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A gravitropic stimulation-induced growth inhibitor, β-(isoxazolin-5-on-2yl)-alanine, is a possible mediator of negative gravitropic bending of epicotyls in etiolated *Pisum sativum* seedlings

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Abstract Negative gravitropic bending and its possible mediator in etiolated Alaska pea seedlings were intensively studied in comparison with seedlings of an agravitropic mutant, ageotropum. When 3.5-day-old etiolated Alaska seedlings were horizontally placed, the growth suppression at the upper side of the epicotyls began 10 min after the onset of the gravitropic stimulation, whereas the growth acceleration at the lower side began at 30 min, resulting in negative gravitropic bending. In contrast, no gravitropic bending was observed in the etiolated *ageotropum* seedlings, for which the epicotyls show an automorphogenesis-like growth. Strenuous efforts to identify a possible mediator that induces the gravitropic bending resulted in successfully identifying β-(isoxazolin-5-on-2yl)-alanine (βIA). The unilateral application of βIA to the etiolated Alaska epicotyls substantially induced epicotyl bending toward the application site, indicating that βIA could act as a growth inhibitor. Analyses of the distribution of βIA in the upper and lower flanks of the etiolated Alaska epicotyls revealed that its content rapidly increased twice in the upper flanks compared with that in the lower ones in response to gravitropic stimulation, whereas its content in the lower flanks was almost equal to that in the vertical control. In etiolated *ageotropum* epicotyls, an almost equal amount of βIA was distributed in the upper and lower flanks of epicotyls. These results suggest that a gravitropic stimulation increases βIA in the upper flank, resulting in the negative gravitropic bending of epicotyls via the suppression of the growth rate at the upper side of epicotyls in the etiolated Alaska pea seedlings.

Keywords *Ageotropum* pea · Alaska pea · Bruinsma—Hasegawa theory · Gravitropism · Growth-inhibiting substance · β-(isoxazolin-5-on-2yl)-alanine

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Introduction

Gravitropism, the developing curvature of a growing organ in response to some asymmetric external gravity, is easily observed, and thus, is open to obvious experiments. Not surprisingly, gravitropism has been a subject of investigation since the earliest times of plant physiology, as has as phototropism. The mechanism of phototropism can be explained in terms of perception of a stimulus vector, signal transduction, and the lateral distribution of a plant growth substance. This growth-promoting substance distributed somehow asymmetrically, and asymmetric growth causing a curvature in the elongating tissue was observed. The study of phototropism led to the discovery of auxin. In gravitropism of coleoptiles that were horizontally placed, it has been generally believed that the negative (upward) gravitropic bending is caused by gravity perception in the tip, thus leading to auxin transport to the lower side, followed by basipetal auxin translocation to the elongation

zone below the tip where the differential flank growth results in upward growth (Cholodny–Went theory; see Went and Thimann 1937).

However, the growth-regulating mechanism underlying the negative gravitropic bending in dicotyledonous shoots remains unclear. If the Cholodny–Went theory is correct, gravity may cause negative gravitropic bending in a horizontal shoot by increasing the rate of cell elongation at the lower side of the shoot, accompanied by a decreasing the rate of elongation of cells at its upper side (Firn et al. 2000). A redistribution of growth such that the lower side starts elongating while the upper side simultaneously suppressed following applied gravitropic stimulation has been observed in both etiolated sunflower hypocotyls (Firn et al. 1978) and etiolated cucumber hypocotyls (Cosgrove 1990), thus lending support to the main prediction of the Cholodny–Went theory. Digby and Firn (1979) also reported that the suppression of growth on the upper side of etiolated maize coleoptiles, de-etiolated sunflower hypocotyls, and etiolated cucumber hypocotyls is a major factor bringing about gravitropism. Considering these facts alongside the results of phototropism, Bruinsma and Hasegawa (1990) proposed a new theory of phototropism, it being the consequence of the regulation by a light-induced gradient of auxin-inhibiting substances (Bruinsma–Hasegawa theory). Tokiwa et al. (2006) also demonstrated that a major factor in gravitropism in radish hypocotyls is the suppression of growth at the upper side of the hypocotyls, and the possible involvement of 4-methylthio-3-butenyl isothiocyanate. The apparent discrepancies among these theories suggest the necessity to obtain a clearer picture of the changes in growth rate across a range of species before attempting to set up a detailed general model of how the process of gravitropism is controlled in plants.

