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Lepidimoide Promotes Light-Induced Chlorophyll Accumulation in Cotyledons of Sunflower Seedlings

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Abstract. The effect of disaccharide lepidimoide on light-induced chlorophyll accumulation was studied in cotyledons of sunflower (*Helianthus annuus* L.) seedlings and detached cucumber (*Cucumis sativus* L.) cotyledons. From studies on the structure-activity relationships of lepidimoide, its analogs, and sucrose with respect to light-induced chlorophyll accumulation in the cotyledons of sunflower seedlings, both lepidimoide and the free carboxylic acid of lepidimoide (lepidimoic acid) showed the highest promoting activity, whereas the hydrogenated lepidimoide, which lacks a double bond in the C4, 5 position in uronic acid, showed lower activity than lepidimoide; however, sucrose exhibited very weak activity. These results suggest that lepidimoide acts as a new type of plant growth regulator, not simply as a carbon source providing energy. Lepidimoide promoted not only light-induced chlorophyll accumulation in sunflower cotyledons but also light-induced 5-aminolevulinic acid content, which is considered to be a rate-limiting step in chlorophyll biosynthesis. Lepidimoide with cytokinin stimulated the accumulation of chlorophyll and 5-aminolevulinic acid additively. In detached cucumber cotyledons, lepidimoide also promoted light-induced chlorophyll accumulation. These results indicate that lepidimoide, in cooperation with cytokinin, causes light-induced chlorophyll accumulation in the cotyledons of several dicot plant species by affecting the level of 5-aminolevulinic acid.

Key Words. 5-Aminolevulinic acid accumulation—Chlorophyll accumulation—*Cucumis sativus*—Cytokinin—*Helianthus annuus*—Lepidimoic acid—Lepidimoide—Structure-activity relationship

Lepidimoide, an allelopathic substance that promotes the shoot growth of different plant species, was first isolated from the mucilage of germinating cress (*Lepidium sativum* L.) seeds and identified as sodium 2-*O*-rhamnopyranosyl-4-deoxy-α-L-*threo*-hex-4-enopyranosiduronate (Hasegawa et al. 1992, Kosemura et al. 1993). Thus far, it has been shown that lepidimoide occurs in the exudates of germinating seeds of various plant species (Yamada et al. 1995), suggesting that lepidimoide is widespread in the plant kingdom. Lepidimoide promotes not only shoot growth but also leaf development, flowering, and seed production in *Arabidopsis* (Goto et al. 1995). Lepidimoide also inhibits the loss of total chlorophyll in excised *Avena* leaf segments during leaf senescence (Miyamoto et al. 1997a) and delays abscission in bean petiole explants (Miyamoto et al. 1997b), suggesting that lepidimoide may be a natural hormone-like substance that controls various physiological development in plants. It has been shown recently that lepidimoide also markedly promotes greening in cotyledons of sunflower seedlings grown in the light. The present publication attempts to clarify the effect of lepidimoide on light-induced chlorophyll accumulation in cotyledons of sunflower seedlings. The mechanism of enhancement of chlorophyll accumulation is also investigated.

Materials and Methods

Chemicals

Lepidimoide and its analogs, the free carboxylic acid of lepidimoide (lepidimoic acid) and the hydrogenated lepidimoide (Fig. 1), were syn-

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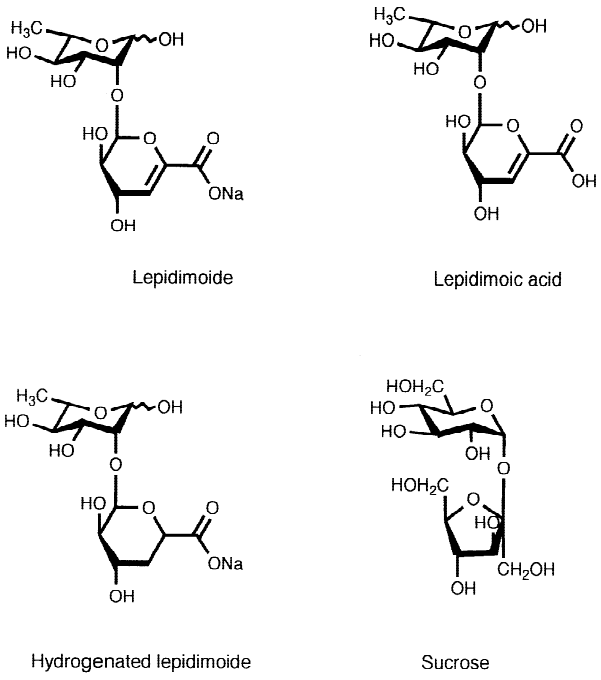

Fig. 1. Chemical structure of lepidimoide and its analogs.

thesized from D-glucose and α-L-rhamnose as described by Kosemura and others (1993).

