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ORIGINAL RESEARCH PAPER

Dehydrocostus lactone, a naturally occurring polar auxin transport inhibitor, inhibits epicotyl growth by interacting with auxin in etiolated *Pisum sativum* seedlings

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Abstract
We have isolated germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety, dehydrocostus lactone (DHCL), costunolide, santamarine, and a novel compound denoted artabolide [3-hydroxy-4,6,7(H)-germacra-1(10),11(13)-dien-6,12-olide] from oriental medicinal Asteraceae plants as novel naturally occurring inhibitors of polar auxin transport detected by the radish hypocotyl bioassay. To investigate the mode of action of natural sesquiterpene lactones on the inhibition of polar auxin transport as well as its relation to the growth of seedlings, the function of DHCL on growth and auxin dynamics in etiolated pea seedlings was studied intensively. DHCL reduced polar auxin transport in a dose-dependent manner together with the inhibition of the accumulation of mRNA of *PsAUX1* and *PsPIN1* genes encoding influx and efflux carrier proteins of auxin, respectively. DHCL applied to the apical hook region as a lanolin paste substantially inhibited elongation growth in the subapical region of epicotyls in intact etiolated pea seedlings, coupled with a significant reduction of endogenous levels of indole-3-acetic acid (IAA). DHCL also revealed the inhibition of IAA-induced cell elongation in etiolated pea epicotyl segments by affecting IAA-induced changes in the mechanical properties of cell walls. These facts suggest that germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety affect the expression of *PsAUX1* and *PsPINs* genes, and then inhibit polar auxin transport and reduce endogenous levels of IAA necessary for stem growth in etiolated pea seedlings. These compounds are also suggested to show the inhibitory effects on auxin action in pea stem growth.

Keywords
auxin; cell wall mechanical properties; endogenous IAA level; IAA-induced elongation; inhibitor; pea epicotyls; polar auxin transport

Introduction

The plant hormone group, auxins, of which indole-3-acetic acid (IAA) is the most common, are mainly synthesized in the shoot apex and actively transported between cells along physiological apical–basal direction in shoots. The directional auxin fluxes generate an auxin gradient within tissues that plays an important role in the diverse regulation of various plant developmental stages in vegetative tissues [1–4]. Auxin transport has been demonstrated to be required for stem elongation [5], and has to be controlled to regulate shade-avoidance syndromes [6,7]. In the early growth stage of etiolated seedlings, the growth rate has been reported to be strongly correlated with

polar auxin transport [8]. Directional auxin fluxes are also important for reproductive tissues such as in floral formation and its development [9–11]. This polar-directional transport designated as polar auxin transport has been regulated by the function of the plasma membrane proteins of AUXIN RESISTANT1/LIKE AUXIN RESISTANT1 (AUX1/LAX) proteins as influx carriers and PIN-FORMED (PIN) proteins as efflux carriers in polar auxin transport [1–4,11–14].

Chemicals such as 2,3,5-triiodobenzoic acid (TIBA), *N*-(1-naphthyl)phthalamic acid (NPA), 9-hydroxyfluorene-9-carboxylic acid (HFCA), and related compounds are useful tools to investigate the mechanisms of polar auxin transport. NPA-binding proteins and actin cytoskeleton have been demonstrated to be deeply involved in polar auxin transport by studies using NAP and TIBA, respectively [4,15–18]. These compounds are not naturally occurring but artificial/synthetic inhibitors of polar auxin transport. Almost no plant-derived compounds showing activities regulating polar auxin transport have been found although flavonoid compounds [19], chromosaponin I [20] and 3,4-(methylenedioxy)cinnamic acid and *cis*-cinnamic acid [21,22] have been reported to inhibit it. In view of the importance of chemical tools, we have surveyed naturally occurring compounds inhibiting polar auxin transport in oriental medicinal Asteraceae plants using the radish (*Raphanus sativus* L.) hypocotyl segment bioassay system and [^{14}C]-labeled indoleacetic acid ([^{14}C]-IAA) [23]. As a result, we have fully succeeded in isolating several germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety such as dehydrocostus lactone [decahydro-3,6,9-trismethylene-azulenol(4,5-b) furan-2(3H)-one; DHCL], costunolide [3a*S*,6*E*,10*E*,11a*R*]-6,10-dimethyl-3-methylene-3,3a,4,5,8,9-hexahydrocyclodeca[b]furan-2(11a*H*)-one], and santamarine [(3,5,6,9a,9b)-6-hydroxy-5a,9-dimethyl-3-methylidene-3a,4,5,5a,6,7,9a,9b-octahydronaphtho[1,2-b] furan-2(3H)-one] from *Saussurea costus* roots [23,24] and a novel compound named artabolide [3-hydroxy-4,6,7(H)-germacra-1(10),11(13)-dien-6,12-olide] from *Artemisia absinthium* shoots, as polar auxin transport inhibitors [25].

A number of germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone derivatives obtained from natural sources have been demonstrated to exhibit pharmaceutically interesting biological activities in animals and plants. DHCL and costunolide have been demonstrated to show antitumor activity, anti-inflammatory activity, and the induction of apoptosis [26–32]. These compounds are also demonstrated to act as germination stimulants for the root parasitic broomrapes *Orobanche cumana* Wallr. and *O. minor* Sm., but not *Phelipanche aegyptiaca* [33–35]. However, little is known the mode of action of DHCL in inhibiting polar auxin transport relevant to cell elongation.

In this paper, as a representative of germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety, we investigated the effect of DHCL on the epicotyl growth in etiolated 'Alaska' pea epicotyls with respect to auxin dynamics, including endogenous IAA levels and polar auxin transport, since etiolated pea seedlings have long been used in the study of mode of action of auxin. To investigate the possible mode of action of DHCL, an IAA-induced elongation system using etiolated pea epicotyl segments was also introduced in this study. The effects of DHCL on osmotic water absorption and cell wall mechanical properties are described, since cell elongation is primarily controlled by these two parameters. A possible mode of action of DHCL on polar auxin transport and growth in etiolated pea seedlings is discussed.

