Circumnutation in adzuki bean epicotyls: maintenance by asymmetric distribution of hormones and induction by light

アズキ上胚軸の回旋運動：
ホルモン不等分布による維持と光による誘導

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Abbreviations

ABA: abscisic acid

A. thaliana: *Arabidopsis thaliana*

GA_1: gibberellin A_1

GA_3: gibberellin A_3

IAA: indole-3-acetic acid

JA: jasmonic acid

LC–MS: liquid chromatography–mass spectrometry

SA: salicylic acid

TIBA: 2,3,5-triiodebenzoic acid

Abstract

Circumnutation is a unique plant growth movement in which the tips of axial organs draw a circular or elliptic orbit. Although it has been studied since the 19th century, its mechanism and significance are still unclear. Auxin has been supposed to play important roles in circumnutation, but it has not yet been uncovered where auxin is and what auxin does in organs during circumnutaion. On the other hand, although there are several reports demonstrating that light regulates circumnutation, no one examined the whole pathway from the perception of light to the induction or termination of circumnutation.

First, I investigated the mechanism how circumnutation is maintained. Greened adzuki bean (*Vigna angularis*) epicotyls exhibited a clockwise circumnutation in the top view with a constant period of 60 min under continuous white light. The bending zone of circumnutation on the epicotyls was always located in the region 1–3 cm below the tip, and its basal end was almost identical to the apical end of the region where the epicotyl had completed elongation. Therefore, epidermal cells that construct the bending zone are constantly turning over with their elongation growth. Since exogenously applied auxin transport inhibitors and indole-3-acetic acid (IAA) impaired circumnutation without any effect on the elongation rate of epicotyls, I attempted to reveal the distribution pattern of endogenous auxin. Taking advantage of its large size, I separated the bending zone of epicotyls into two halves along the longitudinal axis, either convex/concave pairs in the plane of curvature of circumnutation or pre-convex/pre-concave pairs perpendicular to the plane. By liquid chromatography–mass spectrometry, I found, for the first time, that IAA and gibberellin A_1 were asymmetrically distributed in the pre-convex part in the region 1–2 cm below the tip. This region of epicotyl sections exhibited the highest responsiveness to

exogenously applied IAA and gibbrerellin, and the latent period between the hormone application and the detection of a significant enhancement in elongation was 15 min. These results suggest that circumnutation in adzuki bean epicotyls with a 60 min period is maintained by differential growth in the bending zone, which reflects the hormonal status 15 min before, and which is shifting sequentially in a circumferential direction.

Next, I investigated the mechanism how circumnutation starts. Although etiolated epicotyls under infrared light did not exhibit any sign of circumnutation, after exposure to continuous red or blue light, they started circumnutation with sequential changes in the mode of movement. First, they opened the apical hook. Second, they exhibited oscillatory movements that are not organized well. Third, they started circumnutation from 39 h of the illumination. The periods of circumnutation under red and blue light were similar and approximately 60 minutes, whereas the amplitudes of circumnutation under red light were significantly smaller than that under blue light. I also examined the elongation pattern of etiolated epicotyls under different light conditions. Upon exposure to red or blue light, etiolated epicotyls changed their elongation pattern prior to the onset of circumnutation to complete the elongation earlier than etiolated epicotyls under infrared light. The bending zone of circumnutation was always located in the region 2–4 cm below the tip, and its basal end was located above the region where the epicotyl had completed elongation. Finally, I investigated what happened in etiolated epicotyls before the onset of circumnutation. The epicotyls exposed to red light for 36 h exhibited a reduced gravitropism, preceding the onset of circumnutation. The size and mobility of amyloplast in the endodermis were lower than those of the epicotyls kept in darkness. Since the shoot of plants elongates vertically by negative gravitropism, the repression of gravitropism by light may tilt the de-etiolating adzuki bean epicotyls and provide the cue to start circumnutation.

Introduction

Axial organs of elongating plants, such as hypocotyls, epicotyls, coleoptiles, tendrils, and roots, exhibit a revolving movement and their tips draw a circular or elliptic orbit. This growth movement was first described in the 19th century, and Charles Darwin (1880) termed it circumnutation. It has long attracted many plant researchers, who have taken multiple approaches in their studies using various plant species including oat, rice, pea, French bean, *Arabidopsis thaliana*, and sunflower (Stolarz 2009). Since Israelsson and Johnsson (1967) proposed that gravity induces oscillatory movements in plant organs, the relation between gravity and circumnutation has been extensively investigated using clinostat or space flight experiments (Chapman and Brown 1979; Brown and Chapman 1984; Brown 1993). It was recently verified genetically that gravity sensing is essential for circumnutation. Inflorescence stems of the *scr* mutant of *A. thaliana*, which cannot sense gravity due to the defect of stem endodermal cells containing amyloplasts (Fukaki et al. 1998), did not exhibit circumnutation (Kitazawa et al. 2005). This abnormal phenotype was restored by introducing the morning glory *SCR* into the mutant concomitantly with the recovery of endodermis tissue. However, the mechanism and significance of circumnutation are not yet completely understood.