In the present study, we report on detailed analyzes of the lengths of the upper and lower sides of etiolated Alaska and an agravitropic mutant, *ageotropum*, pea epicotyls just prior to and following their gravitropic stimulation (studied with an infrared-imaging system). In addition, the successful identification of β-(isoxazolin-5-on-2yl)-alanine (βIA, Fig. 1) as a possible mediator of gravitropism increasing quantitatively in response to the gravitropic stimulation in etiolated Alaska seedlings is also described. Possible mechanisms of gravitropism as mediated by the compound βIA are also briefly discussed.

Materials and methods

Plant materials

Two kinds of pea plants (*Pisum sativum* L.), cv. Alaska and an agravitropic mutant, *ageotropum*, were used in our

Fig. 1 β-(Isoxazolin-5-on-2yl)-alanine, a gravitropism-regulating substance, in the etiolated pea epicotyls
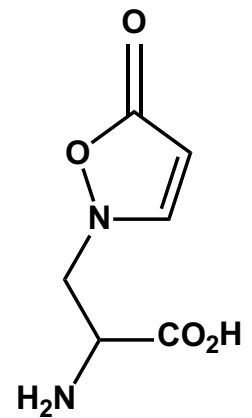
study. The Alaska seeds were purchased from Watanabe Seeds Co., Ltd. (Miyagi, Japan), and the *ageotropum* seeds were propagated in the experimental field of the laboratory at Osaka Prefecture University (these seeds were kindly supplied by Prof. Hideyuki Takahashi, Tohoku University, Japan). The a*geotropum* mutant, an X-ray radiation-induced mutant of the pea cv. Weibull's Witor (Blixt et al. 1958), is known to display agravitropic behavior in both its shoots and roots (Schöldéen and Burström 1960; Olsen and Iversen 1980a, b; Takahashi and Suge 1991; Takahashi et al. 1991), and to show growth that is similar to the automorphogenesis observed in space and under simulated weightlessness conditions in a 3-dimensional clinostat (Ueda et al. 1999, 2014; Miyamoto et al. 2011, 2014).

Plant culture

The pea seeds were imbibed at 23 °C for 8 h in running tap water, then sown in moistened vermiculite in a plastic tray, where they were allowed to germinate and grow at 23 °C for 3.5 days in the dark. To measure the growth rates at the upper and the lower sides, and of the right and the left sides of the epicotyl in seedlings kept horizontally and vertically, respectively, ion exchange resin beads (Amberlite XAD-2) with lanolin were positioned on the proximal and distal sides to the cotyledons ca. 2 and 12 mm below the hook of 12 etiolated seedlings. These seedlings were kept in a vertical position for 30 min to determine the growth rate prior to the gravitropic stimulation. They were then placed horizontally for 100 min for gravitropic stimulation, or left vertically as the control.

The epicotyl bending and the distance between the beads at the both sides were measured at 5-min intervals using an infrared (IR)-imaging system. This system consisted of two separated stations: one is a recording station that used an IR-sensitive MiniDV Handycam (DCR-TRV9 NTSC, Sony, Japan), and the other is a monitoring station that included a digital video application (DVBK-W2000, Sony, Japan)

and Photoshop 5.5 software (Adobe Systems Incorp., USA). Images of the seedlings were recorded throughout the experiment which was monitored in real time. These manipulations were performed under dim green light $(0.03 \ \mu mol \ m^{-2} \ s^{-1})$. The experiments were repeated at least three times.