Material and methods

Growth experiment in intact etiolated pea seedlings

Seeds of pea (*Pisum sativum* L. 'Alaska') were purchased from Watanabe Seed Co., Japan. Rock wool blocks (9 cm × 4.8 cm × 1.5 cm) cut out from a large sheet of rock wool (Chibikko Ace Mat, Nippon Rockwool Corp., Japan) were placed individually in an acrylic resign box (9 cm × 4.8 cm × 6 cm). Each box had four holes (1 cm in diameter) covered with MilliSeal (Millipore, Merck, Japan) on the top for ventilation. Twelve seeds were placed in each box so that the seed axis was normal to the upper surface of the block. After supplying 40 mL of distilled water, each box was maintained

at 23.5°C in the dark for 4 days. Four-day-old etiolated pea seedlings were subjected to further experiments. An application of dehydrocostus lactone [decahydro-3,6,9-trismethylene-azulenol (4,5-b)furan-2(3H)-one; DHCL] (purchased from Waka Pure Chemical Industries, Ltd., Japan) to intact 4-day-old etiolated pea seedlings was made as follows. Five-mm subapical elongation regions below the apical hook (2–7 mm) of etiolated pea seedlings were marked with Indian ink, since this region of the epicotyl has been demonstrated to mainly elongate for the following 24 h in the dark [36]. Aqueous lanolin paste (30%, w/w) containing different amounts of DHCL (0, 0.3, 1, 3, and 10 μg/plant) was applied to the tip of apical hook, and then the seedlings were incubated for another 24 h in the dark at 23.5°C. The changes in length of the subapical region of the epicotyls marked with Indian ink were determined.

For analyses of gene expression and endogenous auxin levels, the apical region (0~5-mm region below the hook) and elongation region (5–15-mm region below the hook) of epicotyls were excised from the DHCL (10 μg/plant)-treated or nontreated pea seedlings. Then the segments were immediately frozen in liquid N_2, and kept at −80°C prior to extraction of total RNA or endogenous IAA. All manipulations of these growth experiments were conducted under dim green light.

Measurement of polar auxin transport

Measurements of polar auxin transport in the segments of etiolated pea epicotyls were determined by the methods already described, with some modifications [23]. DHCL dissolved in ethanol was put into the bottom of 1.5-mL Eppendorf plastic tubes, dried in vacuo, and then dissolved with 30 μL of [^{14}C]-IAA (1 μCi/mL, American Radiolabeled Chemicals Inc., USA). Epicotyl segments (30 mm in length) excised from 5 mm below the apical side of 4-day-old etiolated pea seedlings, were placed into the Eppendorf tubes in downward orientation of the apical side. Almost no auxin transport was observed when IAA was applied at the basal side of the segments in the same manner, indicating that the [^{14}C]-IAA applied to the apical side was transported to the physiological apical–basal direction in this assay system. After incubation at 23.5°C for 18 h, a 3-mm piece of the opposite side of the segment was cut. This piece was put directly into a vial containing the liquid scintillation cocktails (Universol-ES, MP-Biomedicals, USA), and then its radioactivity determined using a liquid scintillation counter (Tri-Carb2200CA, Packard Instrument Co., Ltd., USA). Experiments were made in triplicate with six segments. The results were expressed as percentage of the mean values of control with standard errors attached (n = 3).

Determination of gene expression of *PsAUX1* and *PsPINs*

Semiquantitative RT-PCR was introduced to determine gene expression of *PsPINs* and *PsAUX1* according to the method reported previously [37–39]. Extraction of total RNA, the synthesis of first-strand cDNAs and PCR reactions were carried out using Isogen (Nippon Gene Co., Ltd., Japan), PrimeScript RTase and an Oligo-dT adaptor primer of the RNA PCR kit (TaKaRa PrimeScript^{R} RT-PCR Kit, Takara Bio. Inc., Japan), and GoTaq^{R} Green Master Mix (Promega Co., USA), respectively, according to the manufacturer's instructions. Primers used for the amplification of *PsPIN1*, *PsPIN2*, and *PsAUX1* were designed from *PsPIN1* (accession No. AY222857), *PsPIN2* (accession No. AB112364), and *PsAUX1* (accession No. AB107919) in 'Alaska' pea seedlings were used, respectively (Tab. 1). After agarose gel electrophoresis and EtBr staining, the density of corresponding bands in agarose gel were determined. The amount of 18S ribosomal RNA was used as an internal standard for quantification. Results were expressed as mean values with standard errors of the mean attached (n = 5).

Determination of endogenous free IAA levels in intact etiolated pea epicotyls

Extraction and solvent fractionation procedures for the determination of endogenous free IAA were carried out in the usual way [40]. Frozen epicotyl segments were extracted

Tab. 1 Primers designed for the amplification of *PsPIN1*, *PsPIN2*, and *PsAUX1* for the semiquantitative analyses.

Gene	Primer sequence		Ref.
	Forward	Reverse	
PsAUX1	5′-ctgaaattggttctccacat-3′	5′-gaaggttgagtattactact-3′	[37,38]
PsPIN1	5′-ctatgatgggtggtggaagaaactc-3′	5′-gaataaaccgagactaaacatggcc-3′	[46]
PsPIN2	5′-atgttgtgtggagctcaag-3′	5′-ttgctacatgaaggagggtaccac-3′	[37,38]
PsPIN3	5′-acaatccttatgccatgaac-3′	5′-aactcatcgtgccaaattc-3′	[39]
18s rRNA	5′-ctggcaccttatgagaaatc-3′	5′-ccacccatagaatcaagaaa-3′	[37,38]

twice with aqueous ethanol at 4°C in the dark. The alcoholic extracts were concentrated in vacuo to give an aqueous residue. Indole-2,4,5,6,7-d_5-3-acetic acid (d_5-IAA) was added to the aqueous residues as an internal standard at a rate of 50 ng g^{-1} fresh weight. The aqueous residue adjusted to pH 3 with HCl was partitioned with diethyl ether. The diethyl ether-soluble fraction was then concentrated to dryness in vacuo. After methylation with ethereal diazomethane, methylated materials were introduced into a Finnigan GCQ gas-liquid chromatography-mass spectrometer equipped with a DB-5MS glass capillary column (0.251 mm × 30 m; J & W Scientific, USA) according to the method reported previously [23]. Quantification of IAA was estimated by the ratio of peak area of *m*/*z* 130 and 135, and 189 and 194. Experiments were performed in triplicate with 20 segments, and the results expressed as mean values with the standard error of the mean attached ($n = 3$).

Effects of DHCL on cell elongation in etiolated 'Alaska' pea epicotyl segments

Epicotyl segments (14-mm long) were excised from the region 3~17 mm below the hook of 4-day-old etiolated pea seedlings, and then incubated in distilled water for the starvation of endogenous IAA. After 2-h incubation, 10-mm epicotyl segments were prepared from the central region of the segments with a double-bladed cutter. The central segments were floated on IAA solution (10 μM) with or without DHCL (30 μM). After 6-h incubation at 23.5°C in the dark, the lengths of the segments were measured under a binocular microscope (×5) equipped with an ocular micrometer. Then, the segments were killed in boiling methanol for the analysis of cell wall mechanical properties or frozen and kept at −80°C for the analysis of osmotic properties. All manipulations of the growth experiments were conducted under dim green light at 23.5°C.