Not only gravity but also light, which is another important environmental signal for plants, affects circumnutation. Yoshihara and Iino (2005) reported that etiolated rice coleoptiles under infrared light exhibited circumnutation, whereas they ceased it when exposed to red light. The coleoptiles grown under white or red light did not exhibit circumnutation. phytochrome A, which is one of the three isoforms in rice, is involved in these responses. In *A. thaliana*, 10% of etiolated hypocotyls exhibited

circumnutation under infrared light and no hypocotyls exhibited circumnutation after pre-illumination with red light (Orbovic and Poff 1997). In greened *A. thaliana* hypocotyls, however, almost all seedlings exhibited circumnutation under white light and the period lengths increased under green or red light (Schuster and Engelmann 1997). Someya et al. (2006) demonstrated that inflorecence stems of *A. thaliana* also exhibited circumnutation under white light, and when transferred into darkness, the amplitude increased and the period became longer. In the case of pea, etiolated epicotyls did not exhibit nutation, while they started it when exposed to red light (Galston et al. 1964, Britz and Galston 1982a). Accordingly, the mode of regulation of circumnutation by light is quite complex and seemingly heterogeneous in different plant species and in different organs even of the same species. Photoreceptors involved in the regulation of circumnutation are not identified except in rice as described.

Auxin, one of the major phytohormones involved in the regulation of plant growth and development, is known to promote cell elongation through two pathways (Badescu and Napier 2006; Velasquez et al. 2016). The faster pathway is by activation of the plasma membrane H^+-ATPase, which leads to acid growth (Hager 2003; Takahashi et al. 2012), and the slower one is through the regulation of gene expressions (Chapman and Estelle 2009). Auxin is mainly produced in the shoot apex and young leaves (Ljung et al. 2002) from where it flows down to the basal regions via the phloem and/or by cell-to-cell transport mediated by carrier proteins (Michniewicz et al. 2007). In both gravitropism and phototropism, after seedlings sense gravity or light stimulus, auxin is asymmetrically distributed in the organs, leading to the differential growth of epidermal cells in the opposing sides (Morita 2010; Liscum et al. 2014). Auxin is also supposed to play important roles in circumnutation. Britz and Galston (1982a) reported that α-naphtylphthalamic acid (NPA), an established inhibitor for polar auxin transport

(Murphy et al. 2002), inhibited the nutation of pea epicotyls. NPA also blocked circumnutation of morning glory shoots (Hatakeda et al. 2003) and of pea roots (Kim HJ et al. 2016). Britz and Galston (1982b) further demonstrated that de-capped pea epicotyls did not exhibit nutation, but resumed nutation after the exogenous application of indole-3-acetic acid (IAA), an endogenous auxin. Finally, inflorescence stems of the auxin-resistant *axr-2* mutant of *A. thaliana* exhibited little circumnutation (Hatakeda et al. 2003). However, the distribution pattern of auxin as well as its mode of action associated with circumnutation has not yet been clarified.

In dissecting the regulation mechanism of circumnutation by light and hormones, plant organs with large size may enable me to analyze the dynamic movements with higher spatial resolution. Furthermore, it may also be effective to focus on the time point when the organ switches the mode of its movement from static to dynamic, or from dynamic to static. From such a view point, in this study, I determined to use adzuki bean (*Vigna angularis*) epicotyls because of the large size and high growth rate. The epicotyl sections exhibit high responsiveness to exogenously applied phytohormones (Shibaoka 1994). Moreover, previous research in our laboratory showed that greened adzuki bean epicotyls exhibit circumnutation (Takano master's thesis 2002; Wakamatsu master's thesis 2003). Adzuki bean epicotyls grown in darkness exhibit etiolated phenotypes, with the apical hook and small pale leaves (Kendrick and Kronenberg 1994). When exposed to light, the epicotyls terminate etiolation and start photomorphogenic development. Since greened adzuki bean epicotyls exhibit circumnutation, I expected to specify the time point of the induction of circumnutation by analyses of de-etiolating epicotyls.