Isolation and identification of a possible mediator of gravitropism

Elongating epicotyl regions [95 g fresh weight (FW)] of the 3.5-day-old etiolated Alaska seedlings placed vertically were harvested and immediately frozen in liquid N_2, and then stored at $-40 \ °C$ until extraction. The plant materials were homogenized in a blender and extracted with 1 L of 80% cold aqueous acetone. After filtration, the residues were re-extracted with 100 mL cold acetone. All the extracts were combined and concentrated to dryness at $40 \ °C$ in vacuo. The residue (4.72 g) was dissolved in MeOH and applied to a C_{18} Sep-Pak cartridge column (Waters, USA), and eluted with 0, 10, 20, 30, 50, and 100% MeOH in water (30 mL/step). The 0% MeOH fraction, which contained the peak responsible for the gravitropic stimulation (see Supplementary Fig. 2), was evaporated to dryness at $40 \ °C$ in vacuo. The residue (2.78 g) was dissolved in MeOH and applied to a NH_2 Sep-Pak cartridge column (Waters, USA), and eluted with 80, 70, 60, 50, and 0% MeOH in water (25 mL/step). The 60% MeOH fraction was evaporated to dryness at $40 \ °C$ in vacuo, and then finally purified by a reversed-phase HPLC [column: ODS-120A, 7.8×300 mm, Tosoh, Japan; elution: linear gradient from 0 to 100% CH_3CN in water for 20 min; flow rate: 2 mL/min, detector: PD-8020 multi-wavelength detector (Tosoh, Japan) absorbance at 195, 205, and 280 nm]. Eluate of the peak (retention time 5.5–7.5 min) corresponding to the quantitative increase in the epicotyls in response to the gravitropic stimulation in Alaska were obtained, and evaporated to dryness at $40 \ °C$ in vacuo, which yielded 2 mg of substance.

The ^{1}H-NMR spectra of the compound were measured on a Bruker AVANCE-500 spectrometer (500 MHz) in $D_2O + AcOD$. The 2D NMR $^1H-^1H$ correlation spectroscopy, heteronuclear multiple-bond correlation experiment, and NOE correlation spectroscopy analyzes were carried out to identify the compound's chemical structure. The ESI-MS was recorded on a Waters Synapt G2 mass spectrometer.

Quantification of β-(isoxazolin-5-on-2yl)-alanine

Etiolated Alaska and *ageotropum* seedlings grown for 3.5 days were divided into two groups: one was incubated vertically as the control, while the other group was subjected

to gravitropic stimulation for 60 min. To survey the growth regulator(s) showing quantitative changes in the epicotyls of Alaska seedlings, but not in *ageotropum* ones, in response to the gravitropic stimulation, 10 mm of the elongating region of the epicotyls ca. 2 mm below the hook was harvested, immediately frozen in liquid N_2, and then stored at $-40 \ °C$ until use.

For the analysis of the endogenous levels of the growth inhibitor, βIA, a 10-mm epicotyl region 2 mm below the hook of the Alaska seedlings positioned vertically and horizontally was harvested; the epicotyl segments were divided into halves (i.e., upper and lower halves, with the longitudinal halves as the control, ca. 20 mg FW) with a razor blade and immediately frozen in liquid N_2, and then stored at $-40 \ °C$ until use.

The frozen samples were homogenized in 10 mL of 80% cold aqueous acetone using a mortar and pestle. The filtrated extracts were concentrated to dryness at $40 \ °C$ in vacuo. The samples were dissolved in MeOH and applied to a reversed-phase HPLC, as mentioned previously. To quantify βIA, the area under the peak was measured. This experiment was repeated five times.