Plants

Sunflower (*Helianthus annuus* L.) seeds were sterilized in a 1% NaOCl solution for 30 min and rinsed with distilled water. The rinsed seeds were spread evenly on wet double layered Toyo filter paper (no. 1) in a Petri dish and germinated for 4 days in the dark at 25°C. Seven germinated seeds were transferred under a dim green light into a 9-cm Petri dish containing 2 mL of test solution and kept in the dark for 6 h. Etiolated seedlings were cultured under white light (23 μmol m^{-2} s^{-1}) at 25°C. After irradiation, cotyledons were excised from the seedlings with a razor blade, and chlorophyll and 5-aminolevulinic acid contents were determined. Ten mM levulinic acid, a competitive inhibitor, was added to the test solution to prevent 5-aminolevulinic acid dehydratase activity.

The greening assays using detached cucumber (*Cucumis sativus* L. cv. Tokiwa-zibai) cotyledons were carried out according to the method of Lew and Tsuji (1982) with some modifications. After the seed coat was removed, seeds were sterilized, rinsed with distilled water, and grown in the dark at 25°C. Cotyledons excised from 4-day-old etiolated seedlings were incubated in the test solution in the dark for 6 h and then in the light for 8 h at 25°C to induce greening. After further incubation in the dark for 1 h at 25°C, the cotyledon chlorophyll and 5-aminolevulinic acid contents were determined. All manipulations were carried out under a dim green light.

Determination of Chlorophyll and 5-Aminolevulinic Acid

Cotyledon samples were homogenized in a mortar and pestle in 8 mL of 80% cold acetone, and chlorophyll was estimated according to the method of Arnon (1949). The extract was centrifuged at 12,000 × *g* for 5 min at 4°C. The resulting pellet was resuspended in 8 mL of 80% cold acetone and centrifuged at the same speed for 5 min twice. The three supernatants were combined and adjusted to 25 mL in a volumetric flask. The absorbances at 645 and 663 nm were measured spectrophotometrically, and the chlorophyll content was calculated.

The 5-aminolevulinic acid content was determined according to the method of Mauzerall and Granick (1956). The cotyledons were homogenized in 5 mL of 4% trichloroacetic acid and centrifuged at 12,000 × *g* for 20 min at 4°C. The pH of the supernatant was adjusted to 4.6 by adding 1 M sodium acetate, and then acetylacetate was added. The samples were heated to 100°C for 10 min. Equal aliquots of the samples and Ehrlich's reagent were mixed, and the absorbance at 553 nm was measured 15 min later.

All experiments were repeated at least three times.

Table 1. Effects of lepidimoide, lepidimoic acid, hydrogenated lepidimoide, and sucrose on light-induced chlorophyll accumulation in the cotyledons of sunflower seedlings. Sunflower seedlings were cultured for 7 days with 1 or 3 mM test solution in the light at 25°C. Data represent the means ± S.E. of three replicates. FW, fresh weight.

Treatments	Chlorophyll contents (μg/g FW)
Control	0.15 ± 0.01
Lepidimoide	
1 mM	0.34 ± 0.02
3 mM	0.46 ± 0.02
Lepidimoic acid	
1 mM	0.36 ± 0.01
3 mM	0.48 ± 0.01
Hydrogenated lepidimoide	
1 mM	0.18 ± 0.01
3 mM	0.28 ± 0.01
Sucrose	
1 mM	0.19 ± 0.02
3 mM	0.22 ± 0.01

Results and Discussion

The structure-activity relationships of lepidimoide, its analogs (Fig. 1), and sucrose were studied with respect to light-induced chlorophyll accumulation in sunflower cotyledons (Table 1). The free carboxylic acid of lepidimoide (lepidimoic acid) was as highly promoting of chlorophyll accumulation as lepidimoide, but the hydrogenated lepidimoide had lower activity than lepidimoide. Sucrose was inactive compared with the water control. These results are in good agreement with the structure-activity relationships of growth-promoting activities obtained by the cockscomb hypocotyl test (Yamada et al. 1996), in which lepidimoide acted as a carbon source to provide energy.

As shown in Fig. 2, lepidimoide at concentrations higher than 100 μM significantly promoted light-induced chlorophyll accumulation in the cotyledons of sunflower seedlings. Relatively high concentrations of lepidimoide may be needed to promote chlorophyll accumulation because lepidimoide occurs in markedly large amounts in

