Plant Physiol. (1990) 93, 131–140
0032-0889/90/93/0131/10/$01.00/0

Received for publication October 4, 1989
and in revised form January 10, 1990

Chloroplastic Regulation of Apoplastic α-Amylase Activity in Pea Seedlings[1]

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ABSTRACT

Photobleaching of pea (*Pisum sativum* L.) seedling leaves by treatment with norflurazon (San 9789) and 7 days of continuous white light caused a 76- to 85-fold increase in the activity of the primary α-amylase, a largely apoplastic enzyme, over normally greening seedlings. Levels of chlorophyll were near zero and levels of plastid marker enzyme activities were very low in norflurazon-treated seedlings, indicating severe photooxidative damage to plastids. As levels of norflurazon or fluence rates were lowered, decreasing photobleaching of tissues, α-amylase activity decreased. Levels of leaf β-amylase and starch debranching enzyme changed very little in norflurazon-treated seedlings. Infiltration extraction of leaves of norflurazon-treated and normally greening seedlings indicated that at least 57 and 62%, respectively, of α-amylase activity was in the apoplast. α-Amylase activity recovered from the apoplast of photobleached leaves of norflurazon-treated seedlings was 18-fold higher than that for green leaves. Inhibitors of photosynthesis (DCMU and atrazine) and an inhibitor of chlorophyll accumulation that does not cause photooxidation of plastid components (tentoxin) had little effect on levels of α-amylase activity, indicating norflurazon-caused loss of chlorophyll and lack of photosynthesis did not cause the large induction in α-amylase activity. An inhibitor of both abscisic acid and gibberellin synthesis (paclobutrazol [PP333]) and an analog of norflurazon which inhibits photosynthesis but not carotenoid synthesis (San 9785) caused only moderate (about fivefold) increases in α-amylase activity. Lincomycin and chloramphenicol increased α-amylase activity in light grown seedlings to the same magnitude as norflurazon, indicating that the effect of norflurazon is probably through the destruction of plastid ribosomes. It is proposed that chloroplasts produce a negative signal for the regulation of the apoplastic α-amylase in pea.

In photosynthetic tissues, normal gene expression involves the interplay of nuclear and chloroplast genomes. There is a significant body of evidence supporting the hypothesis that plastids produce a positive signal, yet unidentified, which regulates nuclear genes of proteins destined for both chloroplasts and extrachloroplastic sites (for reviews *cf.* 33, 40). Photooxidation of plastid constituents in carotenoid deficient plastids has been found to reduce or prevent the expression of nuclear encoded genes for the chloroplastic proteins, *Cab*[2] and SSu (40). Similarly, NR and peroxisomal enzymes, extraplastidic enzymes that are integral to plastid metabolism are greatly decreased in activity by plastid photooxidation (33). Such observations have led researchers to postulate that a positive signal originating from plastids is involved in controlling the nuclear genes for plastid proteins and extrachloroplastic enzymes that are integral to chloroplast function (33, 40). Photooxidation of chloroplasts appears to have little or no effect on the expression of nuclear encoded genes for enzymes which are not integral to chloroplast function, such as phenylalanine ammonia lyase and chalcone synthase (21, 35). The only reported example to date of an enzyme that is greatly enhanced by the photooxidation of carotenoid deficient plastids is the so-called inducible form of NR in norflurazon-treated soybean cotyledons (11, 23).

In this study we have examined plastid control of the most abundant α-amylase in pea tissues. In pea, this enzyme is largely apoplastic (2, 3) and, as is pea β-amylase, is totally or almost totally extrachloroplastic (2, 25, 43). α-Amylase is also primarily extrachloroplastic in other plant species (22, 28). To date, no apoplastic substrate for the pea α-amylase has been identified (3). NF (San 9789), a powerful inhibitor of carotenoid synthesis that causes photooxidation of chloroplasts in white light and inhibition of the expression of certain nuclear encoded genes (34, 40), was found to enhance greatly the activity of the apoplastic α-amylase in pea, whereas other factors which limit plastid functions other than protein synthesis did not.

MATERIALS AND METHODS

Plant Material

Seedlings of pea (*Pisum sativum* L. cv Laxton's Progress No. 9), used in all treatments, were initially grown in darkness for 5 d, followed by either cWL or continuous darkness. White light for most experiments had a fluence rate of ca 370 μmol m^{-2} s^{-1} (160-W Sylvania cool-white fluorescent F72 T12/CW/VHO and 40-W Sylvania incandescent lamps) at

[1] This material is based on work supported by the Cooperative State Research Service, U.S. Department of Agriculture under Agreement 87-CRCR-1-2324, by the College of Agricultural and Life Sciences, University of Wisconsin-Madison and by the Ministry of Education, Government of Pakistan.

[2] Abbreviations: *Cab*, light harvesting Chl *a*/*b* binding protein; SSu, small subunit of ribulose-1,5-bisphosphate carboxylase; NR, nitrate reductase; NF, norflurazon [San 9789, 4-chloro-5-(methylamino)-2-(α,α,α-trifluoro-*m*-tolyl)-3(2*H*)-pyridazinone]; cWL, continuous white light; PP333, paclobutrazol [(2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-1,2,4-triazol-1-yl-)pentan-3-ol]; IE, infiltration extraction; NAD(P)-MDH, NAD(P)-malate dehydrogenase; NAD(P)-GAPDH, NAD(P)-glyceraldehyde-3-phosphate dehydrogenase; CAP, chloramphenicol.

plant height. In fluence rate response studies with NF treatments, cWL fluence rate was varied with neutral density filters. Temperature was constant at 21±1°C.

Herbicide Treatments

Seeds were treated with NF as before (23) and in the same manner with DCMU, San 9785 [4-chloro-5-(dimethylamino)-2-phenyl-3(2*H*)-pyridazinone], or atrazine [2-chloro-4-(ethylamino)-6-(isopropylamino)-*s*-triazine]. Each herbicide was dissolved in acetone to a final concentration of 100 μM and 10 mL of each herbicide solution was applied directly onto 60 dry seeds placed in a 9 cm Petri dish containing a 9 cm diameter filter paper. After the acetone evaporated from seeds they were imbibed for 6 h on 5 layers of filter paper soaked in distilled H_2O. Control treatment seeds were treated with acetone without herbicide and then imbibed with distilled H_2O. After this treatment seeds were either placed about 2 cm deep in vermiculite in flats (for time course studies) or rolled in germination paper soaked in distilled H_2O and germinated for 5 d in a growth chamber in continuous darkness at 21±1°C.

