Occurrence of Lepidimoide-like Substance in Growing Plant Tissues and its Physiological Activity in *Arabidopsis thaliana**

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Abstract: Lepidimoide, which was first identified as an allelopathic substance, was roughly confirmed to be included in growing tissues (rosette leaves, stems + inflorescences and roots) of *Arabidopsis thaliana* by means of HPLC analysis. Peaks of retention time in HPLC of the lepidimoide fractions extracted from the above organs completely coincided with that of authentic lepidimoide.

The lepidimoide-like substance, which was contained in the fraction below MW 3000 in the molecular exclusion chromatography, promoted hypocotyl growth and inhibited root growth of cockscomb (*Celosia cristata*). The substance also promoted the growth of hypocotyls and cotyledon-petioles, increased the area of cotyledons, but inhibited the root growth of *A. thaliana* seedlings. These facts suggest that the lepidimoide-like substance may play some role as an endogenous plant growth regulator in growing plants of *A.*

thaliana.

Key words: *Arabidopsis thaliana*, lepidimoide, plant growth regulator, growth promotion, growth inhibition

Introduction

Lepidimoide was first identified as an allelopathic substance in germinated cress seeds by the mixed culture test⁵⁾. When cress seeds were placed near cockscomb, tomato or lettuce seeds for germination in a seed bed, the hypocotyl growth in the latter seedlings was promoted, but their root growth was inhibited. In this case the growth of the cress seedlings was not affected by the other plants^{5,6)}. Thereafter, germinated seeds of various dicots, sunflowers and buckwheat, in particular^{8,15)}, as well as monocots were found to secrete a lepidimoide-like substance. Generally, allelopathic substances have an inhibitory effect on plant growth, for example, 2-*cis*-dehydromatricaria ester from seeds of *Solidago altissima*¹⁰⁾, and 5-hydroxynaphthoquinone (juglone) from the stone fruits of walnuts¹⁾. However, lepidimoide is a unique substance in the respect that it promotes the seedling growth of other plants⁷⁾.

Lepidimoide, named after the scientific name of cress, *Lepidium sativum* L., is a disaccharide composed of glucose and rhamnose (Fig. 1). The chemical structure of the substance has been determined and syntheses

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Fig. 1 Chemical structure of lepidimoide.

ized from glucose and rhamnose¹²). There is a report that ceratin oligosaccharides (rafinose and stachyose) promoted the *Arabidopsis* growth in leaf number and dry weight⁹). Since lepidimoide belongs to the disaccharide group, there may be a common process of growth promotion functioning similar to the oligosaccharide action.

Lepidimoide promotes hypocotyl growth but inhibits root growth, particularly of plants belonging to *Amaranthaceae*, such as *Celosia cristata* and *Amaranthus caudatus*⁶). Several other physiological effects have been investigated. Promotion of hypocotyl growth, rosette leaf development, plant height, plant mass, and acceleration of flowering in *Arabidopsis thaliana*⁴); inhibition of senescence in *Avena* leaf segment¹³); and inhibition of abscission of bean petiole explants¹⁴) have been reported. These studies suggest that lepidimoide is not only an allelopathic substance but also a common plant growth regulating substance. We have found that germinated seeds of *A. thaliana* contain lepidimoide¹⁷).

In this article we report the occurrence of a lepidimoide-like substance from various organs of growing plants of *A. thaliana* and

also on its physiological activities during seedling growth.

Materials and Methods

Chemicals

Authentic lepidimoide (Na 2-O-L-rhamnopyranosyl 4-deoxy- α -L-threo-hex-4-enopyranosiduronate, Fig. 1) was synthesized from D-glucose and α -L-rhamnose as described by Kosemura et al.¹²).

Extraction and isolation of lepidimoide-like substance

Arabidopsis thaliana L. Heynh. (Shokei line) was cultivated in a field in Sendai, Japan, from October 1997 to April 1998. The plants that were about to flower and those whose flower stalks were 2-10 cm high with just-opened flowers were collected. The plants were separated into roots, rosette leaves and stems + inflorescences.

Each organ was frozen with liquid nitrogen and ground by mortar and pestle. The frozen powder of the roots (ca. 10g in fresh weight), rosette leaves (ca. 130g in fresh weight) and stems + inflorescences (ca. 190g in fresh weight) was extracted in boiling water (11 in roots and 31 in rosette leaves and in stems + inflorescences) for 30 min. The water extract was evaporated in a vacuum at 40°C to 200ml, and the concentrate was centrifuged (10000 rpm, 15min). The supernatant was evaporated to dryness in a vacuum at 40°C. The residue was dissolved in water (100ml) and fractionated by chloroform 3 times. The water phase was evaporated to dryness in a vacuum at 40°C. The residue dissolved in water was fractionated successively into four parts (MW above 10⁵, from 10⁵ to 10⁴, from 10⁴ to 10³, and below 10³) by molecular exclusion chromatography (Novacell, Filtron Technology Co., USA).