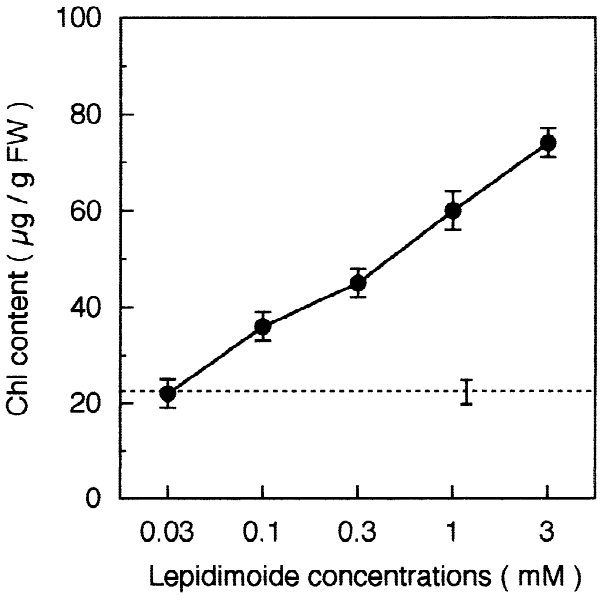

Fig. 2. Effect of lepidimoide on the chlorophyll content in cotyledons of sunflower seedlings. Four-day-old etiolated sunflower seedlings were either not pretreated or were pretreated with lepidimoide at concentrations from 0.03 to 3 mM for 6 h in the dark and then incubated for 6 h in the light at 25°C. The *dotted line* indicates the level of control. Data represent the means ± S.E. of four replicates. FW, fresh weight.

sunflower (Yamada et al. 1995). The concentrations of exogenous lepidimoide needed to induce biological activities may be ascribed to the endogenous lepidimoide level. The hypocotyl growth of cockscomb, in which there is a low level of endogenous lepidimoide, was promoted with low concentrations of exogenous lepidimoide (Yamada et al. 1995, 1996).

The similarity between the effects of lepidimoide and cytokinin in detached cotyledons was demonstrated by the detached cucumber cotyledon test, which is used widely as an assay for cytokinin (Table 2). Lepidimoide increased the chlorophyll content in detached cucumber cotyledons substantially.

The promotive mechanism of light-induced chlorophyll accumulation by lepidimoide was investigated further by examination of the kinetic changes induced by 300 μM lepidimoide (Fig. 3*A*). The difference between lepidimoide treatment and the control in the cotyledons of sunflower seedlings was first observed 4 h after the start of illumination. The chlorophyll content of the lepidimoide-treated cotyledons became twice that of the control after 8 h. In continuous darkness, the chlorophyll contents did not change with lepidimoide treatment or in the controls. Fig. 3*B* shows the variation in illuminated cotyledons treated with lepidimoide of 5-aminolevulinic acid content, which is considered to be a rate-limiting step in the formation of chlorophyll (Beale and Weinstein 1990). A significant difference in the 5-aminolev-

Table 2. Effects of lepidimoide and benzyladenine on the chlorophyll content in detached cucumber cotyledons. Cotyledons of 4-day-old etiolated cucumber seedlings were either not pretreated or were pretreated with the test solution for 6 h in the dark and then incubated for 8 h in the light at 25°C. Data represent the means ± S.E. of three replicates. FW, fresh weight.

Treatments	Chlorophyll contents (μg/g FW)
Control	212 ± 19
Lepidimoide	
1 mM	356 ± 41
3 mM	438 ± 37
Benzyladenine	
30 μM	441 ± 12
100 μM	477 ± 33

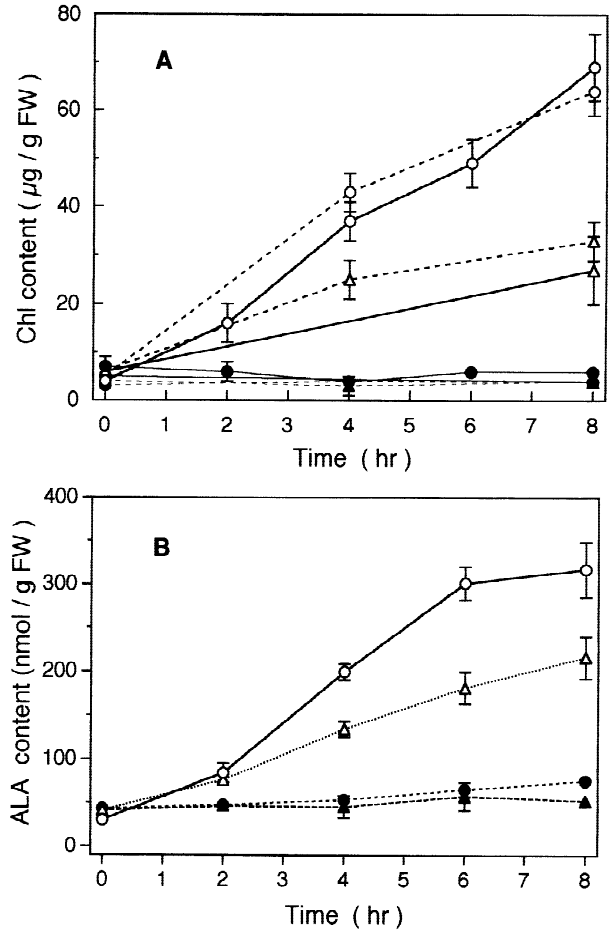

Fig. 3. Effects of lepidimoide on the chlorophyll (*A*) and 5-aminolevulinic acid content (*B*) in the cotyledons of sunflower seedlings. Four-day-old etiolated sunflower seedlings were pretreated with 300 μM lepidimoide (*circles*) or not (*triangles*) for 6 h in the dark and then incubated in the light (*open symbols*) or in the dark (*solid symbols*) at 25°C. The different lines indicate independent experiments. Data represent the means ± S.E. of three replicates.