Determination of osmoregulation

To obtain cell sap from the epicotyl segments, the centrifugation method with some modifications was introduced [36]. Ten pea epicotyl segments were incubated with or without 30 μM DHCL in the presence or in the absence of 10 μM IAA. After incubation, epicotyl segments were carefully wiped with filter paper, then frozen/thawed to obtain cell sap by centrifugation. The osmotic concentration of cell sap obtained from frozen/thawed epicotyl segments by centrifugation was determined with a vapor pressure osmometer (Wescor model 5300, USA). The total amount of osmotic solutes in the epicotyl segments was estimated as the product of the osmolarity and length of the epicotyl segment according the previous method by Miyamoto and Kamisaka [36].

Measurements of mechanical properties of cell walls

The mechanical properties of the cell walls were determined by the creep extension analysis reported by Tanimoto et al. [41] with a minor modification. Boiling methanol-killed epicotyl segments were rehydrated with distilled water. The segments were secured

between upper and a lower clamps, 5 mm apart, of a creep meter (Rheoner RE-33005, Yamadan, Japan). The creep extension was measured by applying a constant load of 40 g to the a segment by driving the lower clamp down at the maximum speed at 1.0 mm sec^{-1}. The extension was recorded by a computer at 0.1 s^{-1} intervals for 1 min and then the load was released to record the shrinkage of the segments for 1 min. The creep curve was analyzed by a computer program using Burger's viscoelastic model, and then simulated by the Kelvin-Voigt–Burger viscoelastic (five elements) model, which is composed of two viscoelastic components (E1, η_1 and E2, η_2) and one Newtonian dashpot (η_n). Calculation of two elastic and three plastic parameters was conducted with a simulation program kindly provided by Dr. Ryoichi Yamamoto, Professor Emeritus at Tezukayama University, Japan.

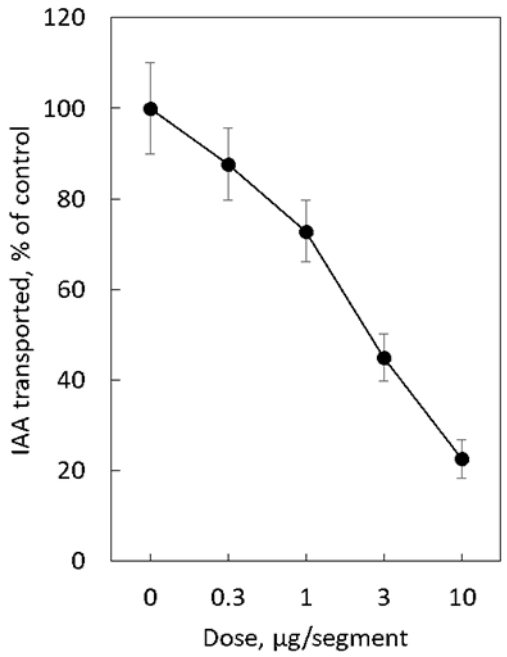

Fig. 1 Inhibitory effect of DHCL polar auxin transport in etiolated pea epicotyls. Epicotyl segments (30 mm in length) excised from 4-day-old etiolated pea seedlings were incubated with [^{14}C]-IAA for 18 h, and then radioactivity in a 3-mm piece of the opposite side of the segment was determined. Experiments were made five times with six segments. The results are expressed as percentages of the mean value of the control with standard errors of the mean attached (n = 5).

Results

Effects of DHCL on polar auxin transport and gene expression in etiolated pea seedlings

As has been already reported by other workers in the radish hypocotyl bioassay system [23], DHCL at doses of >1 µg/segment also substantially reduced the polar auxin transport in etiolated pea epicotyls in a dose-dependent manner (Fig. 1).

Gene expressions of *PsAUX1* and *PsPIN1*, *PsPIN2*, and *PsPIN3* were analyzed by using semiquantitative RT-PCR and estimation relative to the amount of 18S ribosomal RNA. When DHCL was applied at the apex as a lanolin paste, DHCL downregulated gene expression and, as a result, the accumulation of mRNA of *PsAUX1* in 4-day-old etiolated pea seedlings was substantially reduced (Fig. 2). On the other hand, gene expression of *PsPIN1* in these seedlings was also substantially reduced by the application

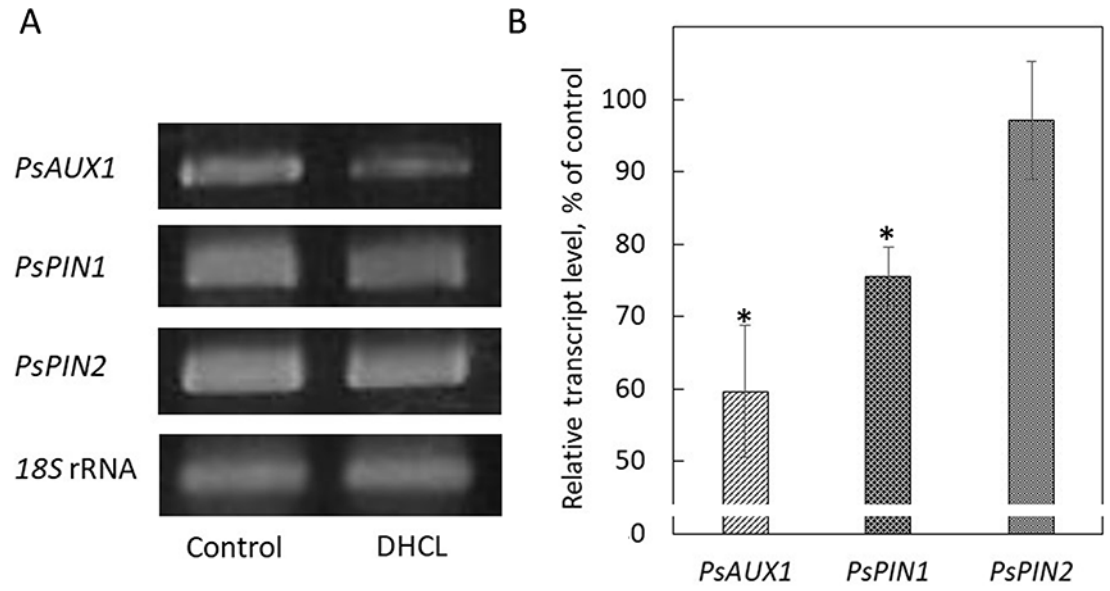

Fig. 2 Effects of DHCL on the expression of *PsPINs* and *PsAUX1* genes in the apical region of epicotyls of etiolated pea seedlings. (**A**) Semiquantitave RT-PCR analysis of *PsAUX1* and *PsPINs*. (**B**) Effect of DHCL on relative transcript levels of *PsAUX1*, *PsPIN1*, and *PsPIN2*. The amounts of *PsAUX1* and *PsPINs* mRNA were quantified relative to the amount of 18S ribosomal RNA. Results are expressed as percentages of the value of the control. Values are means with *SE* attached (n = 5). * Indicates significant difference against the control value at $p < 0.05$ by Student's t test.