We used the fraction below MW 10³, in

which lepidimoide was included. This fraction, called 'lepidimoide-like substance', was concentrated to dryness in a vacuum at 40°C, and separated it into 2 parts for HPLC (high performance liquid chromatography) analysis and for bioassay.

HPLC analysis

The lepidimoide-like substance dissolved in water was applied to three solid-phase extractions with Sep-Pak Plus Cartridges (Waters Co., USA): C18, Accell CM and Accell QMA, in that order. In the first step, the 10-ml sample was applied to a reverse-phase cartridge column (Sep-Pak Plus, C18) and washed with 5ml water. The combined sample was applied to a cation-exchange cartridge column (Sep-Pak Plus, Accell CM). The unadsorbed fraction was applied to an anion-exchange cartridge column (Sep-Pak Plus, Accell QMA), washed with 5ml each of water and 0.01% trifluoroacetic acid, and then eluted with 20ml of 0.1% trifluoroacetic acid. The effluent was evaporated to dryness in a vacuum at 40°C. In some cases, to obtain a more purified sample, the residue dissolved in water was fractionated below MW 5000 by molecular exclusion chromatography (Mol Cut, UFP2 LCC24, Nihon Millipore, Ltd. Japan). The sample was freeze-dried and stored at -26°C.

The stored sample was dissolved in water and was then purified by HPLC (Tosoh, SCX (H+); ϕ 7.8mm I. D. $\times$ 300mm; water, flow rate 0.8ml/min, 214nm detector). The eluate retained for 5 to 7 min was collected in which lepidimoide was contained¹⁶⁾. For an assay of biological activity, this fraction was collected more than 20 times. The combined effluent was evaporated to dryness in a vacuum at 40°C. This final sample was applied to the biological assay. After confirmation of the biological activity, the sample was reapplied

to HPLC singly and/or mixed with authentic lepidimoide.

Bioassay

Cockscomb test: Fifteen cockscomb seeds (*Celosia cristata* L.) were placed on filter paper (Advantec Toyo, No. 101) moistened with 0.5ml of test solution in a 3-cm petri dish. The petri dishes were kept in darkness at 25°C for 4 days. The lengths of the hypocotyls and roots were then measured.

***Arabidopsis* test:** Thirty seeds of *Arabidopsis thaliana* L. Heynh. (WS and/or Columbia line) were sown on 1% agar with 3 ml of test solution in a 3-cm petri dish. The petri dishes, placed in a moistened transparent plastic tray, were kept in darkness at 5°C for 2 days and then allowed to grow under continuous light (ca. 10W/m²) at 23°C for 7 days. The lengths of the hypocotyls and cotyledon-petioles, and the areas of the cotyledons were measured with a photographic enlarger with 0.1mm accuracy. To measure root length, seeds were sown on a 1%-agar plate with 10ml of test solution in a square plastic dish (8 $\times$ 7 $\times$ 2cm) and kept in darkness at 5°C for 2 days. The dish was then placed in a vertical position so as to let the roots grow downward along the agar surface. The root length was measured after incubation under continuous light at 23°C for 7 days.

Results

Biological activity of lepidimoide-like substance on cockscomb seedlings

The lepidimoide-like substance from roots, rosette leaves and stems + inflorescences were applied to the cockscomb test (Fig. 2). As shown in Fig. 2A, the samples from rosette leaves and those from stems + inflorescences showed marked promotion of the cockscomb hypocotyls, which grew to approximately twice the length of the control

Fig. 2 Effects of lepidimoide-like substance from rosette leaves, stems + inflorescences and roots of *Arabidopsis thaliana* (Shokei line) on hypocotyl and root growth of cockscomb. A: Hypocotyl length. B: Root length. Bars indicate SE of the mean ($n=12$). Horizontal bars on concentration 0 indicate the effect of authentic lepidimoide (1000mg/l).

at 100mg/l. The fraction of rosette leaves at 1000mg/l showed about 3-fold elongation compared with the control. The fraction of roots showed a small promoting effect at 1000mg/l. Figure 2B shows the effect of the lepidimoide-like substance on the growth of cockscomb roots. All three fractions showed a remarkable inhibitory effect on cockscomb root growth at 1000mg/l; many short lateral roots occurred instead of elongated main roots. No significant effect was observed in the cockscomb hypocotyl test of gibberellin A_3 (GA_3), indoleacetic acid (IAA) and benzyladenine (BA) at a concentration of $10^{-4}M$ (data not shown).