Unilateral application of β-(isoxazolin-5-on-2yl)-alanine

Ten 3.5-day-old etiolated Alaska seedlings were treated unilaterally with the growth-regulating substance, βIA, administered as a lanolin paste. The treated seedlings were placed vertically in the dark at $23 \ °C$. The extent of epicotyl bending in the direction of the site of unilateral application at 20, 40, and 60 min after the application was determined. All manipulations were carried out under dim green light as mentioned above.
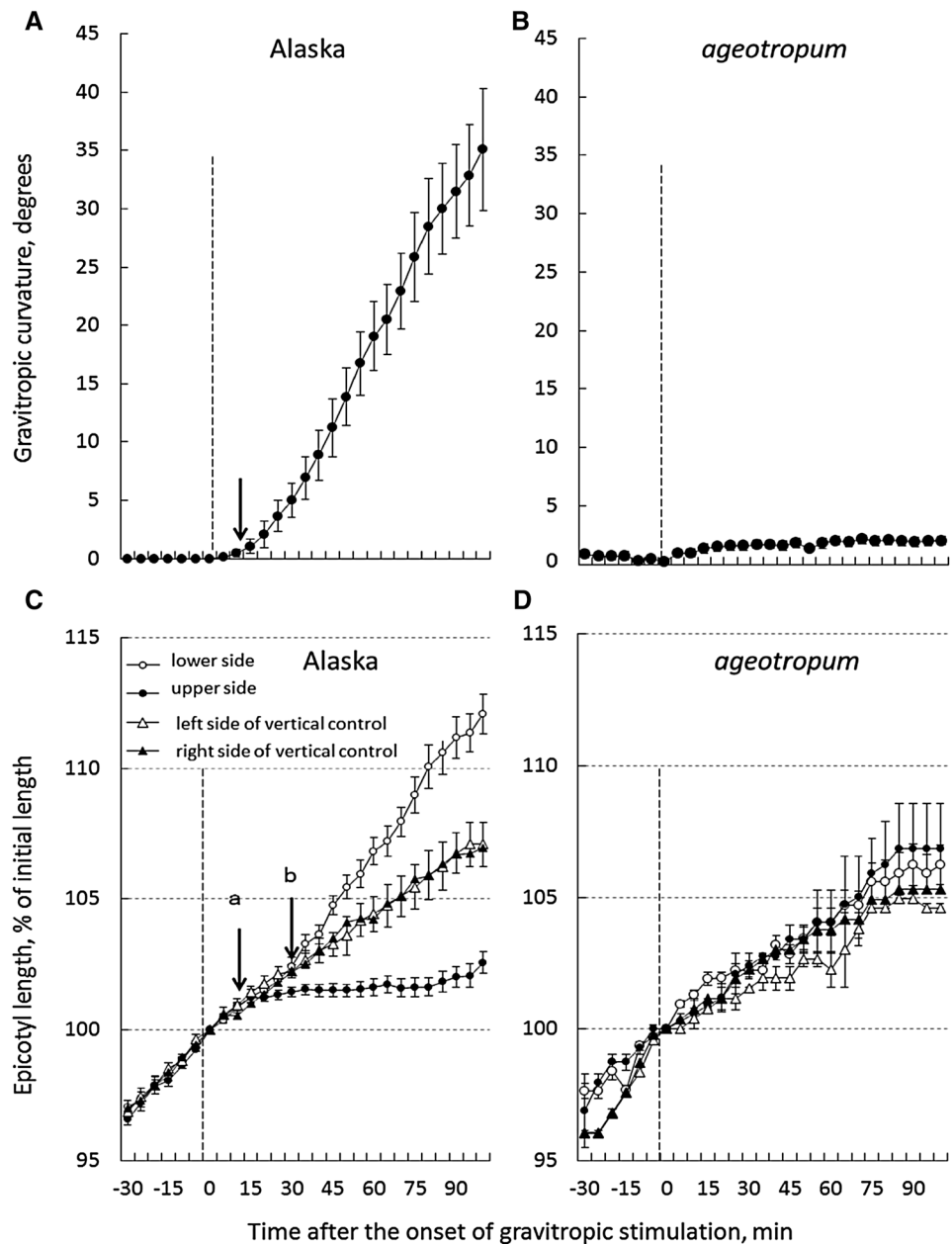
Results

Kinetics of negative gravitropic bending in the etiolated Alaska and ageotropum pea epicotyls

The negative gravitropic bending and the growth rates at both the upper and the lower sides in the horizontally placed epicotyls of the etiolated Alaska and *ageotropum* pea seedlings were measured prior to, and following, the gravitropic stimulation.

As shown in Fig. 2 and Supplementary Fig. 1, the epicotyls of the etiolated Alaska seedlings showed typical negative gravitropic bending when they were horizontally placed. The negative gravitropic bending in the etiolated Alaska epicotyls steadily increased from 10 min after the onset of gravitropic stimulation (as indicated by the arrow in Fig. 2a). The growth suppression at the upper side in the etiolated Alaska epicotyls began at least

10 min after the onset of gravitropic stimulation (as indi-
cated by arrow a in Fig. 2c) and it continued during the
period of gravitropic stimulation. Growth acceleration at
the lower side was not observed within 30 min after the
onset of gravitropic stimulation (as indicated by arrow b
in Fig. 2c). These results indicated that the growth sup-
pression at the upper side is important for generating
the negative gravitropic bending in the etiolated Alaska
epicotyls.

