozymes on native PAGE, maize α-amylases were numbered as α-amylase-1,2,3,4. The peak I of DEAE-cellulose consisted of α-amylase-1,2 and peak II consisted of α-amylase-3,4, respectively. The antiserum against α-amylase-1,2 and α-amylase-3,4, were named α-1,2- and α-3,4-antiserum, respectively. β-Amylase was purified to homogeneity from the unbound fraction obtained from starch affinity chromatography by glycogen precipitation and DEAE-cellulose chromatography (Subbarao, 1992). Pure α-amylases and β-amylase were injected into rabbits to obtain respective polyclonal antibodies using standard protocols. The antibodies against one particular amylase group did not cross-react with other amylases. Double diffusion against α-amylase was carried out using the protocol of Ouchterlony (1949). Immunoprecipitation of α-amylase activity in plastidic homogenate was carried out using the above antibodies following the protocol described by Subbaramaiah and Sharma (1989).

Electrophoresis

Nondenaturing PAGE was performed using the procedure described by Davis (1964) and SDS-PAGE was carried out by following the procedure of Laemmli (1970). After SDS-PAGE (10% gel) the amylase activity in the gel was renatured using the procedure described by Segundo et al. (1990). After SDS-PAGE the gel was submerged in 200 mL of 1% (v/v) Triton X-100 at room temperature, and the solution was changed at 30-min intervals during the next 2 h. The amylase bands after native PAGE and SDS-PAGE were visualized using an incubation mixture containing 0.5% (w/v) β-limit dextrin and 10 mM $CaCl_2$ as described earlier (Vally and Sharma, 1991). The molecular mass of the renatured protein and also that of in vivo labeled α-amylase immunoprecipitated with α-1,2-antiserum was determined after SDS-PAGE by running standards of known molecular mass. The molecular masses of the standards used were: α-lactoalbumin, 14.2 kD; trypsin inhibitor, 20.1 kD; trypsinogen, 24 kD; carbonic anhydrase, 29 kD; glyceraldehyde-3-phosphate dehydrogenase, 36 kD; egg albumin, 45 kD; and BSA, 66 kD.

Western blotting after native PAGE and SDS-PAGE was conducted using α-1,2-antiserum following the procedure of Renart and Sandoval (1984). The bands were visualized by using a peroxidase-conjugated anti-rabbit donkey antibody.

In Vivo Labeling

Isolated bundle-sheath strands (about 2.8×10^7 strands) were incubated at 25°C in 150 μL of 10 mM, pH 7.4, Tris-HCl buffer containing 10 mM $CaCl_2$ and 40 μCi of [^{35}S]Met (>1000 Ci $mmol^{-1}$) in plastic tubes. After 4 h the tubes were transferred to an ice bath, and the bundle-sheath strands were washed three times with an ice-cold homogenization buffer containing 25 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 10 mM Met. The bundle-sheath strands were homogenized in 200 μL of the above homogenization buffer in a precooled pestle and mortar. The homogenate was clarified by centrifugation in a microfuge at 14,000*g* for 10 min at 4°C, and the supernatant was supplemented with SDS (0.8%, w/v) and boiled for 4 min. The supernatant was subjected to immunoprecipitation, SDS-PAGE, and fluorography using the procedure described by Subbaramaiah and Sharma (1989).

RESULTS

To specifically detect amylases in pearl millet chloroplasts, only the chloroplast preparations that were judged highly intact (>95%) were used in this study. Isolated chloroplasts on disruption released a significant amount of amylolytic activity that could degrade polymeric carbohydrates such as amylose and β-limit dextrin. Disruption of chloroplasts by osmotic lysis or sonication released amylolytic activity in the homogenates, and inclusion of $CaCl_2$ (10 mM) in the lysis mixture significantly stabilized the α-amylase activity. By contrast, inclusion of EDTA (10 mM) in the lysis mixture completely abolished amylolytic activity (Table I). When the homogenate was subjected to heat treatment (70°C, 10 min) in the absence of $CaCl_2$, amylolytic activity was completely abolished, whereas inclusion

Table I. *Characterization of α-amylase present in chloroplast homogenate obtained after sonication of intact chloroplasts*

Chloroplasts were obtained from leaves of 10-d-old red light-grown pearl millet seedlings.