Immediately after the onset of cWL (after 5 d darkness), seedlings germinated in rolls of germination paper were removed from old rolls and wrapped in new germination paper soaked in distilled H_2O (NF treatment) or 100 μM herbicide (DCMU, San 9785, or atrazine). From this time until harvest, seedlings were continuously supplied with either distilled water (NF treatment) or 100 μM herbicide. Seedlings were oriented so that the leaves were exposed at the top of the rolled germination paper.

For NF dose response experiments, NF treatments were the same as outlined above, except that NF concentrations varied from 10 μM to 2 mM.

Tentoxin and Paclobutrazol Treatments

Seeds were imbibed in 30 μg tentoxin [cyclo(*N*-methyl-L-alanyl-L-leucyl-*N*-methyl-*trans*-dehydrophenylalanyl-glycyl), Sigma] mL^{-1} distilled H_2O for 8 h. Seedlings were grown in 20 μg tentoxin mL^{-1} distilled H_2O for 24 h before the onset of the cWL treatment until the time of tissue harvest.

Seeds were treated with 10 mL of 100 μM PP333 dissolved in acetone as described for herbicide treatments. Seedlings were grown under conditions described for NF treatments except that 100 μM PP333 was continuously supplied to seedlings from just before the onset of cWL until harvest.

Protein Synthesis Inhibitor Treatments

Seeds were imbibed for 6 h in solutions containing 323 μg CAP or 443 μg lincomycin mL^{-1} distilled H_2O. Seeds were germinated and grown in the same solutions until harvest. Inhibitor solutions were replaced every 24 h starting from the onset of the light period until harvest (after 7 d of cWL). Cycloheximide-treated seedlings were grown for 5 d in continuous darkness followed by 72 h of cWL in distilled H_2O only. Thereafter, they were treated with 200 μg cycloheximide mL^{-1} distilled water until harvest after 7 d of cWL. Aluminum foil was wrapped around beakers containing inhibitor solutions, as well as the upper half of each rag doll containing seedlings, to avoid exposure of inhibitors to light.

Enzyme Extraction

Leaves were homogenized in an ice chilled mortar in chilled (about 3°C) grinding buffer A (50 mM Hepes-KOH [pH 6.9], 20% [v/v] glycerol, 3 mM $MgCl_2$, 3 mM $CaCl_2$, 1% [v/v] Triton X-100, 20 μM leupeptin, 1 mM DTT) using a tissue to buffer ratio of 1:10. Grinding buffer A for extrachloroplastic and chloroplastic marker enzymes and starch debranching enzyme did not contain glycerol and Triton X-100, and the DTT concentration was increased to 6 mM. Leupeptin and DTT were added to all grinding buffers immediately before use. Homogenates were centrifuged at 21,000*g* for 15 min. Supernatants were decanted and frozen at −20°C until use, except those for starch debranching enzyme assays which were used immediately after preparation.

Infiltration Extraction of Leaf Apoplastic Enzymes

Six 1 g samples from both NF-treated photobleached and normally greening leaves of 12 d old (5 d continuous darkness followed by 7 d cWL) seedlings were harvested, rinsed in distilled H_2O, and blotted dry. Leaves of NF-treated seedlings were not fully expanded and were excised near the apical meristems of seedlings, whereas leaves from normally greening seedlings were well expanded and 1 cm diameter discs were cut from leaves and pooled to obtain 1 g replicate samples. Three 1 g samples from each treatment were subjected to 10 cycles of IE. The IE procedure used was similar to that described by Beers and Duke (2). Tissues were vacuum infiltrated in buffer B (50 mM Hepes [pH 6.9], 3 mM $CaCl_2$, 3 mM $MgCl_2$, 6 mM DTT, 100 mM KCl) for 2 min followed by 3 min at atmospheric pressure. This was repeated three times for each IE cycle. Tissues were then blotted dry and centrifuged to remove buffer infiltrated into tissue as previously described (2). For each IE cycle, buffer centrifuged from tissue and buffer used in vacuum infiltration were stored at −20°C until used for enzyme assays. The remaining three leaf samples from each treatment that were not subjected to IE and tissues subjected to IE were homogenized in a chilled mortar in buffer B to which leupeptin (20 μM) was added. The tissue to grinding buffer ratio was 1:10 (w/v). Homogenates were centrifuged at 21,000*g* for 15 min and supernatants were stored at −20°C.

Enzyme Assays

Total amylolytic activity was measured by assaying production of reducing sugars from Lintner soluble starch (15 min assay) and starch debranching activity by measuring production of reducing sugars from pullulan (3 h assay), as previously described (25). The starch debranching enzyme assay also contained 4 mM DTT. α-Amylase activity was determined by the β-amylase saturation starch-azure technique as described previously (8). All amylolytic assays were performed at pH 6.0. NAD-MDH (EC 1.1.1.37), PEP carboxylase (EC 4.1.1.3.1), and NAD-GAPDH (EC 1.2.1.12) were assayed as described by Beers and Duke (22). NADP-MDH (EC 1.1.1.82)

and NADP-GAPDH (EC 1.2.1.13) were assayed under the same conditions as for NAD-MDH and NAD-GAPDH, respectively, except that NADPH was substituted for NADH. All assays were conducted at 30°C.

Protein and Chl Determinations

Levels of protein in the 21,000*g* supernatants were determined by the method of Bradford (5), using crystalline BSA as a standard. Blanks contained equal amounts of Triton X-100, which causes interfering color in the Bradford assay. Levels of Chl were measured by the method of Harborne (18), immediately following homogenization of leaf tissues.

Electrophoresis

Native gel electrophoresis and electrophoretic transfer through starch containing gels were performed as described by Kakefuda and Duke (24) and as modified by Beers and Duke (2). A 7 to 15% (w/v) linear gradient polyacrylamide gel was used for enzyme separation. A 50 mM imidazole buffer (pH 6.5), containing 0.5 mM $CaCl_2$, and 1 mM DTT was used during electrophoretic transfer.

RESULTS AND DISCUSSION

Effects of Chloroplast Photooxidation on Seedling Growth and Development

In seedlings treated with 100 μM NF, Chl concentrations were less than 1% of that in normally greening tissues at time points of 1 to 7 d after the onset of cWL (370 μmol m^{-2} s^{-1}) (data not shown). The concentration of Chl in seedling leaves after 7 d cWL decreased linearly to nearly undetectable levels as NF concentration was increased from 10 to 40 μM (Fig. 1). These results are characteristic of NF-treated plants grown under white light (11, 36). The lack of carotenoids in NF-treated plants is thought to ultimately result in the formation of triplet Chl which leads to the photooxidation of various plastid components, including Chl, membranes, and proteins (33). This, in turn, reduces number of plastid ribosomes, levels of plastid transcription, and the expression of nuclear genes coding for plastid proteins and for extraplastidic proteins with functions that are required for proper plastid function (33, 40).