Biological activity of lepidimoide-like substance on *A. thaliana* seedlings

The *Arabidopsis* test was applied to the lepidimoide-like substance extracted from rosette leaves. Figure 3 shows the effects of the substance on four growth processes of

WS and Columbia lines. The hypocotyl length of the WS line was promoted in proportion to rising concentration between 10 and 10^3 mg/l, but at 10^4 mg/l the seedlings showed no further growth (Fig. 3A). In the Columbia line the hypocotyl growth was enhanced at 10^3 mg/l, but the degree of growth promotion decreased at 10^4 mg/l. Root growth in both lines was severely inhibited at concentrations over 10^2 mg/l and did not occur at 10^4 mg/l (Fig. 3B). Cotyledon area and petiole length increased at a concentration of 10^3 mg/l in the WS line (Fig. 3C, 3D and Fig. 4) and of 10^3 and 10^4 mg/l in the Columbia line (Fig. 3C and 3D).

These results indicate that the Columbia line is less sensitive to the growth inhibiting action of a high concentration of the lepidimoide-like substance compared with the WS line. Similar activity was recognized in the substance from stems + inflorescences (date not shown).

Sucrose (1%) and glucose (1%) did not

Fig. 3 Effects of lepidimoide-like substance from rosette leaves of *A. thaliana* (Shokei line) on some growth indicators of *A. thaliana* seedlings. A: Hypocotyl length. B: Root length. C: Cotyledon area. D: Petiole length. Circles: WS line. Squares: Columbia line. Bars indicate SE of the mean ($n=25$). The error bars that are not visible are contained within the symbol.

affect any growth process after 7 days incubation (data not shown). This shows that the lepidimoide-like substance does not act as a nutrient source.

HPLC analysis of lepidimoide-like substance

The three samples (rosette leaves, stems + inflorescences and roots) dissolved in water were purified by HPLC. Eluate from 5 to 7 min in which lepidimoide was contained and that from 7 to 10 min were collected. The retention time of authentic lepidimoide was 5.

Fig. 4 Effect of lepidimoide-like substance from rosette leaves of *A. thaliana* (Shokei line) on cotyledon area of WS line. Seedlings were incubated on an agar plate in a small petri dish for 7 days under continuous light at 23°C. Left: Control. Right: lepidimoide-like substance 1000mg/l. Bar=10mm.

5min. The biological activity of the eluates was assayed by the cockscomb test. The sample of rosette leaves revealed growth promotion of hypocotyls and growth inhibition of roots in cockscomb seedlings (Fig. 5). Samples from stems + inflorescences and roots showed similar effects to the rosette leaves (data not shown).

The eluate containing the lepidimoide-like substance was evaporated to dryness in a vacuum at 40°C. The residue dissolved in water was finally applied to HPLC (column and conditions were the same as in Materials and Methods). The retention time of each sample from the 3 organs (leaves, stems + inflorescences and roots) was compared with authentic lepidimoide (Fig. 6). The peaks of the extracted sample completely coincided with that of authentic lepidimoide in all cases of the rosette leaves (Fig. 6, left), stems + inflorescences (Fig. 6, middle) and roots (Fig. 6, right). The peaks also coincided with those of the chromatograms of authentic lepidimoide (Fig. 6, top row), extracted sample (Fig. 6, middle row), and the mixture of authentic lepidimoide and extracted sample

Fig. 5 Effects of HPLC fraction prepared from rosette leaves of *A. thaliana* (Shokei line) on hypocotyl and root growth of cockscomb. Biological activity was assayed for fractions with retention times of 5-7 and 7-10 min. A: Hypocotyl length. B: Root length. Bars indicate SE of the mean (n=12).

(Fig. 6, bottom row). From this result we concluded that the lepidimoide-like substance in the growing tissues almost certainly coincided with or contained lepidimoide.

Discussion

Lepidimoide is an allelopathic substance at the germination stage^{5,6,8)}. It can also be considered as a growth regulating substance in growing plants⁴⁾. Lepidimoide is a unique allelopathic substance because of having a two-faced effect: a growth promoting and a growth inhibiting effect. Most allelopathic substances have growth-inhibiting effects on plants, but lepidimoide shows growth-promoting effects on hypocotyls^{7,8)} and flower stalks⁴⁾, and growth-inhibiting effects on roots⁷⁾.