Treatment	α-Amylase Activity	
	Mesophyll	Bundle sheath
	nkat/mg protein	
Homogenate (H)	5.0	2.7
H + 10 mM $CaCl_2$ (70°C, 10 min)	4.7	2.6
H − 10 mM $CaCl_2$ (70°C, 10 min)	0	0
H + 10 mM EDTA + 10 mM $CaCl_2$	0	0
pH optimum (Na-acetate)	6.2	6.2
Molecular mass (kD)	46	46

of $CaCl_2$ (10 mM) in the medium protected it from heat inactivation (Table I). The pH optimum of plastidic amylase in Na-acetate buffer was 6.2 and different from that of β-amylase in pearl millet leaves (pH optimum 4.6).

Since these properties are largely characteristics of α-amylases, it is evident that chloroplastic amylolytic activity consists of α-amylase. The above conclusion is further supported by the observation that, after the chloroplastic homogenate was subjected to SDS-PAGE, the denatured amylase protein could be renatured in situ by exchanging SDS in the gel with Triton X-100 (Fig. 1, lanes 1 and 2). The molecular mass of plastidic amylase was found to be 46 kD using renaturation of α-amylase after SDS-PAGE as a tool (Table I).

The α-amylase present in pearl millet chloroplasts was antigenically similar to α-amylase-1,2 (pI 6.30, 5.70) present in maize seed (Subbarao, 1992) and in pearl millet seed but was absent in maize leaf (Fig. 2A). In contrast, α-amylase-3,4 of maize seed was antigenically similar to that of maize leaf and pearl millet seed but could not be detected in pearl millet leaf (Fig. 2B). The pearl millet leaf α-amylase generated a single precipitin line against maize α-1,2-antiserum on double diffusion (Fig. 2A) and immunoelectrophoresis (data not shown). Moreover, the immunocomplex between pearl millet α-amylase and maize α-1,2-antiserum could also be specifically stained for α-amylase activity after double diffusion (Fig. 2C). Native PAGE of the plastidic homogenate revealed the presence of a single isozyme of α-amylase, which could degrade β-limit dextrin (Fig. 1, lane 4). Western blotting of the plastidic homogenate after native PAGE and probing membranes using maize α-1,2-antiserum revealed a single band at a position identical with that of the α-amylase isozyme detected with activity staining (Fig. 1, lane 5).

The plastidic homogenate from both mesophyll cells and bundle-sheath strands did not possess detectable antigenic activity of β-amylase (Fig. 2D) and that of α-amylase-3,4 (pI 6.14, 5.50) (data not shown). When the plastidic amylolytic activity was immunoprecipitated by antisera specific for α-amylases and β-amylase from maize, only α-1,2-antiserum could immunoprecipitate plastidic amylolytic activity. Although maize antiserum against β-amylase could not immunoprecipitate any plastidic amylase activity, the same antiserum could immunoprecipitate β-amylase activity from crude homogenate, signifying the presence of only cytosolic β-amylase in pearl millet leaves (data not shown).

Since pearl millet is a C_4 plant, the transitory starch generated during photosynthesis is mainly localized in bundle-sheath cells. However, α-amylase activity was present in chloroplasts isolated from both mesophyll and bundle-sheath cells (Table II). On an average, a bundle-sheath chloroplast possessed 1.75-fold higher α-amylase activity on a chloroplast basis than that present in mesophyll chloroplasts. In contrast to mesophyll cells, bundle-sheath cells did not possess any cytosolic β-amylase activity. In etioplasts isolated from dark-grown leaves no α-amylase activity could be detected. Similarly, bundle-sheath cells isolated from dark-grown leaves did not possess any α-amylase activity (Table II).