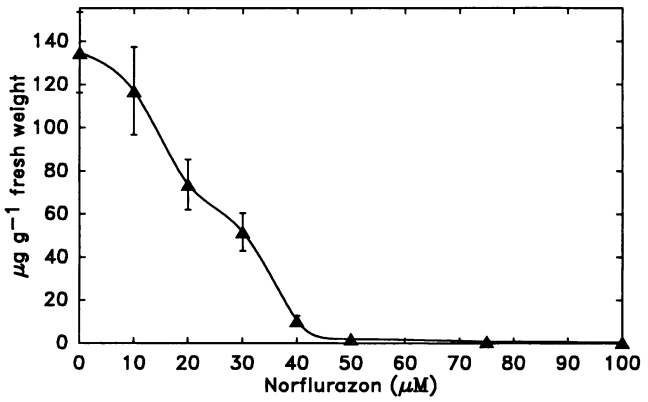

Figure 1. Effect of norflurazon concentration on the leaf Chl concentration of pea seedlings grown for 5 d in dark followed by 7 d of continuous white light. Values are mean ± SE for three separate extractions.

In darkness, NF treatments had no significant effect on the fresh weight of pea seedling epicotyls. After 7 d of cWL, epicotyl fresh weight of control treatments were 3.7-fold higher than those of NF treatments (data not shown). Under cWL, leaf protein concentration in NF and control treatments increased at the same rate for the first 24 h (Fig. 2). After 7 d of cWL, total leaf protein concentration of NF-treated seedlings was 53% that of control seedlings. However, in constant darkness, NF-treated seedlings had slightly higher protein concentrations than control seedlings. Leaves of control seedlings grown either in cWL or constant darkness had similar protein concentrations. The decrease in leaf protein concentration in seedlings treated with NF is not pronounced unless the initial application of NF is 40 μM or greater (data not shown). The decrease in both fresh weight and soluble protein levels in photobleached plants appears to be due to the absence of functional chloroplasts (4).

Effects of Chloroplast Photooxidation on Hydrolytic-Amylolytic Enzymes

Total amylolytic activity assays measured the combined activities of both α- and β-amylases. Starch azure assays measured only α-amylase activity. Starch debranching enzyme activity was not detectable in the 15 min assays for either total amylolytic or α-amylase activity with the frozen enzyme preparations. Total amylolytic activity increased 3.9-fold on a specific activity basis (data not shown) and 3.3-fold on a fresh weight basis (data not shown) in photobleached pea seedlings treated with 100 μM NF and grown under cWL for 7 d. This increase in total amylolytic activity was primarily due to a 28-fold (specific activity, Fig. 3) and a 24-fold (fresh weight basis, data not shown) increase in α-amylase activity over rates of activity in control and NF-treated seedlings at

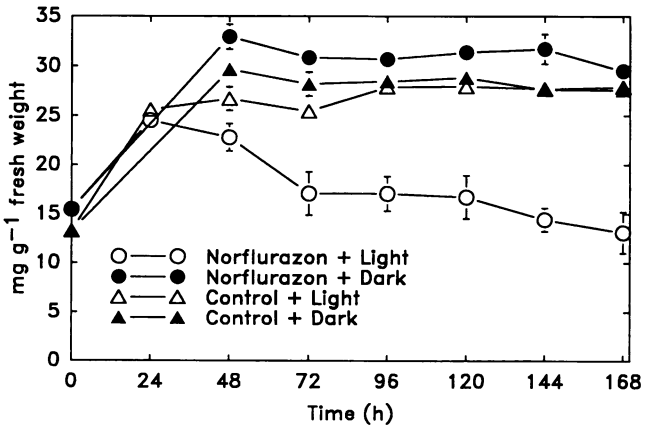

Figure 2. Effect of photobleaching (norflurazon treatment) on the protein concentration in leaves of pea seedlings grown for 5 d in dark followed by 7 d of continuous white light or dark. Leaf protein concentrations were determined after every 24 h starting from the onset of light period. Values are mean ± SE for 3 separate extractions.

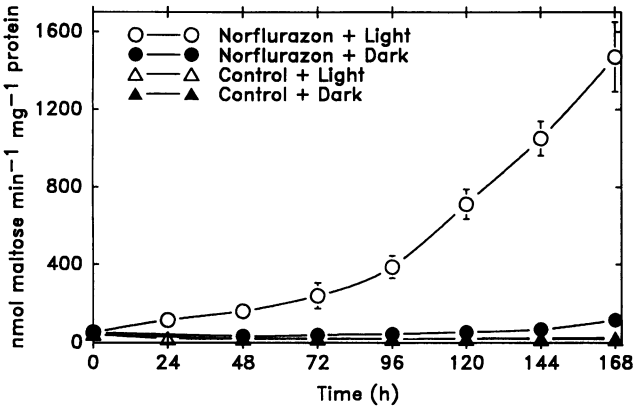

Figure 3. Effect of photobleaching (NF treatment) on α-amylase activity in pea leaves. Seedlings were grown as described in Figure 2. α-Amylase activity was determined at 24 h intervals starting from the onset of light period. Values are means ± SE for three separate extractions.

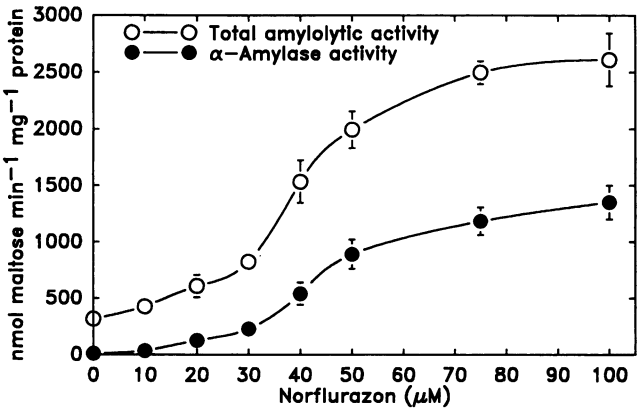

Figure 4. Effect of norflurazon concentrations on the extractable total amylolytic and α-amylase activity in pea leaves. Seedlings were grown as described in Figure 1. Values are means ± SE for three separate extractions.

the beginning of the light treatment. In contrast, in darkness, NF treatments resulted only in a 2.2-fold increase in the α-amylase activity (Fig. 3). The largest increase in both total amylolytic and α-amylase activities in NF-treated seedlings was observed with treatments of 30 to 50 μM NF (Fig. 4), the same range of NF concentrations in which Chl photooxidation became complete (Fig. 1). Seedlings treated with 1 mM NF under cWL had only a slight increase in both the total amylolytic and α-amylase activity over those treated with 100 μM NF, whereas those treated with 2 mM NF had slightly decreased amylolytic activity compared with seedlings treated with 1 mM NF (data not shown).