In chapter I, I investigated how circumnutation is maintained in greened adzuki bean epicotyls. By combining careful handling of intact plants and liquid

chromatography–mass spectrometry (LC–MS), I succeeded in quantifying endogenous hormones in greened adzuki bean epicotyls exhibiting circumnutation. I found, for the first time, that IAA and gibberellin A_1 (GA_1) were asymmetrically distributed in the bending zone of circumnutation. The epicotyl region where the asymmetric distributions of hormones were detected exhibited the highest responsiveness to these hormones in terms of elongation enhancement. These results suggest that the differential growth of epidermal cells induced by the asymmetrically distributed hormones drives circumnutation.

In chapter II, I investigated how circumnutation starts in de-etiolating adzuki bean epicotyls. By long-lasting observation, I found that circumnutation is induced by red or blue light, and that the gravitropic response is reduced preceding the onset of circumnutation. Thus, I propose that the reduction of gravitropism by light provides the cue to start circumnutation for adzuki bean epicotyls.

Chapter I: Maintenance of circumnutation by asymmetric hormone distribution

Materials and Methods

Plant materials

Adzuki bean (*V. angularis* cv. Erimowase) seeds stored at 4°C were transferred to a growth chamber (LH-60FL12-DT; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) for 1 h to adapt to 27 ± 1°C. Thereafter, the seeds were sown in 200 ml pots filled with vermiculite and warm water. The pots were covered with plastic wraps to maintain high humidity and exposed to continuous white light (30 μmol m^{-2} s^{-1}, FL10D; NEC Co., Tokyo, Japan) from above. Three days later, the plastic wraps were removed so as not to interfere with epicotyl growth.

Recording and analysis of epicotyl circumnutation and elongation

Vinyl tape 1 mm in width was carefully pasted on each 5-day-old greened epicotyl every 3 mm from the tip (Fig. 1a). Thereafter, epicotyls were put in a clear, colorless acrylic box maintained at 27°C with an electric panel heater (MP-916; TRiO Co., Osaka, Japan) under continuous white light (30 μmol m^{-2} s^{-1}). Epicotyls were photographed every 10 min in the plane parallel (x view) or perpendicular (y view) to the first leaves (Figs. 1a, c), by programmable cameras (Ltl-5210B; Little Acorn Outdoors, Green Bay, WI, USA). Circumnutation and epicotyl elongation were analyzed from sequentially recorded images using the Tracker Video Analysis and Modeling Tool (http://www.cabrillo.edu/~dbrown/tracker/).

The angles between the apical region and the basal region of the epicotyl were

independently measured in the x and y views (Fig. 1b) and processed with fast Fourier transform to obtain the periods and amplitudes. Since the number of samples processed with fast Fourier transform is restricted to 2^x, I determined to process every 2^5 samples, at an interval of 10 min, for 320 min (5.3 h) in total, to keep balance between the time-resolution and the precision of the analysis. The integrated periods (P) and amplitudes (A) were obtained from the periods in the x view (P_x) and the y view (P_y), and the amplitudes in the x view (A_x) and the y view (A_y) by the following equations, respectively.

$$P = \frac{P_x + P_y}{2}$$

$$A = \sqrt{A_x^{\,2} + A_y^{\,2}}$$

Scanning electron microscopy

Sections 1 cm in length were taken from 6-day-old greened epicotyls and buried in silicone resin (Extrude Wash; Kerr, Romulus, MI, USA). After the resin had dried up, the sections were removed and the silicone molds were filled with epoxy resin (DEV-TUBE S-208; ITW, Inc., Glenview, IL, USA) to make replicas. Prepared replicas were coated with gold and observed with a scanning electron microscope (SU6600; Hitachi, Ltd., Tokyo, Japan).

Spray treatments of intact epicotyls

After the 5-day-old greened adzuki bean epicotyls had been observed for 24 h to confirm that they exhibited normal circumnutation, they were gently sprayed with test solutions containing auxin transport inhibitors or hormones from four directions at right angles to each other. Tween 20 was added to the test solutions at 0.1% to reduce the surface tension of the solutions and facilitate the efficient spread of the applied

solution drops onto the surface of the epicotyls.