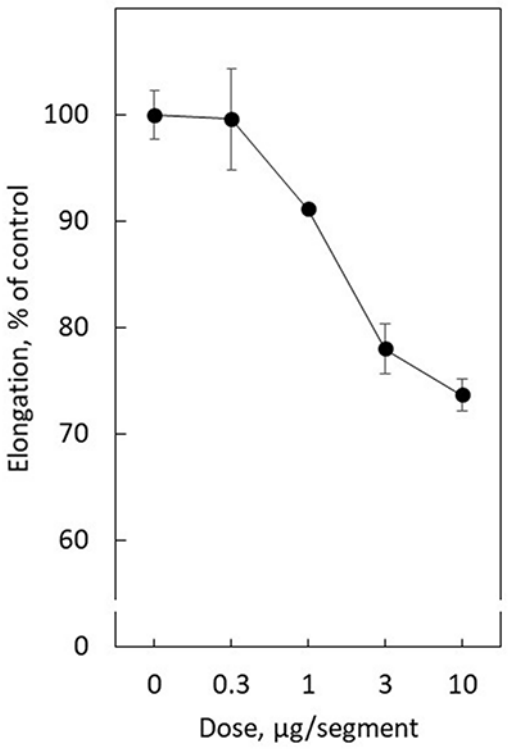

Fig. 3 Effect of DHCL applied at the apex on the growth of the subhook region of epicotyls of etiolated pea seedlings. The elongation of this region was determined, and the results are expressed as percentages of the control value. Vertical lines represent *SE* values of the means ($n = 10$).

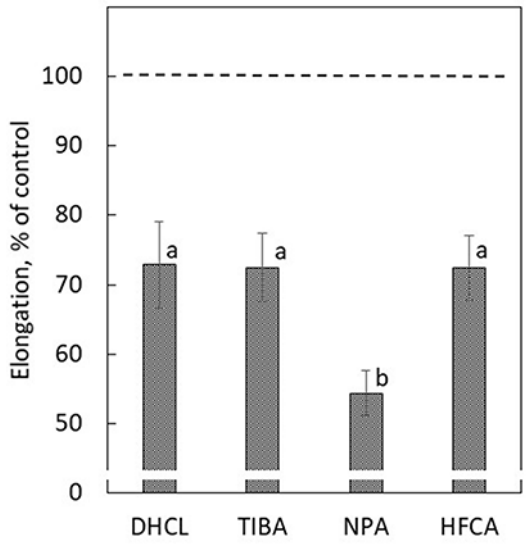

Fig. 4 Effect of DHCL and synthetic inhibitors of polar auxin transport on IAA-induced cell elongation of etiolated pea epicotyl segments. Pea epicotyl segments (10 mm long) were incubated with inhibitors at 30 µM together with IAA (10 µM) for 6 h. Values are expressed as percentage to IAA treatment. Vertical lines represent *SE* values ($n = 10$). Different letters indicate a significant difference at $p < 0.05$ from Student's *t* test.

Tab. 2 Effect of DHCL on endogenous IAA levels in apical and elongating regions of epicotyls of etiolated pea seedlings.

	IAA (ng g^{-1} FW)	
	Apical region	**Elongating region**
Control	28.6 ±4.3 (100%)	37.6 ±8.4 (100%)
DHCL at 10 µg/plant	29.7 ±5.8 (104%)	17.0 ±2.1 (45%)*

DHCL was applied to the apical hook as a lanolin paste, and then incubated at 23.5°C for 24 h in the dark. Twenty epicotyl segments excised from the apical and subapical elongating region (5~15-mm region below the hook) were used for extraction of IAA. Values in the parentheses are expressed as % of the control. Results are expressed as the mean with standard error attached ($n = 3$). * Indicates significantly different from the control value at $p < 0.05$ by Student's *t* test.

of DHCL, whereas that of *PsPIN2* was little affected (Fig. 2). The accumulation of mRNA of *PsPIN3* was not clearly detected in this experiment due to the low expression (data not shown).

Effects of DHCL on growth and endogenous IAA levels in epicotyls of intact etiolated pea seedlings

When DHCL was applied at the apex as a lanolin paste, it substantially inhibited the elongation growth of our intact etiolated pea seedlings (Fig. 3). As shown in Tab. 2, DHCL applied at 10 µg/plant to the apical hook of intact etiolated pea seedlings substantially reduced the endogenous levels of IAA in the elongating region (5~15 mm region below the apical hook) but not in the apical hook of the epicotyls of etiolated pea seedlings.

Effect of DHCL on cell elongation in etiolated pea epicotyl segments and parameters regulating cellular water absorption

DHCL at 30 µM inhibited IAA-induced cell elongation in etiolated pea segments, the inhibition being almost the same as that of TIBA and HFCA at 30 µM (Fig. 4). NPA at 30 µM was more effective than DHCL.

A kinetic study of the inhibitory effect of DHCL on IAA-induced cell elongation of etiolated pea epicotyl segments revealed that DHCL began to inhibit it without a lag period (Fig. 5). Pretreatment with DHCL for 2 h was also effective in inhibiting IAA-induced elongation at 2 h after transferring to IAA, but less thereafter (Fig. 6). Continuous application of DHCL even after application of IAA was more effective at 2 h after IAA application compared to that in IAA application alone.