In contrast, the *ageotropum* epicotyls did not show any
negative gravitropic bending, nor did they exhibit changes
in growth rate at the upper and the lower sides in the hori-
zontally positioned seedlings (Fig. 2b, d, Supplementary

Fig. 1). The growth rate of the *ageotropum* epicotyls posi-
tioned vertically was similar to that of the Alaska ones.

Survey of possible compound(s) involved in the negative gravitropic bending in pea epicotyls

The results shown in Fig. 2 suggest the possible involve-
ment of a plant growth regulator(s) which show quan-
titative changes in the upper flank of etiolated Alaska
epicotyls, but not in the lower one, in response to the
gravitropic stimulation. We also expected little quantita-
tive differences in the plant growth regulator(s) between
the *ageotropum* epicotyls positioned vertically and

horizontally. To investigate this, we compared the HPLC chromatograms of the extracts from the horizontal- and vertical-positioned pea epicotyls of 3.5-day-old etiolated Alaska and *ageotropum* seedlings at 60 min after the start of the treatment. The peak A was higher in the horizontal-positioned Alaska epicotyls than in the vertical-positioned ones (Supplementary Fig. 2A, B), whereas the peak went unchanged in the *ageotropum* epicotyls (Supplementary Fig. 2C, D). This result suggested that the compound corresponding to the peak A may be considered a candidate for regulating the gravitropic response observed in the etiolated pea epicotyls.

Strenuous efforts to find the growth regulator showing a quantitative increase in the epicotyls in response to the gravitropic stimulation were successful. We were able to isolate the peak A compound from the elongating epicotyl region of the 3.5-day-old etiolated Alaska seedlings based on its retention time on HPLC. This compound was identified as βIA (Fig. 1) based on the spectra of the ^{1}H NMR and 2D NMR analyzes, in complete agreement with the data in the reported literature (Lambein et al. 1992).

Inhibitory effect of β-(isoxazolin-5-on-2yl)-alanine on growth in the pea epicotyls

To evaluate the possible role in negative gravitropic bending played by βIA, its biological activity was examined. The unilateral application of βIA caused substantial epicotyl bending toward the direction of the application site in vertically placed etiolated Alaska epicotyls (Table 1), thus indicating that it can function as a growth inhibitor.

Quantitative changes in β-(isoxazolin-5-on-2yl)-alanine in the pea epicotyls in response to gravitropic stimulation

The content of βIA in the elongating region of the etiolated Alaska epicotyls increased almost twice when subjected to gravitropic stimulation for 60 min compared with the vertically placed control (Table 2). On the other hand, the content in etiolated *ageotropum* epicotyls was not affected by gravitropic stimulation. Since these suggest that βIA is responsible for gravitropic stimulation, detail changes in epicotyls were analyzed.

The content of βIA was increased almost twice in the upper flank, but not in the lower flank of the epicotyls within 30 min after the onset of gravitropic stimulation (Table 3). The endogenous level of the compound in the lower flank was almost equivalent with that in the right and the left haves of epicotyl in etiolated Alaska seedlings positioned vertically. The local gradient of the compound was continued until at least 60 min after the onset of gravitropic stimulation.

Discussion

Gravitropic bending of the epicotyl in pea seedlings

Curvatures due to growth are irreversible, resulting in a tropism. The growth-regulating mechanism underlying the negative gravitropic bending in dicotyledonous shoots has not been settled yet. As described in the "Introduction" section above, if the Cholodny–Went theory relating to auxin redistribution is correct, gravity should induce a negative gravitropic curvature in a horizontal shoot by a deceleration of elongation at the upper side in tandem with an acceleration at the lower side (Firn et al. 2000). The negative gravitropic bending of the epicotyl in the etiolated Alaska

Table 1 Time course of the epicotyl bending in the etiolated Alaska pea seedlings after unilateral application of the growth inhibitor, β-(isoxazolin-5-on-2yl)-alanine

β-(isoxazolin-5-on-2yl)-alanine	Epicotyl bending toward the direction of unilateral application, degree		
	20 min aai*	40 min aai	60 min aai
10 µg	0.5 ± 0.1	3.5 ± 0.1	8.5 ± 0.2
30 µg	2.0 ± 0.1	4.5 ± 0.1	8.5 ± 0.2
100 µg	3.0 ± 0.1	5.0 ± 0.1	8.7 ± 0.2