Under the same conditions and over the same period, normally greening seedlings (controls) decreased in both total specific amylolytic and α-amylase activities. After 7 d of cWL, total amylolytic and α-amylase activities in photobleached leaves were, respectively, 9.3-fold (data not shown) and 76-fold (Fig. 3) higher than in normally greening seedling leaves. On a fresh weight basis total amylolytic and α-amylase activities were respectively, 4.4-fold and 36-fold higher in photobleached leaves than in normally greening leaves after 7 d of cWL (data not shown). The difference in the magnitudes of specific and fresh weight basis activities is due to differences in protein levels of the two treatments (Fig. 2). Leaves of dark-grown NF-treated seedlings had 4.4-fold higher α-amylase activity than that of control seedlings (Fig. 3).

In vitro incubation of crude enzyme preparations with 100 μM to 1 mM NF had little or no effect on either total amylolytic or α-amylase activity (data not shown), indicating no direct effect of this herbicide on α-amylase activity. Also, mixing extractions from light grown seedlings grown with and without NF resulted in the expected cumulative α-amylase activity, indicating that there were no soluble α-amylase inhibitors or activators in either preparation and that the NF-caused increase in α-amylase activity was probably due to an increase in the total amount of the enzyme.

NF-treated seedlings grown at a fluence rate of 330 μmol m^{-2} s^{-1} had, respectively, 7.3-fold and 19.2-fold higher total amylolytic and α-amylase activity than seedlings grown at a fluence rate of 0.5 μmol m^{-2} s^{-1} (data not shown and Fig. 5). In contrast, normally greening seedlings grown at the highest light intensity had lower α-amylase activity than those grown at the lowest fluence rate. NF-treated seedlings grown at the lowest and highest fluence rates had, respectively, 70% and 0.018% of the Chl concentration of control seedlings grown at the same fluence rates (Fig. 5). These findings indicate that plastid photooxidation is required for the large induction of α-amylase activity in NF-treated seedlings.

NF-mediated photobleaching of seedlings did not induce starch debranching enzyme activity. In contrast, starch debranching enzyme activity was 1.5-fold higher in normally greening seedling leaves than in photobleached leaves (Table I).

Identification of the α-Amylase Induced by Chloroplast Photooxidation

Following electrophoretic blot transfer of proteins from a native polyacrylamide gel through a starch containing gel, KI-

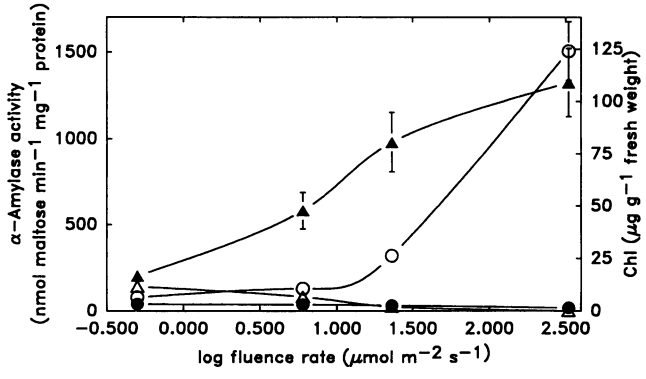

Figure 5. Effect of fluence rate on the development of α-amylase activity (○, ●) and Chl concentration (△, ▲) in leaves of NF-treated (○, △) and normally greening (●, ▲) pea seedlings. Seedlings were grown as indicated in Figure 1. Values are means ± SE for three separate extractions.

Table I. *Starch Debranching Enzyme Activity in Photobleached (Norflurazon-Treated) and Normally Greening (Control) Leaves of Pea Seedlings*

Seedlings were grown for 5 d in continuous dark followed by 7 d of continuous white light. Values are means ± SE for three separate extractions.

Treatments	Starch Debranching Enzyme Activity nmol Maltose min^{-1}	
	g^{-1} fresh wt	*mg^{-1} protein*
Photobleached leaves	117 ± 9 (45.2)[a]	9.89 ± 0.7 (66.4)
Green leaves (control)	259 ± 19	14.9 ± 1.2

[a] Numbers in parentheses = % control.

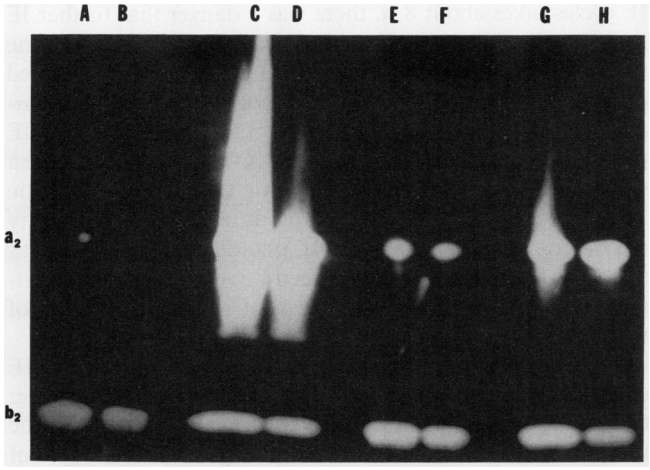

Figure 6. Detection of amylolytic enzymes in the leaves of photobleached (norflurazon-treated, lanes C, D, G, and H) and normally greening (control, lanes A, B, E, and F) leaves of pea seedlings. Seedlings were grown for 7 d either in continuous white light (lanes A, B, C, and D) or continuous dark (lanes E, F, G, and H) after germination for 5 d in continuous dark. Amylolytic enzymes were detected from crude leaf extracts by native polyacrylamide gel electrophoresis followed by electrophoretic transfer through a polyacrylamide gel containing Lintner soluble starch and staining with KI/I_2 solution. α-Amylase (a_2) produces low mol wt dextrins which diffuse from the gel, leaving unstained bands. β-Amylase (b_2) produces β-limit dextrins which stain orange to red. This β-amylase (b_2) has also been identified from the disappearance of this band from a gel containing β-limit dextrins instead of soluble starch (2) and the supersensitivity of b_2 band to *in vitro* heat treatment at 70°C (see Fig. 7). Lanes were loaded on a protein equivalent basis (50 μg-lanes A, C, E, and G; 25 μg-lanes B, D, F, and H).