Water absorption of cells for elongation growth is primarily controlled by the osmotic concentration of cell sap and the mechanical properties of cell walls. The effect of DHCL on the osmolarity of the cell sap of etiolated pea segments was examined (Fig. 7). IAA substantially decreased the osmolarity of the cell sap during incubation for 6 h. DHCL did not enhance the IAA-induced decrease in the osmolarity of the cell sap, it being higher than in the IAA treatment. The total amounts of osmotic solutes were not affected by either DHCL or IAA, nor by simultaneous

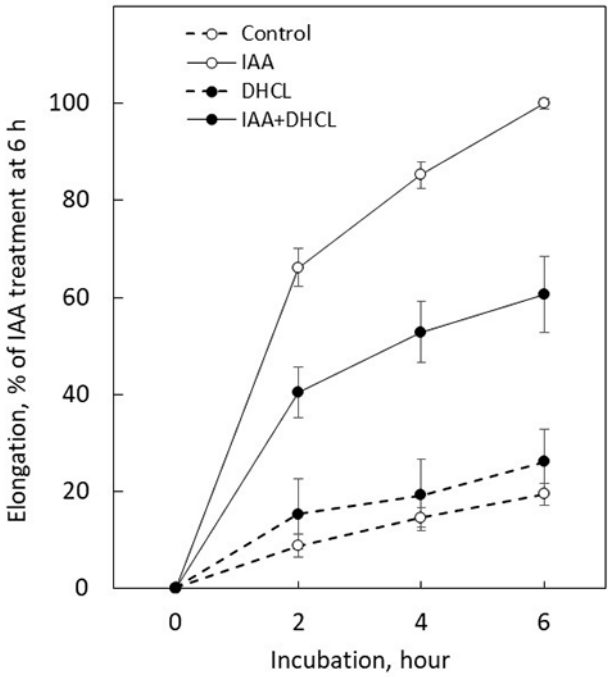

Fig. 5 Effect of DHCL on IAA-induced cell elongation of etiolated pea epicotyl segments. Pea epicotyl segments (10 mm long) were incubated with or without DHCL (10^{-4} M) in the presence or absence of IAA (10^{-5} M). Data are expressed as percentage of elongation values induced by IAA for 6 h. Vertical lines represent *SE* values (n = 10).

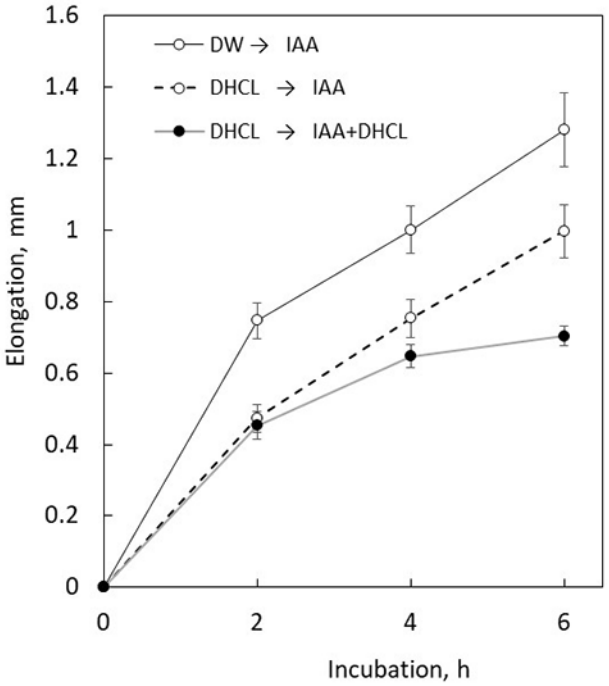

Fig. 6 Effect of pretreatment of DHCL on IAA-induced cell elongation of etiolated pea epicotyl segments. Pea epicotyl segments (10 mm long) were preincubated with or without DHCL (30 μM) for 2 h, then the segments were transferred to IAA (10 μM) in the presence or absence of DHCL. Initial lengths of the segment were those after 2 h preincubation. Data are mean values with *SE* values attached (n = 10). DW – distilled water.

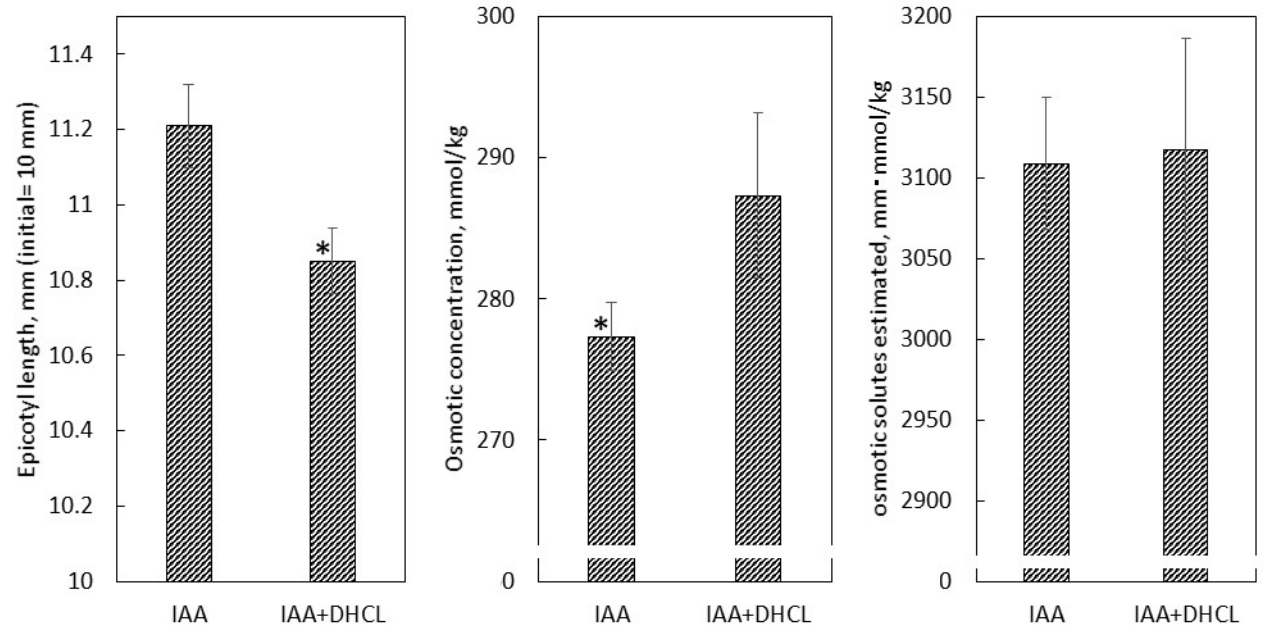

Fig. 7 Effects of DHCL and/or IAA on the osmolarity and the amount of osmotic solutes in etiolated pea epicotyl segments. Ten pea epicotyl segments were incubated with or without 30 μM DHCL in the presence or absence of 10 μM IAA. After incubation, cell sap was obtained from frozen-and-thawed epicotyl segments by centrifugation, then its osmolarity was determined using a vapor pressure osmometer. The amount of osmotic solutes was estimated as the product of osmolarity and epicotyl segment length. Data are mean values with *SE*s attached (n = 4). * Indicates significant difference from the control value at $p < 0.05$ by a Student's t test.

