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Partial Restoration of the High Rate of Plastid Pigment Development and the Ultrastructure of Plastids in Detached Water-stressed Wheat Leaves[1]

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ABSTRACT

Detached etiolated wheat (*Triticum aestivum* L. cv. Chris) leaves accumulated plastid pigments at a high rate, developed chloroplasts with stacked thylakoids, and stored plastid starch when wetted on filter paper in light. A moderate water deficit of −10 bars markedly reduced the accumulation of chlorophyll and carotenoids in the 8-day-old detached leaves during greening. δ-Aminolevulinic acid treatment of stressed leaf segments resulted in slightly increased pigment accumulations but benzyladenine application restored plastid pigment formation in stressed tissue to within 15% of the pigment content of the nonstressed detached leaves. The addition of δ-aminolevulinic acid to benzyladenine-treated stressed leaf segments improved both chlorophyll and carotenoid formation to nearly the amounts found in nonstressed leaf tissue. Stressed leaf sections developed plastids that were small, lacked starch, contained few thylakoids per granum, and possessed dilated thylakoids. Benzyladenine application to the stressed leaf segments did not restore normal plastid stacking but benzyladenine induced the formation of extended intergranal lamellae and stimulated pigment accumulations in both stressed and nonstressed detached leaves. Starch was absent in plastids of benzyladenine-treated leaf sections.

Mild water deficits have been shown to impair the formation of plastid pigments (6, 9, 33) and modify chloroplast development (7, 13) in intact leaves of seedlings during greening. An imposed stress of −10 bars reduced Chl and carotenoid accumulations early in the development of chloroplasts (9). Plastids of wheat leaves subjected to a moderate stress were small and contained fewer grana per plastid, fewer thylakoids per granum, and more dilated thylakoids (13) than when there was little or no stress.

The stress impairment of Chl production could result from a restricted pool size of ALA,[2] and/or a modified metabolism affecting normal lamellar formation in plastids. Virgin (33) reported that moderate water deficits decreased the formation of Chl *a* by reducing the rate of synthesis of PChl(ide). The lag in Chl production early in the greening process in barley and beans can be eliminated by the addition of ALA (23, 28). Furthermore, the rate of Chl formation has been attributed to the rate of ALA synthesis in leaves (23). The lag phase was eliminated when well watered jack beans were grown at high relative humidity (6). This result can be interpreted to mean that under fully turgid conditions there is an adequate ALA pool size.

It has been known for some time that applications of cytokinins prevent the loss of Chl in detached leaves (25). Differentiation of chloroplasts of cultured tobacco tissue from proplastids seems to require a cytokinin (5, 30). Recently, cytokinins have been reported to stimulate both the synthesis of Chl (10–12, 31) and the development of chloroplasts in detached cotyledons (18).

The present study was undertaken to determine the effect of BA on plastid pigment accumulation and plastid development in detached wheat leaves which were subjected to a moderate water deficit stress.

MATERIALS AND METHODS

Wheat (*Triticum aestivum* L. cv. Chris) seeds were germinated in washed sand in the dark at 25 C and 80% relative humidity. Leaf sections, 5 cm in length were excised from 8-day-old dark-grown plants 1 cm from the leaf tip under a dim green safelight. At least 10 primary etiolated leaf segments were placed in Petri plates on Whatman No. 1 filter paper that had been wetted with one of the following treatment solutions: PEG (−10 bars), BA (10^{-5} M), ALA (10^{-3} M), distilled water, PEG (−10 bars) plus ALA (10^{-3} M), PEG (−10 bars) plus BA (10^{-5} M) and PEG (−10 bars) plus BA (10^{-5} M) plus ALA (10^{-3} M). The detached etiolated leaf segments were treated in the dark at 25 C 7 hr prior and during the exposure to 3000 lux continuous white fluorescent light. Plastid pigments were extracted under dim light in aqueous 80% (v/v) acetone-saturated with $NaHCO_3$. Leaf areas were calculated from length by width measurements of another set of treated leaf sections by using a binocular microscope fitted with an ocular micrometer. PEG 20,000 solutions were prepared by adding 21 g to make a total of 100 ml. The ψ of the PEG solutions was checked at −10 bars by using a Spanner thermocouple psychrometer (29). The quantities of plastid pigments that accumulated during the greening period were determined as reported previously (9). Leaf sections were prepared for transmission electron microscopy after they had been exposed 20 hr to continuous light using techniques previously described (13).