I_2 staining of limit dextrins in the starch containing gel indicates the type of amylolytic activity associated with the bands (24). Both NF-treated and normally greening seedlings grown either in cWL or dark revealed the same pattern of amylolytic enzymes (Fig. 6). However, α-amylase (band a_2) from photobleached leaf extracts produced a much more pronounced band as compared to α-amylase of normally greening tissues. In contrast, β-amylase (band b_2) from NF-treated photobleached leaves produced a band of only slightly higher intensity to the control bands. It is also evident that α-amylase was slightly more active in dark than in cWL-grown leaves of control seedlings. Minor forms of both α- and β-amylase as well as both isoforms of starch debranching enzyme, that have been previously identified (2), were not visible on the gel (Fig. 6) due to low amount of protein loaded on the gel. These results indicate that an α-amylase (a_2), that appears to be apoplastic (based on R_F and ref. 2), was the primary amylolytic enzyme induced by photooxidation of chloroplasts.

A general property of many plant α-amylases is their requirement for Ca^{2+} (15). The α-amylase induced in the NF-treated photobleached pea leaf extracts is readily inactivated by EDTA (Table II). Purified α-amylases from pea cotyledons, leaves, and the stem apoplast require Ca^{2+} for activity and are therefore EDTA/EGTA sensitive (3). Pea β-amylase (29, 39) and starch debranching enzyme (39) do not require Ca^{2+} for activity; hence, α-amylase is the only hydrolytic-amylolytic enzyme in our crude preparations that EDTA inhibits. After incubation with EDTA the total amylolytic activity in the photobleached leaf extracts was lower than the amylolytic activity in extracts of normally greening leaves. Addition of EDTA to the green leaf extracts, which contain very low α-amylase activity as compared to total amylolytic activity (25), only caused a slight decrease in total amylolytic activity (Table II).

In crude enzyme preparations of some species, α-amylase is heat stable in the presence of Ca^{2+}, whereas β-amylase is not (3, 22). Heating crude extracts of control or photobleached leaves at 70°C inactivated β-amylases but not the a_2 α-amylase (Fig 7). β-Amylase was very heat sensitive and was inactivated after 5 min at 70°C (Table III; Fig. 7). Activity of α-amylase(s) decreased slightly in photobleached and green leaf extracts between 5 and 20 min at 70°C. These findings agree with a previous report which showed that the primary α-amylase from pea leaves is heat stable in the presence of Ca^{2+} (3).

Localization of α-Amylase in the Apoplast of Photobleached and Normally Greening Pea Leaves

The major α-amylase in pea has been shown to occur primarily in the apoplast in stem tissues (2). Very little of this α-amylase is present in chloroplasts (2, 3, 25), the site of starch degradation. The apoplastic α-amylase appears to be the same α-amylase induced by plastid photooxidation due to NF treatments (see above). To determine if the photooxidation-induced α-amylase is apoplastic, leaves of NF-treated-seedlings were subjected to IE to remove apoplastic proteins.

Starch debranching enzyme was not detectable in apoplastic fractions; hence, all endoamylolytic activity recovered from

Table II. *Effect of EDTA on the in Vitro Amylolytic Activity of Crude Extracts of Photobleached (Norflurazon-Treated) and Normally Greening (Control) Pea Seedling Leaves*

Crude enzyme samples were prepared from the leaves of seedlings grown as in Table I. Values are means ± SE for three separate extractions.

Treatments	Total Amylolytic Activity	
	Photobleached	Green (Control)
	nmol min^{-1} mg^{-1} protein	
+EDTA	556 ± 66	656 ± 75
−EDTA	3260 ± 328	675 ± 51

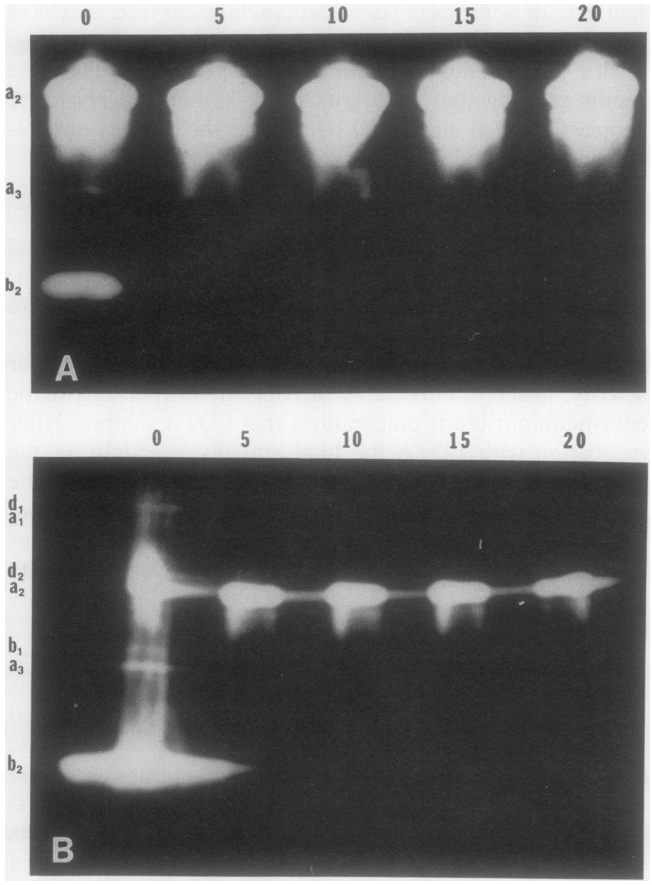

Figure 7. Detection of amylolytic enzymes remaining in crude pea leaf homogenates after heating at 70°C for different periods. Homogenates were prepared from (A) photobleached (norflurazon treatment) and (B) normally greening (control) leaves of pea seedlings grown for 5 d in dark followed by 7 d of continuous white light. Bands are labeled as in Figure 6. Additional α-amylases (a_1 and a_3) were detected. These isozymes were not apparent in Figure 6 due to the low amount of protein that was loaded in lanes. For the same reason, starch debranching enzyme (d_1), which produces amylose that stains blue, was not detected in the gel shown in Figure 6. A second form of starch debranching enzyme (d_2) is not apparent in Figure 7 because α-amylase (a_2) band depleted starch from the location of d_2. Lanes were loaded with equal volumes of crude enzyme.