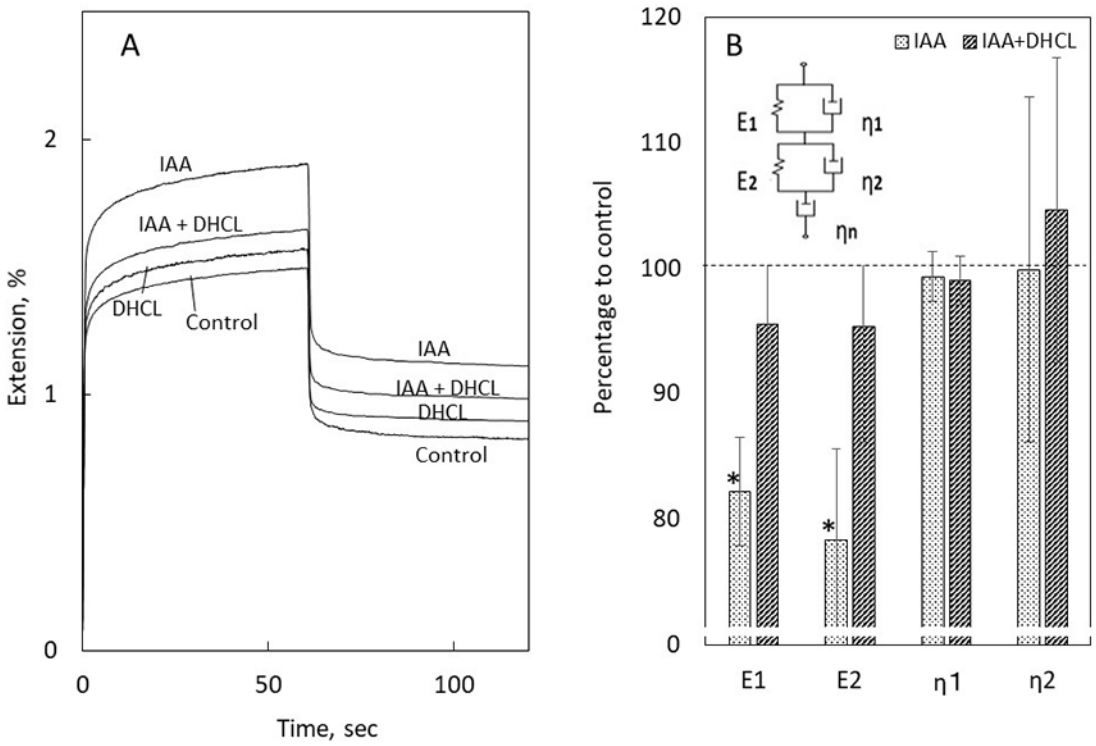

Fig. 8 Effects of DHCL and/or IAA on the creep extension and shrinkage curve of cell walls (**A**) and viscoelastic parameters (**B**) in etiolated pea epicotyl segments. Ten pea epicotyl segments were incubated with or without 30 μM DHCL in the presence or absence of 10 μM IAA, then epicotyl segments were killed in boiling MeOH. After rehydration of methanol-killed segments with distilled water, segments were subjected to a creep meter. The creep curve was analyzed using Burger's viscoelastic model, and then simulated by the Kelvin-Voigt–Burger's viscoelastic (five elements) model. * Indicates significant difference from the control value at $p < 0.05$ by Student's t test.

application of DHCL and IAA. These results suggest that the higher osmolarity due to DHCL seems to be a consequence of inhibition of IAA-induced cell elongation by DHCL in etiolated pea epicotyl segments.

IAA substantially increased cell wall extension, and simultaneous application of DHCL with IAA inhibited the increase in cell wall extension of epicotyl segments of etiolated pea seedlings (Fig. 8A). The creep extension and shrinkage curve of the cell walls measured agreed well with a Kelvin-Voigt–Burger's five-element model, which comprises two viscoelastic components (E1, η1 and E2, η2) and one Newtonian dashpot (ηn). Elastic moduli (E1 and E2) and viscosity coefficients (η1, η2, η_n) calculated by the five-element viscoelastic model of creep extension are indices of rigidity, the lower score indicating a higher extensibility. IAA reduced the value of the elastic moduli, E1 and E2 in viscoelastic components, but little affected that of the viscosity coefficients (Fig. 8B). DHCL substantially inhibited the IAA-induced decrease in the elastic moduli, E1 and E2 in the two viscoelastic parameters. These results indicate that DHCL suppresses IAA-induced elongation growth by suppressing the IAA-induced increase in cell wall extensibility in etiolated pea epicotyl segments, but does not do so by the osmoregulation.

Discussion

We have isolated DHCL, costunolide, santamarine, and a novel compound denoted artabolide [3-hydroxy-4,6,7(H)-germacra-1(10),11(13)-dien-6,12-olide] as naturally occurring inhibitors of polar auxin transport in oriental medicinal Asteraceae plants [23–25] (see Fig. 9). The structure/activity relationships suggest that germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety are potent inhibitors of polar auxin transport, since ketopelenolide a and b, and hydroxypelenolide had little

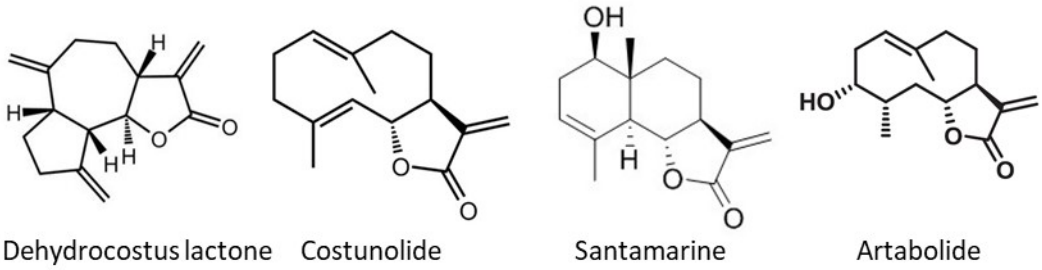

Fig. 9 Chemical structures of dehydrocostus lactone, costunolide, santamarine, and a novel compound named artabolide isolated from oriental medicinal Asteraceae plants.

effect on this transport [24]. As a representative of germacranolide-type sesquiterpene lactones, we studied the effect of DHCL on the epicotyl growth in etiolated 'Alaska' pea seedlings to elucidate auxin dynamics including endogenous IAA levels and polar auxin transport.