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[2] Abbreviations: ALA: δ-aminolevulinic acid; PChl(ide): protochlorophyll(ide); ψ: water potential; PEG: polyethylene glycol.

RESULTS

The amounts of plastid pigments produced in the nonstressed wheat leaf sections and in the osmotically imposed stressed leaf sections after 16 hr continuous light are shown in Table I. The application of a 10^{-3} M ALA solution to detached wheat leaves stimulated a slight increase in the production of plastid pigments over that by nonstressed leaves. Chl and carotenoid accumulation in mildly stressed leaf segments were reduced 60 and 46% of their counterparts in well watered leaf segments. The application of ALA to the detached stressed leaves was associated with about a 10% increase in pigment production. The BA treatment (10^{-5} M) improved plastid pigment production in stressed leaf segments to within 15% of that in the nonstressed leaf sections. A concentration of 10^{-5} M was optimal for the BA-stimulated partial restoration of Chl and carotenoid accumulations in water-stressed wheat segments. A combination treatment of BA and ALA restored plastid pigment accumulation in stressed leaves to values slightly below those of the nonstressed detached leaves.

Table I. *Plastid Pigment Accumulation in Nonstress and PEG-induced Stress Wheat Leaf Sections Treated with ALA and/or BA*

Lots of 10 detached etiolated leaf segments were treated on filter paper in Petri plates 7 hr in the dark and during light with one of the following solutions: ALA, 10^{-3} M; PEG, −10 bars; PEG (−10 bars) plus ALA (10^{-3} M); PEG (−10 bars) plus BA (10^{-5} M); PEG (−10 bars) plus BA (10^{-5} M) plus ALA (10^{-3} M). PEG was prepared by dissolving 21 g in the appropriate solution to make a total of 100 ml. The pigments were extracted from leaf sections after exposure to 16 hr continuous light.

Treatment	Chl (a + b)	Change	Carotenoids	Change
	$\mu g/cm^2$ *leaf area*	%	$\mu g/cm^2$ *leaf area*	%
Control	11.25 ± 0.97		2.69 ± 0.22	
ALA	11.82 ± 0.94	+5	2.74 ± 0.19	+2
PEG	4.49 ± 0.60	−60	1.46 ± 0.11	−46
PEG + ALA	5.80 ± 0.94	−48	1.64 ± 0.23	−39
PEG + BA	9.47 ± 0.76	−16	2.34 ± 0.10	−13
PEG + BA + ALA	10.72 ± 0.95	−5	2.62 ± 0.19	−3

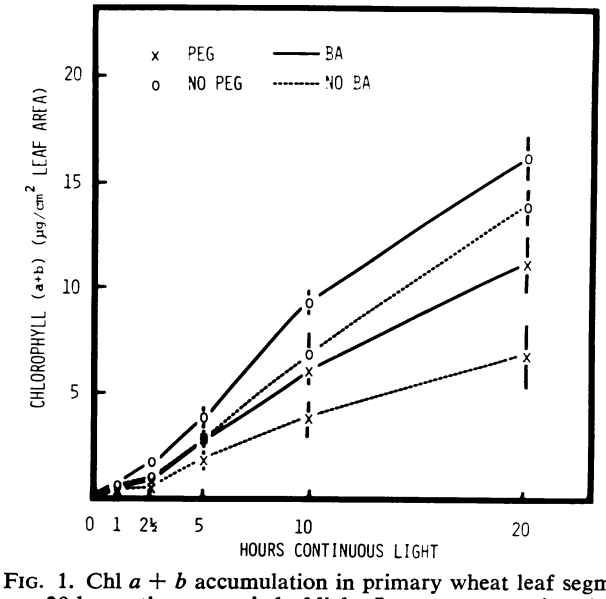


FIG. 1. Chl a + b accumulation in primary wheat leaf segments over a 20-hr continuous period of light. Leaves were sectioned from etiolated 8-day-old seedlings and were placed in Petri plates on filter paper wetted with treatment solution 7 hr prior to light exposure. Vertical bars on the graph are the standard error of the means.