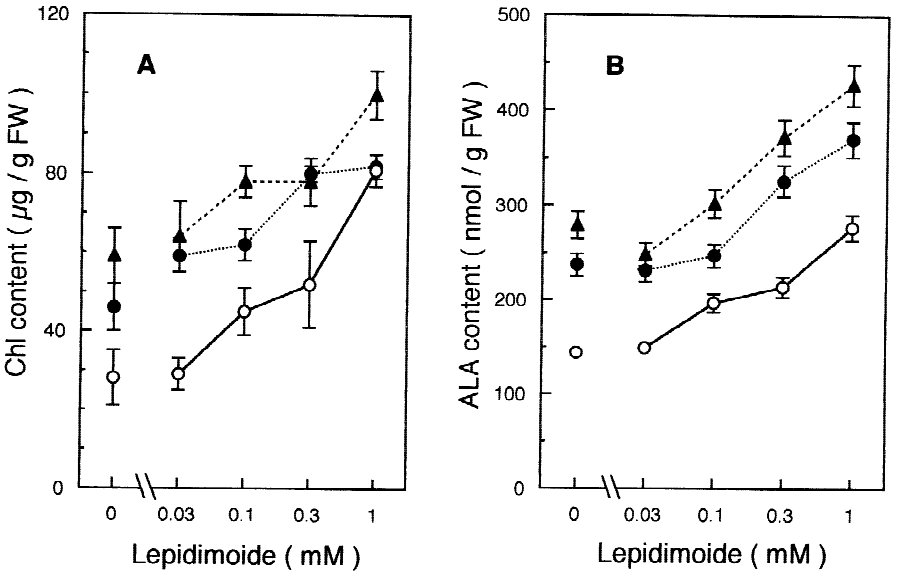

Fig. 4. Effect of lepidimoide on benzyladenine-promoted chlorophyll (*A*) and benzyladenine-promoted 5-aminolevulinic acid accumulation (*B*) in the cotyledons of sunflower seedlings. Four-day-old etiolated sunflower seedlings were pretreated with lepidimoide and/or benzyladenine (*open circles*, control; *closed circles*, 30 μM; *closed triangles*, 100 μM) for 6 h in the dark and then incubated for 6 h in the light at 25°C. The *open circle* at zero concentration of lepidimoide indicates the untreated control. Data represent the means ± S.E. of three replicates.

ulinic acid content between lepidimoide-treated and control cotyledons was observed 4 h after illumination, similar to the effect on chlorophyll accumulation (Fig. 3*A*). These results suggest that lepidimoide-induced enhancement of chlorophyll accumulation is related to the enhancement of 5-aminolevulinic acid synthesis. Two different pathways for the synthesis of 5-aminolevulinic acid have been defined in living organisms. One path via condensation of glycine and succinyl CoA is catalyzed by 5-aminolevulinic acid synthetase (Kikuchi et al. 1958, Shemin and Russell 1983), and another is via the C5 pathway (Wang et al. 1981, Weinstein and Beale 1985). It is not clear which pathway for 5-aminolevulinic acid synthesis is promoted by lepidimoide.

Cytokinin is known to stimulate chlorophyll accumulation in various plants (Davies 1987). Benzyladenine at concentrations of 30 and 100 μM enhanced the levels of both chlorophyll and 5-aminolevulinic acid in the cotyledons of sunflower seedlings (Fig. 4, *A* and *B*). Lepidimoide acted somewhat additively with benzyladenine, but not synergistically, on increasing the contents of chlorophyll and 5-aminolevulinic acid as with the case of lepidimoide alone. These results suggest that lepidimoide, in cooperation with cytokinin, causes light-induced chlorophyll accumulation by affecting the level of 5-aminolevulinic acid. It is known that stimulation of the synthesis of 5-aminolevulinic acid by benzyladenine was caused by increased levels of glutamyl-tRNA reductase in its synthesis system (Masuda et al. 1994, 1995). Further experiments are needed to clarify whether lepidimoide also acts on this enzyme or not.

From the present results, lepidimoide seems to be a new type of plant growth regulator with multiple physiological functions in the regulation of plant growth and development.

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