Function of DHCL in expression of genes related to polar auxin transport in etiolated pea seedlings

DHCL substantially reduced the polar auxin transport in etiolated pea epicotyls (Fig. 1) as in the radish hypocotyl bioassay system [23]. Auxin transport is demonstrated to be required for regulation of stem elongation [5–8]. Endogenous auxin and its manipulation by the auxin transport inhibitor, *N*-(1-naphthyl)phthalamic acid (NPA), have been demonstrated to influence in vitro shoot organogenesis observed in the basal end of epicotyl explants of juvenile citrus seedlings [42]. Thus it is possible that elongation growth of etiolated pea epicotyls is controlled by auxins polar-transported from the shoot apex, a major site of IAA biosynthesis, to the elongating region of epicotyls. Since the mode of action of DHCL in inhibition of polar auxin transport has not been clarified yet, it is worthwhile to investigate the effect of this compound on gene expression related to polar auxin transport.

AUX1 and PINs have been demonstrated to play important roles in polar auxin transport as an influx and efflux carriers, respectively [4,11–13,44–47]. In etiolated pea seedlings, *PsAUX1* and *PsPIN1*, *PsPIN2* and *PsPIN3* have been isolated [27–39,46,47]. Semiquantitative RT-PCR analysis revealed that DHCL substantially reduced accumulation of mRNA both of *PsAUX1* and *PsPIN1* in the etiolated pea seedlings (Fig. 2). Contrarily, DHCL had little effect on *PsPIN2* mRNA, and the gene expression of *PsPIN3* was quite low. Phylogenetic relationships revealed that *PsPIN1* belongs to the same clade as *AtPIN1*, and *PsPIN2* and *PsPIN3* belong to the same subclade including *AtPIN3*, *AtPIN4*, and *AtPIN7* [39]. Immuno-histochemical analysis using specific polyclonal antibodies for PsPIN1 demonstrated the localization of PsPIN1 in the basal side of the plasma membrane of cells in endodermal tissues in etiolated pea seedlings [48], suggesting that PsPIN1 plays a fundamentally important role in directional transport of auxin as does AtPIN1. These results suggest a possible involvement of reduced levels of PsAUX1 and PsPIN1 carrier proteins in auxin influx and efflux, respectively, via a reduction of expression of both *PsAUX1* and *PsPIN1* mRNA, in the reduction of polar auxin transport by DHCL in etiolated pea epicotyls.

Accumulation of *PsPIN1* mRNA has been shown to correlate well with gravity-controlled polar auxin transport in etiolated 'Alaska' pea seedlings [38,49]. In the early growth stages of etiolated maize seedlings, maximum gene expression of *ZmPIN1* was observed just prior to the maximum level of polar auxin transport in the coleoptiles and mesocotyls [8]. Thus, the possible explanation that DHCL inhibits polar auxin transport via a reduction of gene expression relating to polar auxin transport is conceivable. The expression of *PsPIN1* and *PsAUX1* genes has been reported to be auxin-inducible [37,46]. Thus, polar auxin transport regulated by PsAUX1 and PsPIN1 carrier proteins of auxin might be highly regulated by the level of mRNA accumulation affected by endogenous IAA.

NPA inhibits IAA transport by specific binding to a so-called NPA receptor, thereby blocking the carrier-mediated efflux of IAA [48], whereas IAA does not compete with NPA for the binding site [9]. Morphactin has been shown to bind to the NPA receptor, suggesting that it acts by the same mechanism as NPA [50]. TIBA has been demonstrated to be deeply involved in membrane localization of PIN protein by affecting vesicle mobility and actin cytoskeleton dynamics [16–18]. Judging from the above evidence that DHCL affects expression of *PsPINs* and *PsAUX1* genes, the mode of action of DHCL to inhibit auxin polar transport seems to be quite different from that of synthetic auxin polar transport inhibitors. Further studies on the effect of DHCL on auxin efflux carrier/facilitators from the point of view of membrane localization of PsPIN1 and binding to NPA binding protein will be required.

DHCL applied at the apical hook of intact etiolated pea seedlings substantially reduced the endogenous levels of IAA and elongation growth in the elongation region (Fig. 3 and Tab. 2). The application of costunolide, another germacranolide-type sesquiterpene lactone with an α-methylene-γ-lactone moiety, at 10 μg/plant to the apical hook also reduced endogenous levels of IAA in the elongating region of intact etiolated pea seedlings to ca. 58% of the control (data not shown). A close relationship between decreased levels of IAA and phytochrome-dependent inhibition of stem growth in pea seedlings [51], and between an increased level of IAA and elongating hypocotyls under low red:far-red (R:FR) light [6] have been demonstrated. It has also been reported that endogenous IAA contents in mesocotyls changes in parallel with the mesocotyl growth rate in dark-grown maize at different seedling depths, after IAA and TIBA treatments [52]. These results strongly suggest that elongation of etiolated pea epicotyls is controlled by auxins polar-transported from shoot apex, a major site of IAA biosynthesis, to the elongating region of epicotyls. It also suggests that DHCL inhibits elongation growth by altering IAA in the elongating subhook region of epicotyls, due to the suppression of polar auxin transport activity in intact etiolated pea seedlings.

Conjugation in ester and amide forms is considered to be a major mechanism for regulating free IAA levels by light [51,53]. Thus, the level of IAA in the site of biosynthesis of IAA might be regulated at a certain level via conjugation. Further studies on the effect of these inhibitors on the biosynthesis of IAA will be required.

Inhibitory mechanisms of DHCL on cell elongation in etiolated pea epicotyl segments

The synthetic inhibitors of polar auxin transport such as TIBA, NPA, and HFCA are known to inhibit IAA-induced elongation of the stem segments [54]. The fact that synthetic inhibitors of polar auxin transport somewhat (or partially) inhibit IAA-induced cell elongation led us to study the effect of DHCL on IAA-induced cell elongation. As shown in Fig. 4, DHCL inhibited IAA-induced cell elongation as did TIBA, HFCA, and NPA. This fact indicates that not only synthetic inhibitors but also naturally occurring germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety somewhat (or partially) have inhibitory effects on cell elongation. As shown in Fig. 6, IAA-induced elongation was observed in epicotyl segments pretreated with DHCL at 2 h after transferring to IAA. This result strongly suggests that the inhibitory effect of DHCL is not due to toxic and/or nonspecific effects, similar to that of abscisic acid (ABA) [55] and jasmonic acid (JA) [56].

Water absorption of cells for elongation growth is primarily controlled by the osmotic concentration of cell sap and the mechanical properties of cell walls. DHCL did not enhance the IAA-induced decrease in the osmolarity of the cell sap, suggesting that osmotic potential was kept low in the segments treated with IAA and DHCL. The total amounts of osmotic solutes were not affected by either DHCL or IAA, nor by simultaneous application of DHCL and IAA (Fig. 7). However, simultaneous application of DHCL with IAA inhibited the IAA-induced increase of cell wall extension in etiolated pea epicotyl segments (Fig. 8). These results suggest that DHCL inhibits cell elongation growth of etiolated pea segments by reducing cell wall extensibility but not via reduction of osmoregulation, the higher osmolarity due to DHCL being a consequence of inhibition of IAA-induced cell elongation by DHCL.