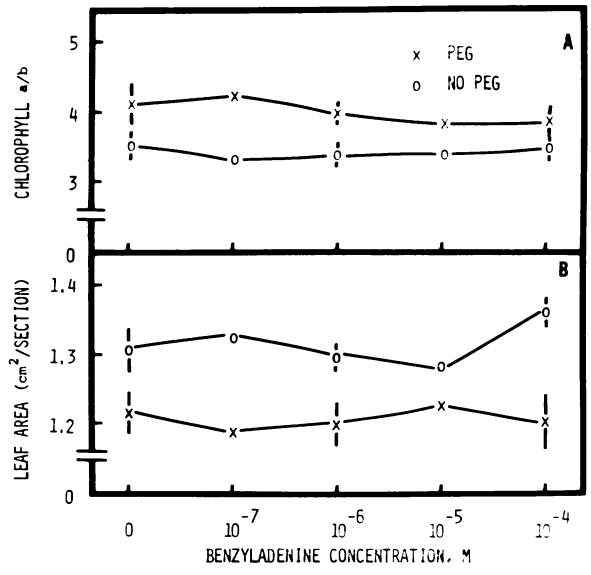


FIG. 2. A: Chl a/b ratio of stressed and nonstressed leaves that had been treated over a range of concentrations of benzyladenine 7 hr prior to light exposure. Chl was extracted and the Chl a/b ratios were determined after leaf sections had been exposed to continuous light for 20 hr. B: Leaf area of stressed and nonstressed wheat sections as affected by benzyladenine over a concentration range. Leaf area was calculated from sections exposed to continuous light after 20 hr. Vertical bars on the graph represent the standard error of the means.

The Chl that accumulated over a 20-hr continuous light period in stressed and nonstressed leaf segments is shown in Figure 1. The nonstressed detached wheat leaves exhibited the typical lag prior to the rapid linear rate of Chl production. The application of BA to nonstressed leaf sections caused an increased rate of Chl production, particularly during the first 10 hr of greening. BA stimulated Chl production slightly during the lag phase of Chl formation. The moderate stress, imposed by the PEG application to detached wheat leaves, caused a marked decline in the rate of Chl production. BA restored the greening rate in stressed leaf segments to a level near that of the rate of Chl accumulation in nonstressed leaf sections, but the Chl differential decreased progressively after 5 hr of continuous light. BA did not eliminate the lag in Chl production in the stressed detached leaves.

The Chl a/b ratio of wheat leaf sections was altered by the imposed osmotic stress (Fig. 2A). Stressed leaf segments exhibited a high Chl a/b ratio, and the ratio appeared to drop more slowly during greening in comparison to the Chl a/b ratios of nonstressed detached leaves. BA caused a rapid drop in the Chl a/b ratio in leaf sections during the first hours of greening; however, the concentration of BA had little effect on the Chl a/b ratio (Fig. 2A). Stressed leaf sections exhibited a Chl a/b ratio near 4 while the Chl a/b ratio of nonstressed leaves was about 3.5.

The leaf areas of the nonstressed wheat segments were unaffected by BA treatment up to a concentration of 10^{-5} M (Fig. 2B). BA applied to nonstressed sections at 10^{-4} M stimulated a 6% increase over the leaf area of control sections. Leaf areas of the stressed detached leaves were about 8% less than those of nonstressed segments. BA was not effective in increasing the areas of stressed leaves.