Table III. *Effect of Heat Treatment (70°C) on Total Amylolytic and α-Amylase Activities in Extracts from Photobleached (Norflurazon-Treated) and Normally Greening (Control) Pea Leaves*

Seedlings were grown as indicated in Table I.

Heat Treatment Duration	Total Amylolytic Activity		α-Amylase Activity	
	Green	Photobleached	Green	Photobleached
min	*μmol min⁻¹ g⁻¹ fresh wt*			
0	5.98	32.8	0.751	22.4
5	0.783	30.6	0.705	21.0
10	0.596	26.8	0.690	20.7
15	0.582	25.1	0.690	19.4
20	0.589	24.0	0.675	17.7

the apoplast was due to α-amylase. About 57 and 63% of the α-amylase activity in leaves was recovered from the apoplast of photobleached and green leaves, respectively, after 10 IE cycles (Table IV), indicating that most of the α-amylase in both treatments was extracellular. The percentage of recovery of α-amylase from the apoplast of leaves was 25 to 30% lower than that previously reported for pea stems (*cf.* Table IV with ref. 2). However, there was some difficulty in removing α-amylase from the apoplast of leaves. This was especially true with photobleached leaves because of the relatively undifferentiated state of these tissues. α-Amylase could still be found in the tenth IE extraction of leaves, although at a lower level than previous IE extractions, indicating that not all of the apoplastic α-amylase had been removed. However, in that 10 IE cycles takes about 8 h, there was a danger that further IE cycles might have resulted in physiological changes to the tissues or denaturation of α-amylase that could have affected results. Recovery of total leaf amylolytic activity and α-amylase activity (total recovered from tissues subjected to IE plus IE cycles) was lower than for leaves that had not been subjected to IE (Table IV), indicating that some amylolytic activity was lost during the IE procedure. α-Amylase activity recovered from the apoplast of photobleached leaves was about 18-fold higher than for green leaves, indicating that α-amylase in the apoplast accounted for a large proportion of the increase due to photooxidation of leaves.

Symplastic contamination of apoplastic fractions from IE cycles was assessed by assaying various intracellular marker enzymes. No activity of PEP carboxylase (Table V), a cytosolic marker enzyme, or starch debranching enzyme (data not shown), a primarily chloroplastic enzyme in pea (2), could be detected in any IE cycle fractions from either treatment. Only very low activity (<1% of total) of NAD-MDH was recovered from 10 IE cycles of either treatment. These findings demonstrate that there was only a very low level of intracellular enzyme contamination in apoplastic preparations prepared by IE.

Effects of Chloroplastic Photooxidative Damage on Cytosolic and Chloroplastic Marker Enzyme Activities

Chloroplastic enzymes exhibit varying degrees of sensitivity to damage caused by NF-induced photooxidation (30, 32). However, extrachloroplastic enzymes, not directly related to chloroplastic functions, have not been reported to be greatly affected by plastid photooxidation (33). To determine if the large increase in activity of the apoplastic α-amylase in NF-treated seedlings was a general phenomenon for extraplastidic enzymes, both cytosolic and plastidic marker enzymes were assayed in NF-treated, photobleached leaves of pea seedlings. After 7 d of cWL, photobleached leaves had only 3.3 and 30%, respectively, of the NADP-GAPDH and NADP-MDH (chloroplastic marker enzymes) activities found in normally greening leaves (Table VI). NADP-MDH activity is apparently less sensitive to the photooxidative damage of plastids (30). Alternatively, the relatively high activity of NADP-MDH in photobleached leaves (30% of the green leaves) may be due to the non-specific activity of extrachloroplastic NAD-MDH with NADPH.

Photooxidative damage to plastids had no adverse effects

Table IV. *Amylolytic Activities in Pea Leaf Apoplast, Symplast, and Noninfiltration Extracted Enzyme Preparations from Photobleached (Norflurazon-Treated) and Green (Control) Seedlings*

Activities of the symplastic and noninfiltration extracted leaf preparations are the sum of the activities of 21,000*g* supernatants and pellets. Apoplastic values include assays for both buffer in which leaves were vacuum infiltrated and buffer removed from leaves by centrifugation. Seedlings were grown as indicated in Table I. Values are means ± SE of three separate extractions.

Enzyme Preparation	Enzyme Activity			
	Photobleached		Green (control)	
	Total amylolytic activity	α-Amylase	Total amylolytic activity	α-Amylase
	μmol min⁻¹ g⁻¹ fresh wt			
A. Infiltration extracted leaves				
Apoplast (10 infiltration-extractions of leaves)	9.63 ± 2.85	9.42 ± 1.16	0.560 ± 0.092	0.538 ± 0.910
Symplast (leaf tissue after 10 infiltration extractions)	19.3 ± 3.7	7.11 ± 1.52	6.30 ± 1.21	0.313 ± 0.043
Total of apoplast plus symplast from infiltration extracted leaves	28.9	16.5	6.86	0.851
B. Noninfiltration extracted leaves				
Apoplast and symplast	37.6 ± 5.6	24.3 ± 3.9	6.89 ± 1.23	0.855 ± 0.168

Table V. *Intracellular Marker Enzyme Activities in Pea Leaf Apoplast, Symplast, and Noninfiltration Extracted Enzyme Preparations from Photobleached (Norflurazon-Treated) and Green (Control) Seedlings*

Activities of symplastic and noninfiltration extracted leaf preparations are the activities of the 21,000*g* supernatants only. Infiltration-extraction values include assays for both buffer in which leaves were vacuum infiltrated and buffer removed from leaves by centrifugation. Seedlings were grown as indicated in Table I. Values are means ± SE of three separate extractions.