The treated seedlings were placed vertically. The changes in epicotyl bending were determined. Each value represents the mean ± SE (n = 10). This experiment was repeated three times and the results among them were similar

*"aai" indicates after the application of β-(isoxazolin-5-on-2yl)-alanine

Table 2 Effects of gravitropic stimulation on the endogenous levels of β-(isoxazolin-5-on-2yl)-alanine (βIA) in the etiolated epicotyls of Alaska and *ageotropum* pea seedlings

	Contents of β-(isoxazolin-5-on-2yl)-alanine, µM/gFW	
	Vertical position	Horizontal position
Alaska	7.00 ± 0.35	13.95 ± 0.80
ageotropum	4.65 ± 0.33	4.77 ± 0.35

Etiolated Alaska and *ageotropum* seedlings grown for 3.5 days were divided into two groups: one was incubated vertically as the control, while the other group was subjected to gravitropic stimulation by positioning horizontally for 60 min. A 10-mm elongating region of the epicotyls ca. 2 mm below the hook was harvested and used to determine the endogenous levels of βIA

Table 3 Kinetics of change in the endogenous levels of β-(isoxazolin-5-on-2yl)-alanine (βIA) in the etiolated Alaska epicotyls up to 60 min after the onset of gravitropic stimulation

	Contents of β-(isoxazolin-5-on-2yl)-alanine, μM/gFW		
	0 min after OGS*	30 min after OGS	60 min after OGS
Right halves*	5.81 ± 0.40	–	–
Left halves*	6.10 ± 0.45	–	–
Upper halves	–	10.64 ± 1.04	11.63 ± 1.00
Lower halves	–	5.87 ± 0.48	6.80 ± 0.55

For the analysis of the endogenous levels of βIA, a 10-mm of the epicotyl region 2 mm below the hook of the Alaska seedlings positioned horizontally for 0, 30, and 60 min was harvested, and the epicotyl segments were divided into halves [upper and lower, with the longitudinal halves serving as the control, ca. 20 mg fresh weight (FW)]. Then, the determination of endogenous levels of βIA was carried out

*Right and left halves indicate the longitudinal ones of the epicotyls that were positioned vertically prior to the gravitropic stimulation

**OGS the onset of gravitropic stimulation by placing the seedlings horizontally

seedlings began 10 min after the onset of gravitropic stimulation, which then increased the negative gravitropic curvature (Fig. 2). The growth suppression at the upper side in the etiolated Alaska epicotyls started at least 10 min after the onset of gravitropic stimulation and was maintained for 100 min, whereas the growth acceleration at the lower side only began 30 min after the onset of the gravitropic stimulation (Fig. 2).

Altogether, these results suggest that the growth suppression at the upper side is first induced by gravity, while the growth acceleration at the lower side is the result rather than a cause of the negative gravitropic bending in the gravitropically stimulated etiolated pea epicotyls. It is therefore possible that a redistribution of auxin played a role in the maintenance of gravitropic upward curvature, but not as an inducer of the negative gravitropic response, even if an asymmetric redistribution of auxin occurred in response to the applied gravitropic stimulation.

The pattern of growth in the etiolated Alaska epicotyls was almost similar to that reported for radish hypocotyls during gravitropic stimulation by Tokiwa et al. (2006). The growth pattern in response to the unilateral gravity vector in both etiolated Alaska epicotyls and radish hypocotyls is also similar to that obtained from the phototropic studies of many plant species in which the measure of phototropic bending depended on the magnitude of growth suppression at the illuminated side and an unchanged growth rate at the shaded side (Franssen et al. 1981; Hasegawa et al. 1987, 2004a, b; Hasegawa and Yamada 1992; Yokotani-Tomita et al. 1999). From the abundant physiological and chemical studies of phototropism, the postulated hypothesis