Carotenoid accumulation in leaf sections during greening oc-

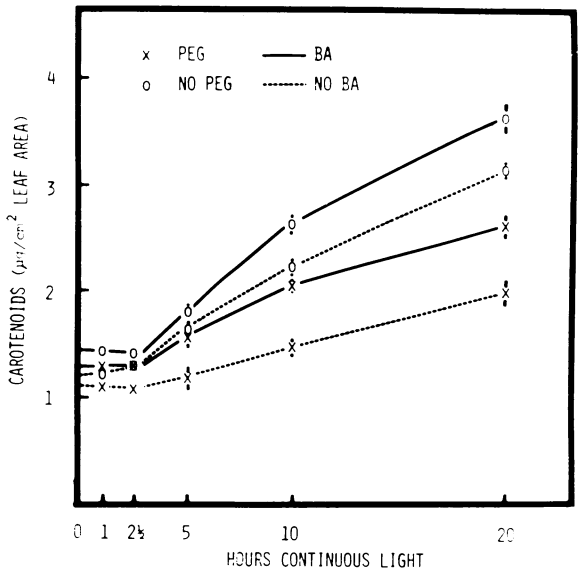


FIG. 3. Production of carotenoids in sectioned primary wheat leaves during greening in continuous light up to 20 hr. The leaves from etiolated 8-day-old seedlings were sectioned in the dark and wetted on filter paper in Petri dishes with treatment solutions 7 hr prior to illumination. The standard error of the means is indicated by vertical bars.

Table II. *Effect of PEG-imposed Stress and/or BA on Plastid Length and Granal Development*

Wheat leaves 5 cm in length, 1 cm from the tip, were removed from plants and treated in the dark 7 hr before being exposed to 20 hr continuous light. Thereafter sections were prepared for electron microscopy and plastid measurements were made from micrographs.

Treatment	Plastid Length	Grana Plastid Section	Thylakoids Granum	Thylakoids/ Plastid Section
	μm		No.	
Control	5.44 ± 0.55	62.40 ± 6.97	4.41 ± 0.21	275
BA	5.32 ± 0.30	54.00 ± 3.31	4.01 ± 0.16	216
PEG	4.94 ± 0.18	41.48 ± 2.14	3.41 ± 0.08	142
PEG + BA	4.32 ± 0.19	40.67 ± 4.02	3.63 ± 0.12	148

curred as shown in Figure 3. BA induced an increase in rate of carotenoid accumulation in nonstressed sections over that in control sections, particularly between 2.5 and 10 hr light. Apparently, BA stimulated carotenoid production in the dark since the carotenoid levels of BA-treated segments were higher than in the nonstressed leaf sections at the time of exposure to light. The BA treatment did not eliminate the 2.5 hr lag in carotenoid accumulation in leaf segments subsequent to exposure to light. Mild water stress reduced the rate of carotenoid accumulation in leaf sections. BA application to the stressed segments improved the carotenoid production rate only after a 2.5-hr illumination period. But BA stimulation of carotenoid biosynthesis did not extend beyond the 10-hr illumination period.

Chloroplasts of nonstressed wheat leaf sections developed substantially during the 20 hr exposure to light. Numerous grana were formed and each granum consisted of several thylakoids (Table II). Ribosomes were abundant (Fig. 4). Fibril inclusions, plastoglobuli, and starch deposits were quite common in the developed plastids. BA treatment resulted in the development of chloroplasts in nonstressed leaf sections that lacked starch and formed numerous elongated intergranal lamellae (Fig. 5). The number of grana per plastid and the number of thylakoids per granum were slightly reduced compared with plastids from the well watered leaf sections (Table II). Ribosomes were abundant and plastoglobuli, and fibril inclusions were observed. Plastids of leaf segments that developed under stress lacked starch and exhibited slightly swollen or dilated thylakoid membranes (Fig. 6). Stress resulted in the development of shorter plastids which contained fewer grana and fewer thylakoids per granum than the developed chloroplasts of the nonstressed wheat leaf segments (Table II). Ribosomes, fibril inclusions, and plastoglobuli were observed in the plastids that developed under stress. BA had little effect on chloroplasts that developed in leaf tissue subjected to a mild stress (Fig. 7). They were short and had a reduced number of grana and granal thylakoids, just as in the stressed leaves (Table II). The chloroplasts lacked starch but contained fibrils and plastoglobuli. BA stimulated the development of numerous elongated intergranal lamellae in plastids of both detached stressed and nonstressed leaves.

DISCUSSION

Plastid pigment production in nonstressed detached wheat leaves was slightly increased when ALA was applied to leaf segments (Table I). Small water deficits markedly reduced the accumulation of Chl and carotenoids in detached wheat leaves and the addition of ALA to stressed leaf sections resulted in nearly a 10% increase. These data could be interpreted to mean that one effect of stress was to restrict the formation of ALA. Acetyl-CoA and mevalonic acid are probable precursors in the biosynthetic pathway of plastid carotenoids (14). Perhaps the addition of exogenous ALA to stressed leaf segments caused a sparing of metabolites resulting in increased mevalonate production that was then used in carotenoid biosynthesis in the leaf tissue.