We have heretofore believed that lepidimoide was exuded from germinated seeds

in cress¹⁵⁾. Additionally, in *A. thaliana* it was produced in the testa and secreted from the 'central elevation' of the testa cells together with mucilage^{2,3,11)}. In the present experiment, we found by HPLC analysis that lepidimoide is also included in growing rosette leaves, flower stalks, inflorescences and roots of *A. thaliana*. The amount of lepidimoide-like substance was roughly estimated by the cockscomb hypocotyl test on the basis of the kinetics of authentic lepidimoide: 1.5mg/g fresh weight in rosette leaves, 0.3mg/g fresh weight in stems + inflorescences, 0.08mg/g fresh weight in roots and 0.6mg/g weight of fully ripened seeds. The order, in descending quantity, was rosette leaves > stems + inflorescences > roots. This suggests that lepidimoide is mainly synthesized in rosette leaves. However, the amounts of the lepidimoide-like substance were beyond our

Fig. 6 HPLC chromatograms of lepidimoide-like substance in rosette leaves, stems + inflorescences and roots of *A. thaliana* (Shokei line). Left to right: Rosette leaves, Stems + Inflorescences, Roots, Top row: Chromatogram of authentic lepidimoide, Middle row: Chromatogram of extracted sample, Bottom row: Chromatogram of mixture of authentic lepidimoide and extracted sample. Arrows indicate peaks of lepidimoide.

expectations. This overestimation may have been caused by the relatively low efficacy of authentic lepidimoide (Fig. 2). We are now investigating the reason for this low efficacy.

For an assay of lepidimoide, the cockscomb test using *Celosia cristata* or *Amaranthus caudatus* is the best at present. Cockscomb seedlings responded negatively to auxin (IAA) and were little promoted by GA_3 ⁷⁾ or sugars¹⁶⁾. We recognized that the lepidimoide-

like substance, as well as IAA and BA, was not effective on lettuce hypocotyl growth in the light, although GA_3 markedly promoted the elongation of lettuce hypocotyls at even a low dose of $10^{-6}M$. (data not shown). From these results we believe that the lepidimoide-like substance acts in a different manner than gibberellin action. In *A. thaliana*, this substance promoted hypocotyl elongation at the somewhat high concentration of 1000mg/l,

but inhibited root elongation at the lower concentration of 100mg/l (Fig. 3). The effective concentrations are much higher than those in other plant hormones or oligosaccharides active in plant growth⁹⁾, but the effect is not that of sugar nutrients, because both glucose and sucrose scarcely promoted the growth of hypocotyls, cotyledons and petioles (data not shown). There are some differences among wild-type lines in regard to the efficacy, i. e. the WS line showed a greater promotion than Columbia line in hypocotyl growth, but the reverse was true in terms of cotyledon growth (Fig. 3). Generally, the sensitivity to lepidimoide differs greatly among wild-type lines⁴⁾.

We could not identify lepidimoide in the growing plant tissues of *A. thaliana*, although the HPLC peaks of the lepidimoide-like substance completely coincided with those of authentic lepidimoide. Studies to identify this substance are in progress.

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シロイヌナズナの成長中の植物組織に含まれる レピジモイド様物質及びその生理活性について

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摘 要

レピジモイド (Fig. 1) は、はじめアレロパシー物質としてクレス発芽種子から単離されたものである。しかし、成長中のシロイヌナズナ (*Arabidopsis thaliana*) のロゼット葉、茎+花序、および根の組織にもレピジモイド様物質が含まれていることが、ケイトウ (*Celosia cristata*) の幼植物を用いた生物試

験 (Fig. 2) および HPLC における合成レピジモイドとのピーク的一致 (Fig. 6) により確かめられた。HPLC において合成レピジモイドと一致する分画は、ケイトウの胚軸伸長を促進し、根の伸長を抑制した (Fig. 5)。また、これらの組織に含まれるレピジモイド様物質 (限外ろ過による分子量3000以下の分画) は合成レピジモイドと同様の生理活性を示した。すなわち、シロイヌナズナ芽生えにおける胚軸と子葉柄の伸長、および子葉の拡大成長を促進し、根の伸長を抑制した (Fig. 3, Fig. 4)。

これらの結果から、レピジモイド様物質はシロイヌナズナの成長中の組織で内生の成長調節物質として働いている可能性が示唆された。

キーワード：シロイヌナズナ、レピジモイド、植物成長調節物質、成長促進、成長抑制

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