The Role of Cytokinins in Chloroplast Lamellar Development

Received for publication December 20, 1974 and in revised form February 19, 1975

RAN DALL S. ALBERTE and AUBREY W. NAYLOR
Department of Botany, Duke University, Durham, North Carolina 27706

ABSTRACT

The accumulation of chlorophyll, production of two specific lamellar chlorophyll-protein complexes, onset of O₂ evolution, and detection of P700 were examined in intact Jack bean (Canavalia ensiformis [L.] DC.) leaves treated with 10⁻² M kinetin or benzyladenine and allowed to green under low (30–35%) and high (80–85%) relative humidity. In contrast to reports of the promotion of chlorophyll accumulation by cytokinin treatment in excised tissue or cotyledons, intact greening leaves showed neither promotion of chlorophyll accumulation nor alteration in formation of the lamellar chlorophyll-protein complexes or development of photosynthetic function. Furthermore, cytokinin was ineffective in relieving the consequences of low relative humidity water stress on chlorophyll accumulation and on the formation of at least one lamellar chlorophyll-protein.

To clarify the biochemical nature of hormonal control of development, investigators have sought well defined plant subsystems in which developmental processes have been reasonably well characterized. The chloroplast is one such system. Several researchers have found distinct biochemical effects of such growth regulators as the cytokinins, GA₃, ABA, and ethylene on various aspects of chloroplast development (1).

Sugiura (25) reported kinetin promotion of Chl synthesis in greening detached primary leaves, while Banerji and Laloraya (7) found enhanced protochlorophyllide formation in detached cucumber cotyledons. It was later shown, however, that kinetin at some concentrations actually inhibited Chl accumulation in cucumber cotyledons (21). In contrast, Fletcher’s laboratory (10–12) recently claimed a dramatic enhancement of Chl accumulation with cytokinin treatment in greening detached cucumber cotyledons. It was suggested (11, 12) that the lag phase in Chl accumulation found in early greening could be overcome by cytokinin treatment through the hormone’s promotion of Chl synthesis.

Since cytokinins appear to be involved in chloroplast development and certainly in maintenance of chloroplasts in senescent tissue (16, 20), kinetin and BA were chosen for examination of their role in development of the chloroplast lamellar system in greening intact leaves. Furthermore, since it appears that cytokinins may play a role in water stress physiology (8, 14, 15), the interactions of low level water stress and cytokinins were examined in relation to the greening process.

MATERIALS AND METHODS

Plant Material. Seeds of Jack bean (Canavalia ensiformis [L.] DC.) were imbibed for 12 hr in running water (30–35°C) and planted in coarse vermiculite. Germination took place in total darkness at 28 to 29°C at either high (80–85%) or at low (30–35%) RH² in controlled environment chambers of the Duke University unit of the Southeastern Plant Environmental Laboratories. All seedlings were illuminated (1000 ft-c) on the 7th day after imbibition. The RH conditions prevailing during development of etiolated seedlings were maintained throughout the greening period.

Cytokinin Treatments. Various concentrations of kinetin, BA (10⁻⁴, 10⁻⁵, 10⁻⁶, and 10⁻⁷ M) and water controls were painted on intact etiolated primary leaves 24 hr before illumination. Aerosol OT was added to test solutions as a surfactant. Dosage response (based on Chl accumulation) analysis revealed that 10⁻⁴ M cytokinin gave the greatest promotion of Chl. Therefore, this concentration was used for all subsequent experiments.

Chlorophyll Determinations. Primary leaves (at least five pair) were harvested at specific times during greening from control and experimental seedlings for the determination of leaf Chl a/b ratios and rates of total Chl accumulation. The leaf pigments were extracted in 80% (v/v) acetone and Chl concentrations were calculated using the equations of Arnon (6).

Separation and Characterization of Chlorophyll-Protein Complexes. Chloroplast lamellae were prepared from greening leaves (2), and these lamellar fragments were solubilized with either SDS or Triton X-100. The Triton extracted lamellae were used for purification of the P700-Chl a-protein of photosystem I following procedures of Shiozawa et al. (24). The SDS-treated lamellae were fractionated into two Chl-protein complexes, CPI and CPII, and free pigment by SDS polyacrylamide gel electrophoresis (2, 26). The distribution and quantity of Chl and protein between CPI and CPII were determined from gel scans (26).

Determination of P700 Content. Triton X-100 solubilized lamellae were used for measurement of light-induced changes of P700 employing the methods of Shiozawa et al. (24).

Detection of Oxygen Evolution. Oxygen evolution was monitored in leaf discs prepared from leaves at various stages of greening using a Clark-type probe (2).

1 Support was provided by the Duke University Research Council, National Science Foundation Grant GB31207 to J.P.T., and Grants GB19634 and GB28950 for Phytotron facilities at Duke.