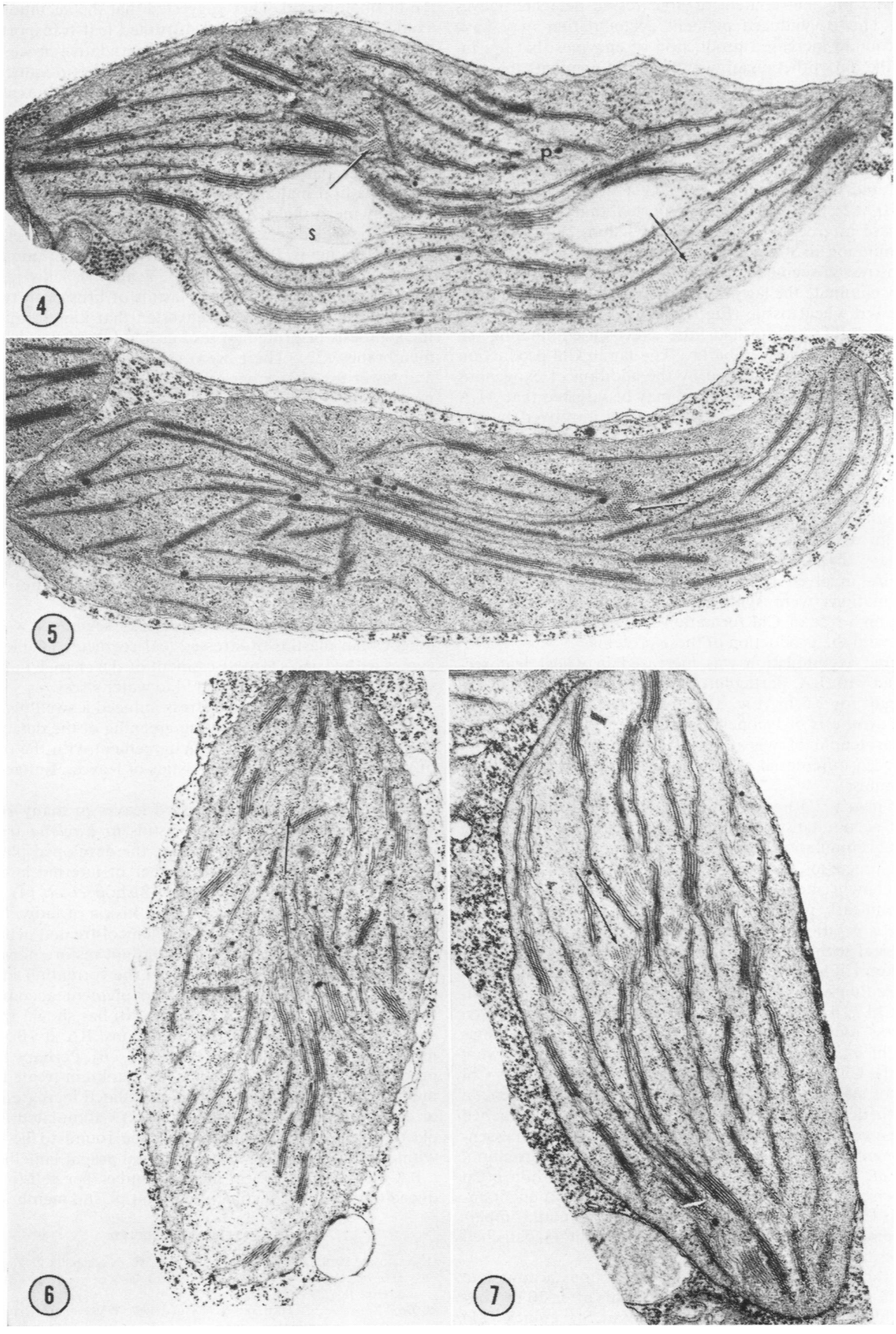
Kinetin has been reported to increase Chl formation in detached leaves by increasing the supply of metabolites for the synthesis of Chl and plastid proteins (31). Recently, Fletcher *et al.* (12) proposed that BA affected Chl formation by inducing the production of proteins, including the enzyme ALA synthase. It was reported that kinetin and BA alleviate, in part, the severe salt stress-induced reduction in rate of amino acid incorporation into protein (2, 17) and in total leaf protein (26). In the present study, BA facilitated plastid pigment accumulation in moderately stressed wheat leaf sections to within 15%