Enzyme Preparation	Enzyme Activity			
	Photobleached		Green (control)	
	NAD-malate dehydrogenase	PEP carboxylase	NAD-malate dehydrogenase	PEP carboxylase
	μmol min⁻¹ g⁻¹ fresh wt			
A. Infiltration extracted leaves				
Apoplast (10 infiltration extractions of leaves)	0.446 ± 0.053	ND[a]	1.47 ± 0.13	ND
Symplast (leaf tissue after 10 infiltration extractions)	159 ± 24	1.16 ± 0.14	167 ± 31	0.907 ± 0.15
Total (apoplast plus symplast from infiltration extracted leaves)	160	1.16	168	0.907
B. Noninfiltration extracted leaves				
Apoplast and symplast	177 ± 29	1.47 ± 0.41	156 ± 28	0.945 ± 0.114

[a] Not detected.

on levels of extraplastidic enzyme activities. The extraplastidic marker enzymes NAD-GAPDH, NAD-MDH, and PEPcarboxylase activities were, respectively, 2.3-, 2.0-, and 1.8-fold higher in photobleached leaves than in normally greening leaves (Table VI). Similar increases of cytosolic enzyme activities with NF-caused photooxidation of plastids (23) and photooxidation caused by other factors (33) have been reported. These results indicate that NF-caused decrease in levels of enzyme activity in photobleached pea leaves is restricted to plastids where photooxidative damage occurs. The about 2-fold enhancement in cytosolic enzyme activities in NF-treated seedlings (Table VI) is much lower than that for α-amylase (Fig. 3). Hence, a general enhancement of extrachloroplastic enzyme activities by NF-caused chloroplast photooxidation appears to be only partially and secondarily involved in the very large increase in α-amylase activity.

Effects of DCMU, Atrazine, Tentoxin, San 9785, and PP333 on Apoplastic α-Amylase Activity

Further experiments were designed to screen for a possible chloroplastic constituent(s) that is photooxidized in NF-treated leaves and which may, when eliminated, result in an increase in activity of the apoplastic α-amylase. Photooxida-

Table VI. *Levels of Extrachloroplastic and Chloroplastic Marker Enzymes in Photobleached (Norflurazon-Treated) and Normal Greening (Control) Leaves of Pea Seedlings*

Seedlings were grown as indicated in Table I. Values are means ± SE for three separate extractions.

Enzyme	Activity	
	Green	Photobleached
	μmol min⁻¹ mg⁻¹ protein	
NADP-glyceraldehyde-3-P dehydrogenase	2.40 ± 0.25	0.078 ± 0.008 (3.25)[a]
NADP-malate dehydrogenase	0.133 ± 0.009	0.040 ± 0.005 (30.1)
NAD-glyceraldehyde-3-P dehydrogenase	2.68 ± 0.23	6.21 ± 0.49 (232)
NAD-malate dehydrogenase	8.19 ± 0.70	16.1 ± 1.25 (197)
PEP carboxylase	0.108 ± 0.010	0.189 ± 0.020 (175)

[a] Numbers in parentheses = % control.

Table VII. *Effects of DCMU, Atrazine, Tentoxin, San 9785, Norflurazon (San 9789), and Paclobutrazol (PP 333) on Total Amylolytic and α-Amylase Activities and Chl Concentration in the Leaves of Pea Seedlings*

Seedlings were grown for 5 d in continuous dark followed by 7 d of continuous white light. Values are means ± SE for three separate enzyme or Chl extractions.

Treatment	Total Amylolytic Activity	α-Amylase Activity	Chl
	nmol min⁻¹ mg⁻¹ protein		*μg g⁻¹ fresh wt*
Control	413 ± 13	18.6 ± 0.3	105 ± 5
DCMU	349 ± 11 (84.5)[a]	16.7 ± 0.1 (89.8)	70.7 ± 7.2 (67.3)
Atrazine	281 ± 11 (68.0)	13.9 ± 0.2 (74.7)	80.3 ± 6.5 (76.5)
Tentoxin	509 ± 25 (123)	22.4 ± 1.6 (120)	4.4 ± 0.94 (4.18)
San 9785	758 ± 25 (183)	102 ± 3.2 (550)	78.3 ± 7.4 (74.2)
San 9789	3170 ± 205 (667)	1590 ± 158 (8450)	0.61 ± 0.05 (0.06)
PP333	689 ± 22 (167)	85.0 ± 3.0 (458)	ND[b]

[a] Numbers in parentheses = % control. [b] Not determined

tive damage to plastids in NF-treated WL grown plants results in the loss of photosynthetic CO_2 fixation (37) and associated metabolism. Both DCMU and atrazine, inhibitors of electron flow from PSII to PSI and thus CO_2 fixation, suppressed total amylolytic and α-amylase activity (Table VII). Similar responses of other cytosolic enzymes, such as NR, to these herbicides have been reported (9). DCMU-and atrazine-treated seedlings had slightly decreased leaf Chl concentrations, apparently due to a localized loss of Chl along the leaf margins. These data indicate that the loss of photosynthesis in NF-treated seedlings did not cause the large increase in α-amylase activity.

Tentoxin, a phytotoxin produced by the fungi *Alternaria tenuis* Auct and *Alternaria alternata* (Fr.) Keissler, causes severe Chl loss in many plant species including peas (12, 41). Tentoxin-induced chlorosis is thought to reflect a selective disruption of chloroplast functions, including reductions in levels of several chloroplastic proteins and galactolipids (26). Tentoxin does not inhibit carotenoid synthesis or cause photooxidation of plastids. Tentoxin-treated seedlings had very low levels of Chl (Table VII). The effect on total amylolytic and α-amylase activity was slightly positive. The slight increase in the specific amylolytic activity in tentoxin-treated seedlings is attributed to the lower protein content (31.8% of control) in this treatment (data not shown). Similar effects of tentoxin on the Chl concentration (10) and α-amylase activity in cucumber cotyledons (17) have been reported. These data indicate that Chl loss in NF-treated seedlings is not the cause of the large increase in apoplastic α-amylase activity and that this increase is due to the photooxidative destruction of another plastid constituent.

Unlike NF (San 9789), its analog, San 9785, is not an inhibitor of carotenoid synthesis, yet it is, as is NF, a potent inhibitor of photosynthesis (20. 27). San 9785 appears to exert its actions on photosynthesis by affecting the amount and

Table VIII. *Effect of Plastid and Nuclear Protein Synthesis Inhibitors on Total Amylolytic and α-Amylase Activities and Chl and Protein Concentrations in Pea Seedling Leaves*

Seedlings were grown as indicated in Table I. Values are the means of two separate experiments.