2 Abbreviations: RH: relative humidity; CPI: P700-Chl a-protein; CPII: light-harvesting Chl a/b-protein.
RESULTS AND DISCUSSION

Chlorophyll accumulation rates of intact Jack bean primary leaves are not responsive to cytokinin treatment. Figure 1 shows the leaf Chl a/b ratio and total Chl accumulation in control plants greened under low (30–35%) or high (80–85%) RH. Under precisely the same conditions, plants treated with cytokinin (kinetin or BA) show essentially identical rates of Chl accumulation and indistinguishable changes in the leaf Chl a/b ratio from control plants during the first 12 hr of greening (Fig. 2). There is no reduction in the lag phase of greening attributable to cytokinin treatment. These observations are in contrast to an earlier report (25) showing kinetin promotion of Chl synthesis in detached greening Phaseolus vulgaris leaves, and in contrast to recent reports (10–12) of BA promotion of Chl accumulation and elimination of the lag phase in greening excised cucumber cotyledons. This discrepancy is most probably due to the differences in excised and intact tissue, in that excised Jack bean leaves respond to cytokinin treatment (R.S. Alberete, unpublished observations; see ref. 17). It is known that excised tissue rapidly undergoes metabolic changes similar to those in senescing tissue (22). Clearly, excised tissue which, for the most part, has had endogenous supplies of hormones removed should respond favorably to exogenous cytokinin; maintainance of chloroplast integrity and Chl in senescing tissue by cytokinins is well-documented (16, 20).

Earlier work from this laboratory (4, 9) demonstrated that leaves greening under low RH conditions were experiencing water deficits in the range of −7 to −8 bars and were showing a 3 to 4 hr lag in Chl accumulation. Similar water deficits could be achieved by using polyethylene glycol (4). When leaf water potentials were maintained at less than −5 bars as under high RH greening conditions, the lag phase in Chl accumu-

**Fig. 1.** Time course of Chl accumulation in control (water-treated) intact primary leaves of Jack bean greening under high (80–85%) (●) or low (30–35%) (○) RH. The leaf Chl a/b ratios are shown for the respective light exposure periods and for high (●) and low (○) RH.

**Fig. 2.** Chlorophyll accumulation during 12 hr of greening in intact Jack bean primary leaves treated with 10−4 m kinetin or BA under high (80–85%) (●) and low (30–35%) (○) RH. The leaf Chl a/b ratios for the respective illumination periods and high (●) and low (○) RH are shown.

lation was eliminated. In light of these observations and previous studies of Itai and Vaadia (14, 15) and Ben-Zioni et al. (8) which provided evidence that exogenously applied cytokinin was capable of relieving the effects of small water deficits, it was thought that cytokinin treatment should relieve the effects of small water deficits on the greening process; namely, reduce or eliminate the lag phase in Chl accumulation. However, cytokinin was neither effective in reducing the 3- to 4-hr lag in Chl accumulation nor in eliminating the delay in the onset of the leaf Chl a/b ratio (Figs. 1 and 2). It appears that exogenous cytokinin is not effective in mitigating the consequences of small water deficits. These observations support the recent findings of Mizrahi and Richmond (19) and Kirkham et al. (18).

Several other parameters of chloroplast development were examined in an attempt to detect other possible effects of cytokinins on rapidly greening tissue. Net O2 evolution (20–25 μmoles mg Chl-min) was detected after 2 hr of greening in both controls and cytokinin treated leaves as previously observed in untreated leaves (2, 4). Initial detection and assembly of the photochemically active photosystem 1 P700 reaction center complex (CPI) was unchanged in treated and untreated leaves. The P700-Chl a-protein was first detected on gels and by hydroxylapatite chromatography after 6 hr of illumination in agreement with previous findings (2, 3). The addition of light-harvesting Chl to the P700 reaction center followed the same pattern reported earlier (3).

The last parameter examined was the rate of formation of the major light-harvesting Chl a/b-protein (CPII) during greening. The production rate of this component was only influenced by the water status of the greening tissue as described earlier (4) and not altered by cytokinin treatment. A 50% reduction (as determined from gels) in the lamellar content of this component during the lag phase in greening (2–4 hr of illumination) corresponded to the delay in lowering the leaf Chl a/b ratio (4). Since a direct relationship between leaf Chl a/b ratio and lamellar content of CPII has been established (5), it was anticipated that a lag in Chl b formation under low RH (Figs. 1 and 2) would be reflected in the lower lamellar content of this component.

In view of the findings reported here, it is difficult to ascertain the significance of the model for chloroplast development proposed by Fletcher and co-workers (11, 12). They suggest that
control of Chl accumulation and the lag phase during greening is regulated by cytokinin through its effect on the activity of δ-amino levulinic acid synthase. Despite the fact that this enzyme has yet to be isolated from green plants (23), it is still not clear whether Chl biosynthesis is the major limiting factor contributing to the lag phase in greening (1, 17). There is evidence which supports the hypothesis that synthesis and/or assembly of specific Chl-binding proteins may be of prime importance (1, 13). Control of early chloroplast development is no doubt dependent on many factors of which hormone balance may be among them. Clearly, further studies are necessary to clarify the role of hormone balance in rapidly developing tissues in the chloroplast developmental process.

Acknowledgment—We wish to acknowledge the helpful discussion and facilities made available by Dr. J. P. Thornber.

LITERATURE CITED