FIG. 4. Chloroplast that developed after 20 hr continuous light in primary wheat leaves of nonstressed sections. Note the fibril inclusions (arrows) starch deposits (S), appressed thylakoids, ribosomes, and plastoglobuli (P). × 22,000.

FIG. 5. BA-treated nonstressed leaf sections produced plastids that lacked starch but contained appressed thylakoids, fibril inclusions, ribosomes, elongated intergranal lamellae, and plastoglobuli. Plastids developed in leaf sections for 20 hr under continuous light. × 25,000.

FIG. 6. A mild water deficit of −10 bars resulted in the development of plastids of detached wheat leaves that possessed ribosomes, fibril inclusions (arrow), plastoglobuli but lacked starch, had dilated thylakoids, and were smaller than plastids of nonstressed leaves. Electron micrographs were taken of plastids after primary wheat leaf sections had been exposed for 20 hr to continuous light. × 21,000.

FIG. 7. Chloroplast of BA-treated stressed primary wheat leaf section after a 20 hr greening period. BA induced the formation of elongated intergranal lamellae but lacked starch. Note the dilation of thylakoids, fibril inclusions (arrow), and numerous ribosomes. × 27,000.



FIGS. 4–7.

of the plastid pigment content of nonstressed detached leaves (Table I). The BA-induced pigment accumulation may have resulted from an increased production of enzymes that are involved in the biosynthetic pathway of plastid pigments together with an increased rate of synthesis of holochrome or plastid lamellae, and/or an increased availability of metabolites utilized in the synthesis of plastid pigments or plastid lamellae.

BA applied to nonstressed wheat sections stimulated Chl synthesis slightly during the lag phase and markedly during the phase of rapid Chl production (Fig. 1). Fletcher and his colleagues (10, 12) have previously reported that BA removed the lag phase in greening cucumber cotyledons. BA increased Chl accumulation in stressed leaf segments nearly to the level of the nonstressed segments early in greening, but BA did not completely eliminate the lag in Chl production in either stressed or nonstressed wheat tissue (Fig. 1). Nadler and Granick (23) have indicated that kinetin had little effect on eliminating the lag phase of Chl synthesis in barley. The lag in Chl production early in greening was eliminated by the addition of exogenous ALA (23, 28). From these findings it may be inferred that ALA production in our wheat leaf segments was improved by the application of BA and ALA could have been limiting in stressed leaves even in the presence of BA.

The synthesis of a protein associated with ALA formation has been suggested as a control mechanism of greening beyond the lag phase in plastid development (22). Remy (24) found that proteins associated with photosystem I, photosystem II, and "primary thylakoids" were present in etioplasts but that new proteins involved in the development of the numerous granal formations were synthesized later. Perhaps the BA-stimulated high rate of Chl formation in wheat leaf sections is associated with the production of these proteins.

Carotenoid accumulation was increased in wheat leaf sections treated with BA, particularly after the segments were exposed to light for 2.5 hr (Fig. 3). Kinetin has been reported to inhibit the synthesis of lycopene in tomato (19). β-Carotene is a major carotenoid of wheat leaves (34). Conceivably, cytokinins induced differential responses in the synthesis of different carotenoids.

A lag of nearly 2.5 hr was observed before the rapid rate of carotenoid accumulation and BA had no effect on the lag in carotenoid accumulation (Fig. 3). This fact suggests that either carotenoid precursor, rate of precursor conversion, or membrane attachment sites were limiting initially and BA did not affect these in early plastid development.