Analysis of the creep extension and shrinkage curve of the cell walls with a Kelvin-Voigt–Burger five-element model revealed that DHCL suppressed the IAA-induced cell wall loosening by affecting elastic moduli in two viscoelastic parameters in the analysis of the creep extension (Fig. 8). Increase in the mechanical extensibility of cell walls by acid treatment and low-pH dependent decrease in viscosity coefficient of creep-extension analysis in the elongating zone in pea roots has been demonstrated by other workers [41,57,58]. The degradation process of cell wall polysaccharides has been considered to cause cell wall loosening, and several hydrolytic enzymes that hydrolyze and/or trans-glycosylate polysaccharide molecules of cell walls have been suggested to participate in the deformation or the reconstruction of cell walls [59–61]. An important role of the synthesis of cell wall polysaccharides in IAA-induced elongation has also been demonstrated in galactose-induced inhibition of IAA-induced elongation in oat coleoptile segments [62,63], in ABA suppression of IAA-induced elongation of squash hypocotyl segments [54,55], and jasmonates-induced inhibition in oat coleoptile segments [56]. Thus, it is possible that DHCL inhibits elongation growth by interfering with the synthesis and/or the degradation of cell wall polysaccharides requited for elongation growth. Further studies of DHCL on cell wall polysaccharide metabolism will be needed.

As shown in our study, DHCL substantially affected auxin dynamics regulating stem growth. Another naturally occurring polar auxin transport inhibitor, chromosaponin I, has been shown to influence the growth of roots in several plants [20]. The allelochemical 4-(methylenedioxy)cinnamic acid has been demonstrated to inhibit lignification and affect auxin homeostasis [21]. DHCL and costunolide are known to act as germination stimulants for the root parasitic weeds, *Orobanche cumana* and *O. minor* [33–35]. Thus it is possible that a group of germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety act as allelochemicals by affecting auxin homeostasis.

In conclusion, as shown in Fig. 10, DHCL, a germacranolide-type sesquiterpene lactone with an α-methylene-γ-lactone moiety, downregulates expression of *PsPINs* and *PsAUX1* genes, and then inhibits polar auxin transport, resulting in a reduction of endogenous levels of IAA required for growth in the elongating region of etiolated pea epicotyls. In addition, this compound shows the inhibitory effects in auxin action in the growth of etiolated pea seedlings. Naturally occurring germacranolide-type sesquiterpene lactones with an α-methylene-γ-lactone moiety are considered to be useful as native plant growth regulators, not only for fundamental studies but also agricultural practice.

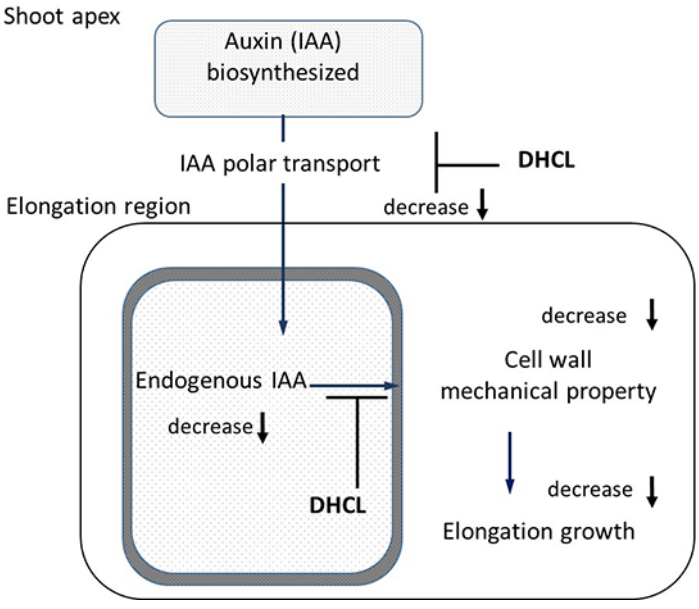

Fig. 10 A possible mode of action by which DHCL inhibits stem elongation growth in etiolated pea seedlings.

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Lakton dehydrokostu, naturalnie występujący inhibitor polarnego transportu auksyny, hamuje wzrost epikotylu poprzez interakcję z auksyną w etiolowanych siewkach *Pisum sativum*

Streszczenie

Wyizolowano germakranolidowe laktony seskwiterpenowe z ugrupowaniem α-metyleno-γ-laktonu, lakton dehydrokostu (DHCL), kostunolid, santamarina oraz nowy związek oznaczony jako artabolid [3-hydroksy-4,6,7(H)-germakra-1(10),11(13)-dien-6,12-olyd] z orientalnych leczniczych roślin Asteraceae jako nowe, naturalnie występujące inhibitory polarnego transportu auksyny, co wykazano za pomocą hipokotylowego testu rzodkiewki. Aby zbadać sposób działania naturalnych laktonów seskwiterpenowych w hamowaniu polarnego transportu auksyny, a także ich związek ze wzrostem siewek, intensywnie badano wpływ DHCL na wzrost i dynamikę auksyny w etiolowanych siewkach grochu. DHCL zmniejszył polarny transport auksyny w sposób zależny od dawki, łącznie z hamowaniem akumulacji mRNA genów *PsAUX1* i *PsPIN1*, kodujących nośniki białkowe regulujące odpowiednio dopływ i wypływ auksyn. DHCL naniesiony na szczytowy obszar haczykowy w postaci pasty lanolinowej istotnie hamował wzrost elongacyjny w strefie podwierzchołokowej epikotyli w nienaruszonych etiolowanych siewkach grochu w połączeniu ze znaczną redukcją endogennego poziomu kwasu indolilo-3-octowego (IAA). DHCL powodował również hamowanie wydłużania komórek indukowane przez IAA w etiolowanych segmentach epikotylu grochu poprzez wpływ na indukowane przez IAA zmiany właściwości mechanicznych ścian komórkowych. Fakty te sugerują, że laktony seskwiterpenowe typu germakranolidu z ugrupowaniem α-metyleno-γ-laktonu wpływają na ekspresję genów *PsAUX1* i *PsPINs*, a następnie hamują polarny transport auksyny i zmniejszają endogenny poziom IAA niezbędny dla wzrostu łodygi etiolowanych siewek grochu. Sugeruje się również, że laktony seskwiterpenowe wykazują hamujący wpływ na działanie auksyny podczas wzrostu łodyg grochu.