These results indicate that plastidic α-amylase activity seems to be associated with chloroplasts only and the enzyme is de novo synthesized during light-mediated chloroplast biogenesis. In vivo labeling of bundle-sheath cells isolated from light-grown leaves with [^{35}S]Met showed that these cells synthesized de novo an α-amylase, which could be detected after immunoprecipitation and SDS-PAGE of bundle-sheath cell homogenates, using a rabbit antiserum against maize α-1,2-antiserum cross-reacting

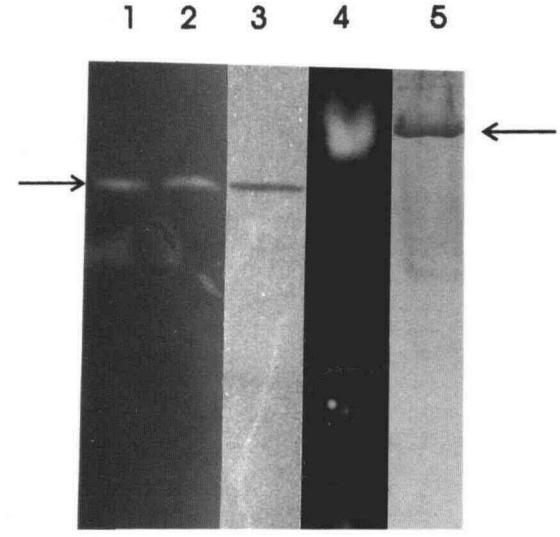

Figure 1. Detection of chloroplastic α-amylase isolated from pearl millet leaves after native or SDS-PAGE. Leaf and bundle-sheath chloroplastic α-amylases after SDS-PAGE were renatured by incubating gels in Triton X-100, followed by detection of α-amylase activity in gels (lanes 1 and 2) by incubating in starch and staining with iodine (negative staining). α-Amylase activity (10 nkat) was loaded in lanes 1, 2, and 4. The in vivo labeling of bundle-sheath cells was carried out by incubating in [^{35}S]Met (lane 3), and labeled proteins were immunoprecipitated using α-1,2-antiserum and were visualized after SDS-PAGE and fluorography. Bundle-sheath chloroplast α-amylase activity after native PAGE was visualized by contact printing of gel on a β-limit dextrin-containing agar gel by negative staining (lane 4). Another gel after native PAGE was blotted on the nitrocellulose sheet and α-amylase isozyme was visualized by western blotting using α-1–2-antiserum (lane 5).

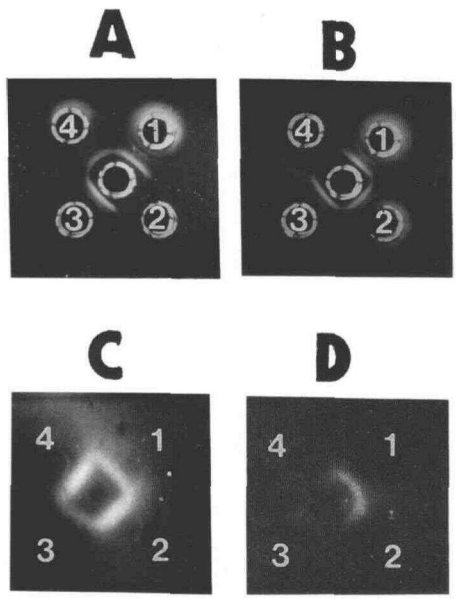

Figure 2. Immunochemical characterization of pearl millet leaf α-amylase using maize amylase antiserums. A, Ouchterlony double-diffusion gel. The central well was filled with maize α-1,2-antiserum and the peripheral well was filled with the samples. The numbers indicate the positions of antigen wells: 1, Maize seed; 2, maize leaf; 3, pearl millet seed; 4, pearl millet leaf. B, The central well was filled with maize α-3,4-antiserum and the peripheral well was filled with the samples. 1, Maize seed; 2, maize leaf; 3, pearl millet seed; 4, pearl millet leaf. C, The staining of precipitin line for α-amylase activity after Ouchterlony double diffusion. 1, Pearl millet leaf; 2 and 4, seed; 3, chloroplasts. D, The staining of precipitin line for β-amylase activity after Ouchterlony double diffusion. The central well was filled with maize β-antiserum and the peripheral well was filled with the samples. The numbers indicate the positions of antigen wells: 1, pearl millet leaf; 2, mesophyll chloroplast; 3, bundle-sheath chloroplasts; 4, blank well.

specifically to plastidic α-amylase (Fig. 1, lane 3). The position of the radioactive band obtained after in vivo labeling of bundle-sheath cells was identical with that of a plastidic α-amylase band renatured after SDS-PAGE. By contrast, no de novo synthesis of apoproteins that are antigenically similar to that of maize β-amylase or of α-amylase-3,4 could be detected in bundle-sheath cells.