The Chl *a*/*b* ratios were consistently highest in the extracts of stressed leaf segments (Fig. 2A). It appears that Chl *b* is synthesized from Chl *a* (27). The Chl *a*/*b* ratio in attached leaves of wheat seedlings subjected to moderate water stress was similar to the Chl *a*/*b* ratio in leaves of nonstressed seedlings even though stress reduced Chl accumulation in stressed seedlings (9). The Chl *a*/*b* ratios in detached nonstressed leaves were similar to the Chl *a*/*b* ratios in attached leaves. The higher Chl *a*/*b* ratios of stressed leaf segments indicate an impairment in the biosynthetic conversion of Chl *a* to Chl *b*. In the detached leaves, stress could have suppressed the availability of an essential component required for the Chl *a* to Chl *b* transformation. Alberte *et al.* (1) suggested the development of a Chl-protein complex rich in Chl *a* prior to the synthesis of a component enriched with Chl *b*. A moderately imposed stress could impair the development of the latter plastid component in detached wheat leaves.

The plastids of well watered wheat leaf sections accumulate much starch and exhibit stacking of thylakoids after 20 hr continuous light (Fig. 4). Mittelheuser and Van Steveninck (21) also observed abundant starch in green detached wheat leaves dipped in water or in a kinetin solution and maintained under a 16-hr photoperiod. They suggested that the accumulated starch could result from either the disrupted leaf transport system or from an impairment of the starch degradative process in leaves. Tetley and Ikuma (32) have reported a kinetin-induced increase of starch accumulation in cultured tobacco pith explants. Little starch was found in plastids that developed in leaf segments wetted with BA on filter paper and kept in continuous light (Fig. 5). Berridge and Ralph (3) reported that kinetin induced starch degradation and increased the flow of sugars into lipids and structural materials in floating cabbage leaf discs. This result may mean that the nutrition of BA-treated detached wheat leaf tissue was directed towards the synthesis of cellular structural components. BA induced the formation of numerous elongated intergranal lamellae (Figs. 5 and 7). This response has been described previously in plastids of brussel sprouts (8) and kohlrabi (22). It has been suggested that kinetin might induce the synthesis of protein(s) responsible for the linking of granal membranes (22). There were slightly fewer grana per plastid and fewer thylakoids per granum in BA-treated wheat leaf segments than in the well watered sections (Table II). BA did not induce a fusion of granal membranes in developing wheat plastids.

Ribosomes were abundant in the plastids of the stressed and nonstressed leaves. Mittelheuser *et al.* (21) reported abundant plastid and cytoplasmic ribosomes in detached wheat leaves maintained up to 48 hr under high light intensity. Stress has been found by Hsiao (15) to cause a shift from the polymeric to monomeric ribosome form. No attempt was made in the present study to correlate the forms of ribosomes with treatment.

Plastids of nonstressed wheat leaf segments were slightly longer than plastids of stressed leaf segments (Table II) which agrees with Hsiao's (16) assessment of the change in chloroplast volume when cells are subjected to water stress.

The moderately imposed stress induced a swelling or dilation of thylakoid membranes during greening of the detached wheat leaves (Fig. 6). This result is in agreement with the observation of thylakoid swelling in the plastids of leaves of intact seedlings subjected to stress (13).

Plastid development of stressed leaves in many respects resembled the development of plastids in *Euglena* treated with chloramphenicol (4). In each case, the developed plastids were smaller, contained a smaller number of internal lamellae, and the lamellae were not appressed. Bishop *et al.* (4) concluded that a protein responsible for granal fusion of individual plastid lamellae was lacking in chloramphenicol-treated cells. The application of BA to stressed leaves did not restore normal plastid stacking (Table II) but BA induced the formation of extended intergranal lamellae and stimulated pigment accumulation in the stressed leaf segments. Letham (20) has shown that kinetin stimulated an enlargement of cotyledons. BA at 10^{-5} M did not increase the area of wheat leaves (Fig. 2B). Perhaps the greater production of plastid pigments in cytokinin-treated leaf segments is not due to the cytokinin stimulated increase in cell size or cell number per leaf section. The BA-stimulated increase in plastid pigment accumulation may be found to be correlated with a BA-induced increase in internal granal lamellae surface, a BA-induced increase in plastid number per cell, or a BA-induced increase in Chl content per unit plastid membrane.

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