Treatments	Total Amylolytic Activity	α-Amylase Activity	Chl	Protein
	nmol min⁻¹ mg⁻¹ protein		*μg g⁻¹ fresh wt*	*mg g⁻¹ fresh wt*
Control	287	12.9	109	29.8
Chloramphenicol	1020 (355)[a]	834 (6470)	1.01	16.4
Lincomycin	1050 (366)	770 (5970)	0.78	19.8
Cycloheximide	281 (98)	8.6 (67)	ND[b]	22.7
Cycloheximide + norflurazon	808 (281)	211 (1640)	ND	10.3
Norflurazon	2610 (909)	1060 (8200)	0.65	13.2

[a] Numbers in parentheses = % control. [b] Not determined.

composition of plastid lipids (16) and by inhibiting photosynthetic electron transport and decreasing the level of chlorophyll-protein complex in photosystem I (27). Seedlings treated with 100 μM San 9785 and grown under cWL had a slight decrease in their leaf Chl concentrations and a slight increase in total amylolytic activity (Table VII). San 9785 increased α-amylase activity 5.5-fold. Under the same conditions, NF (San 9789) treatments resulted in an increase of 85-fold in the α-amylase activity. These data indicate that the induction of α-amylase in the San 9789-treated photobleached pea seedling leaves is much higher than with its analog San 9785, which does not cause photooxidative damage. It is doubtful that the large induction of the apoplastic α-amylase in NF-treated seedlings is due to a general effect of substituted pyridazinones on photosynthesis or any other plastid function.

Other than carotenoid synthesis, NF has been found to inhibit ABA synthesis (38). PP333, an inhibitor of both ABA (42) and GA synthesis (7), was found to increase the level of activity of α-amylase in pea seedlings 4.6-fold (Table VII). GA is known to be required for the induction of α-amylases in certain tissues of some plant species (6). Seedlings treated with PP333 had epicotyls that were only 35% the length of those of control seedlings. These data indicate that the NF-caused large induction of apoplastic α-amylase is not due to inhibition of ABA or GA synthesis.

Effects of Inhibitors of Protein Synthesis on Amylolytic Activity

In our studies, NF was initially used to determine the role of the plastid in regulating apoplastic α-amylase because it disrupts all plastid functions, from protein synthesis to photosynthesis, and it specifically targets the chloroplast in plants grown in white light. Photooxidation as a result of NF treatments causes the destruction of plastidic ribosomes (1, 13, 33) and proteins (14, 19, 31, 35), but does not adversely affect cytosolic ribosomes or proteins (4, 33). However, in that the effects of NF on chloroplastic function are so wide ranging, and because we have found that inhibitors of plastid functions which occur after protein synthesis have little or no effect on apoplastic α-amylase, seedlings were treated with inhibitors of plastid protein synthesis to determine if the effect of NF is through blocking plastid or nuclear genome expression.

After 7 d of cWL and treatment with the plastid protein synthesis inhibitors CAP and lincomycin, 3.6- and 3.7-fold increases in total amylolytic activity were observed, respectively (Table VIII). These increases in total amylolytic activity were due to 65- and 60-fold increases in the α-amylase activity with CAP and lincomycin treatments, respectively. The level of increase of α-amylase activity was similar to that of NF treatments (*cf.* Fig. 3). Concentrations of leaf Chl with both CAP and lincomycin treatments were less than 1% of that of the control treatments, indicating that both treatments had significantly inhibited chloroplastic protein synthesis.

CAP and NF have been reported to prevent the synthesis of the nuclear encoded chloroplastic proteins Cab and SSu by decreasing the amount of translatable mRNA for these two proteins (34). Oelmüller *et al.* (34) proposed that this was due to the prevention of the production of a positive signal from the chloroplast by these two inhibitors of intraplastidic protein synthesis. In contrast, CAP has been reported to induce the activity of other extrachloroplastic enzymes (*e.g.* NR in rice, maize, and pea seedlings [9]).

The nuclear protein synthesis inhibitor cycloheximide inhibited the increase in total amylolytic and α-amylase activity by 69 and 80%, respectively, in NF-treated photobleached leaves (Table VIII). However, in normally greening pea seedling leaves, this inhibitor caused only 2 and 33% decreases in the total amylolytic and α-amylase activity, respectively. Although these data indicate less than total efficacy of these cycloheximide treatments, they do indicate that cytosolic protein synthesis is essential for: (a) full induction of α-amylase in NF-treated photobleached leaves and (b) normal *in vivo* levels of α-amylase in green leaves. Other workers have reported similar effects of cycloheximide on NR activity (9), which like pea α-amylase is an extrachloroplastic enzyme.

CONCLUSIONS

Photooxidative damage due to lack of carotenoid synthesis in WL grown seedlings is restricted to plastids (33), as is indicated in our study from the very low levels of leaf Chl (Fig. 1) and chloroplastic marker enzyme activities (Table VI) and the lack of any adverse effect on extraplastidic enzymes (Table VI) with the NF treatment. This photooxidative damage to plastids results in reduced expression of plastid genes and nuclear genes coding for plastid proteins and extraplastidic proteins that are essential to normal plastid metabolism (33, 40). It has been postulated that such nuclear encoded genes are controlled by a positive signal from the plastid (33, 40). Studies, to date, indicate that nuclear encoded proteins that are not directly linked to plastid function are not greatly affected by photooxidative damage (33). In this study we have demonstrated that the activity of a primarily apoplastic α-amylase is greatly enhanced by plastid photooxidation (Figs. 3 and 6; Tables IV and VII). Factors which cause loss of plastid function, but do not cause photooxidative damage or loss of intraplastidic protein synthesis, either had no effect or had a relatively small positive effect on the activity of this apoplastic α-amylase (Table VII). Thus, it seems likely that the rapid destruction of plastid ribosomes and proteins with photooxidation (*cf.* 33) was the cause of the large increase in apoplastic α-amylase activity. This conclusion is supported by the observation that large increases in α-amylase activity were also caused by CAP and lincomycin treatments (Table VIII). We propose that chloroplasts produce a negative signal for the regulation of the primarily apoplastic α-amylase in pea leaves.

ACKNOWLEDGMENTS

We thank Dr. Stephen O. Duke for his very useful advice concerning the experiments and for critically reading the manuscript. Dr. Eric P. Beers is thanked for his technical advice on electrophoresis and his useful comments concerning the manuscript. We also thank Prof. Cynthia A. Henson, Prof. Judy Callis, Prof. Nelson E. Balke, and Prof. David M. Peterson for their comments and suggestions concerning the manuscript. BASF Corporation is thanked for the generous gift of analytical grade norflurazon, Dr. Judith St. John is

thanked for gift of San 9785, and Prof. Eldon Stang is thanked for the gift of paclobutrazol.

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