DISCUSSION

Although chloroplasts are a logical site for the presence of amylases, the existence of the major part of activity of amylases in the extraplastidic compartment has been a major drawback in conclusively assigning amylase activity to chloroplasts. Isolated pearl millet chloroplasts possess a significant amount of amylolytic activity with properties characteristic of a typical α-amylase. Since amylolytic activity could not be detected in isolated etioplasts, it is likely that α-amylase activity in chloroplasts is acquired during chloroplast differentiation. The dependence of plastidic α-amylase appearance on chloroplast biogenesis is in accord with the observations that light mediates induction of several plastidic enzymes, including Rubisco, during chloroplast development in monocot leaves (Nelson and Langdale, 1992). The presence of α-amylase in bundle-sheath chloroplasts and its de novo synthesis in bundle-sheath strands isolated from light-grown leaves is also positively correlated with its likely role in degradation of transitory starch, because in C_4 plants the bundle-sheath chloroplasts are the principal site of starch synthesis (Spilatro and Preiss, 1987). Moreover, in pearl millet leaves bundle-sheath chloroplasts possess a higher level of α-amylase activity in comparison to mesophyll chloroplasts.

Chloroplast-specific α-amylases have been reported in spinach (Okita and Preiss, 1980), *Arabidopsis* (Lin et al., 1988), and sugar beet (Li et al., 1992). Spinach plastidic α-amylase lacked a few of the characteristic properties of α-amylases, such as loss of activity on incubation with EDTA and thermostability of α-amylase activity in the presence of Ca^{2+} (Okita et al., 1979). By contrast, pearl millet plastidic α-amylase possess properties typical of α-amylase, e.g. its activity is sensitive to EDTA and can be stabilized by Ca^{2+} against heat denaturation (Table I). Moreover, in a fashion similar to maize seed α-amylase (Segundo et al., 1990), pearl millet plastidic α-amylase can also be renatured after SDS-PAGE in situ in gel by exchanging SDS with Triton X-100 (Fig. 1, lanes 1 and 2). In addition, it also possesses antigenic epitopes similar to that of α-amylase-1,2 of maize of 46 kD present in maize seed (Fig. 2A). In contrast to sugar beet leaves, in which four α-amylase isozymes could be detected in chloroplasts (Li et al., 1992), pearl millet chloroplasts possess only a single α-amylase isozyme. Western blotting of plastidic homogenates after native PAGE revealed only a single band corresponding with the above isozyme (Fig. 1, lanes 4 and 5).

Even though our results indicate the presence of α-amylase in chloroplasts of bundle-sheath and mesophyll cells of pearl millet leaf, its role in degradation of transitory starch is still uncertain. In C_4 plants, although the starch is mainly localized in bundle-sheath chloroplasts, the above α-amylase is also present in mesophyll chloroplasts, which normally do not accumulate starch. Although it has been suggested that only α-amylase and α-glucosidase can initiate degradation of native starch grains (Steup et al., 1983; Sun and Henson, 1991), the isolation of an *Arabidopsis* mutant that lacks the capacity to degrade starch, although possessing the normal complement of α-amylase activity,

Table II. *Distribution of α- and β-amylase activity in mesophyll and bundle-sheath cells of pearl millet leaves*

Mesophyll and bundle-sheath cells and chloroplasts/etioplasts from thereof were isolated from leaves of 10-d-old red light-grown and 8-d-old dark-grown pearl millet seedlings, respectively.

Fraction	Amylase Activity			
	Mesophyll		Bundle sheath	
	β-Amylase	α-Amylase	β-Amylase	α-Amylase
	nkat/mg protein			
Homogenate	21.6	4.8	0	2.8
Cytosol	18.1	0.3	0	0
Chloroplast	0	5.0	0	2.7
Etioplasts	0	0	0	0

indicates an additional role of some unknown factor in transitory starch mobilization (Caspar et al., 1991).

In summary, it is evident that, although pearl millet chloroplasts do possess α-amylase activity, its role in the mobilization of transitory starch in bundle-sheath cells can only be speculated upon. A molecular-genetic investigation may be particularly useful in ascertaining whether or not plastidic α-amylase plays a role in the mobilization of transitory starch.

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