is that phototropism is caused by a local gradient of light-induced growth inhibitor(s) at the illuminated side set against a background of an unchanged, even distribution of auxin (so-called Bruinsma–Hasegawa theory, see the Introduction, Bruinsma and Hasegawa 1990; Yamamura and Hasegawa 2001). A lot of papers supporting this mechanistic view have already been published. For example, 8-Epixanthatin (Yokotani-Tomita et al. 1999) and 8-(β-D-glucopyranosyloxy)-3-hydroxy-1,9,14-pentadecatriene-4,6-diyne designated as helian (Hasegawa et al. 2007) from sunflower hypocotyls; benzoxazinoids, such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DMBOA) and 6-methoxybenzoxazolinone from maize coleoptiles (Jabeen et al. 2007); raphanusanin and its precursor 4-methylthio-3-butenyl isothiocyanate and 4-methylthio-3-butenyl glucosinolate from radish hypocotyls (Hasegawa et al. 2000); indolyleacetnitrile from cabbage and *Arabidopsis* shoots (Kosemura et al. 1997; Hasegawa et al. 2004a); and uridine from *Avena* coleoptiles (Tamimi 2004) have all been demonstrated as important in phototropism by functioning as a light-induced growth inhibitor. Therefore, it is not unreasonable to adapt the Bruinsma–Hasegawa theory to gravitropism in the etiolated pea epicotyls of the present study.

β-(Isoxazolin-5-on-2yl)-alanine acts as a negative gravitropic-bending regulating substance

As described above, a gravitropic stimulation-induced growth inhibitor is possibly involved in gravitropism. Much effort led to the successful identification of βIA in our study. βIA from pea seedlings has been described as the first member of a unique group of heterocyclic amino acids that are highly labile toward UV light and alkalinity (Lambein et al. 1969, 1976), and it is derived from O-acetyl-L-serine and isoxazolin via cysteine synthase in higher plants (Ikegami et al. 1991). The compound has also been found to be an inhibitory allelopathic substance in the roots and root exudates of pea seedlings while also showing antimycotic activity (Schenk and Werner 1991), and it is the metabolic precursor for the neurotoxin β-N-oxalyl-L-α,β-diaminopropionic acid (ODPA) (Lambein et al. 1990).

Unilateral application of βIA to the etiolated Alaska epicotyls that were positioned vertically caused their epicotyls to bend toward the site of application (Table 1), indicating it acts as an inhibitor. The analysis of the endogenous distribution of the compound between the upper and lower flanks of the epicotyl revealed that its content rapidly increased twice in the upper flank of etiolated Alaska epicotyls than that in the lower one after the onset of gravitropic stimulation, whereas in the etiolated *ageotropum* epicotyls there was no evident increase of the inhibitor in response to the gravitropic stimulation like for the agravitropic response (Tables 2, 3). This

suggests that the gravitropic stimulation suppressed the growth rate at the upper side of pea epicotyls by increasing the βIA content, resulting in the negative gravitropic bending. A role in gravitropism has been demonstrated for jasmonates in rice coleoptiles (Gutjahr et al. 2005) and likewise for 4-methylthio-3-butenyl isothiocyanate and 3,6-disinapoylsucrose in etiolated radish hypocotyls (Tokiwa et al. 2006; Hasegawa et al. 2010). When these are taken together with our present results, an important role of a growth-inhibiting substance(s) for gravitropism in shoots is strongly suggested.

Edelmann (2001) has proposed a model for the principal mechanism regulating gravitropism, in which the gravitropic stimulation temporarily inhibits the infiltration of auxin-induced secreted wall-loosening factors into the outer cell wall of the epidermal cells, resulting in the inhibition of growth at the upper organ flanks in rye coleoptiles. Clark et al. (2000) have reported that close relationships between the gravity vector and the distribution of annexins, a known family of Ca^{2+}-dependent membrane proteins that participate in a wide array of essential cellular processes including secretion, transport, and DNA replication. Hence, it is possible that gravitropic stimulation results in a redistribution of the annexin-mediated secretion or transport, thus modifying the redistribution of growth-regulating substance(s) or factor(s) in gravi-responsive-elongating cells.

In conclusion, our detailed analysis of growth rates at the upper vs. lower sides in the gravitropic-stimulated etiolated epicotyls of Alaska seedlings revealed an important role for growth suppression at the upper side in negative gravitropic bending. Great efforts to find this growth regulator were rewarded by the identification of βIA as a functional growth inhibitor that is synthesized at the gravitropically-stimulated upper flank of the etiolated pea epicotyls. Further analyses of an agravitropic pea mutant, *ageotropum*, supported the possible involvement of growth-inhibiting substances in gravitropism.

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