Quantification of endogenous phytohormones

Sections 0–1, 1–2, and 2–3 cm below the tip of the 6-day-old greened epicotyls were carefully separated along the longitudinal axis into either convex/concave or pre-convex/pre-concave pairs with respect to the plane of curvature of circumnutation using a razor blade (Fig. 11a). They were then frozen in liquid nitrogen. The contents of five different plant hormones [GA_1; IAA; abscisic acid (ABA); salicylic acid (SA); jasmonic acid (JA)] were simultaneously determined by LC–MS, essentially according to Tsukahara et al. (2015). In brief, approximately 0.1 g (fresh weight) of dissected tissues was ground in liquid nitrogen, followed by extraction in 80% acetonitrile containing 1% acetic acid at 4°C for 1 h. Internal standards of plant hormones were added to the extraction buffer at this step. The composition of internal standards are described elsewhere (Tsukahara et al. 2015). The hormones were fractionated into three parts by a solid-phase extraction comprised of a reverse-phase (Oasis HLB, Waters Corporation, Milford, Massachusetts, USA), cation-exchange (Oasis MCX, Waters Co.), and anion-exchange cartridges (Oasis WAX, Waters Co.), and each fraction was analyzed by triple-quadrupole LC–MS (Agilent 6410, Agilent Technologies, Santa Clara, California, USA) according to Tsukahara et al. (2015).

Effects of phytohormones on the elongation of epicotyl sections

Sections 0–1, 1–2, and 2–3 cm below the tip were taken from the 6-day-old greened epicotyls and were floated in the control solution (10 mM KCl, 2% sucrose, and 2 mM PIPES, pH 7.0) for 1 h to release the endogenous hormones. The sections were then treated with different hormone combinations dissolved in the control solution.

The length of each section was recorded using a stereomicroscope (Stemi DV4; Carl Zeiss, AG, Oberkochen, Germany) and a digital camera (GR DIGITAL; Ricoh, Co., Ltd., Tokyo, Japan), and analyzed using ImageJ software (https://imagej.nih.gov/ij/).

Results

Circumnutation and elongation pattern of greened adzuki bean epicotyls

First, the movement of 5-day-old greened adzuki bean epicotyls grown under continuous white light (Fig. 1) was recorded every 10 min by time-lapse imaging for 48 h (Fig. 2a). The angle between the apical region and the basal region of the epicotyl continued to oscillate during the observation in both x and y views (see Fig. 1 for the definition). I processed these results with fast Fourier transform (Figs. 3a–i). The periods of detected oscillations were maintained more or less constant at around 60 min during the observation (Fig. 2b), while the amplitudes increased from 10° to 25° in the x view and to 35° in the y view in the first 15 h, and then gradually decreased thereafter (Fig. 2c). The mean integrated period and amplitude during the observation was 61.3 min and 24.3° (Table 1), respectively. The trajectory projections of the epicotyl tip on a horizontal plane were reconstructed from two-view measurements of the tip orientation angle (Figs. 3a′–i′). The tip of the greened epicotyl moved in a slightly irregular manner from 0 to 10 h, drawing circles with unfixed orbits. Finally, the tip turned to move in a circular orbit. All of the observed epicotyls eventually exhibited a clockwise movement in the top view, while 40% of the epicotyls exhibited a counterclockwise movement during the early irregular movement. The trajectory of the tip was an ellipse rather than a perfect circle (Fig. 2d), and approximately 20% of the epicotyls exhibited a pendulum-like movement (Fig. 2e). Indeed, the amplitude of oscillation in the y view

was significantly larger than that in the x view (Fig. 2c). The contour of greened adzuki bean epicotyl is like a misshapen hexagon with two vertexes on the X-axis, the center line of the epicotyl cross section parallel to the x view, and two concave edges parallel to the X-axis (Fig. 4). These structural characteristics might provide a preference in the oscillation direction to the epicotyls.

To clarify which epicotyl region was responsible for the curvature of circumnutation, I analyzed the elongation pattern of epicotyls (Fig. 5a). The mean length of the 5-day-old greened epicotyls was approximately 6.5 cm. Over the next 48 h, epicotyls under white light elongated at a constant rate of approximately 0.16 cm/h. Elongating epicotyls could be separated into two regions: the more apical region, in which epicotyls continued to elongate (elongating region), and the more basal region, in which epicotyls had elongated completely (completed region). The elongation of greened epicotyls completed from the most basal region and in regions V, IV, and III (see Fig. 1a for the definition), sequentially, at around 27, 36, and 42 h of observation (gray triangles in Fig. 5a). By tracing the bending zone of circumnutation on each epicotyl (see Fig. 1b for definition), I found that the bending zone was always located in the region 1–3 cm below the tip (regions intercepted by the open diamond and open circle in Fig. 5a). Markedly, its basal end (open circles in Fig. 5a) was always almost identical to the apical end of the completed region (gray triangles in Fig. 5a), and accordingly, moved up toward the tip along the longitudinal axis of the epicotyl. When I calculated the elongation rate of apical regions separately, the suppression of elongation of the region III started at around 27 h (Fig. 5b). I also found that the epicotyls did not exhibit any twisting movement since the orientation of the marking tape did not change during the observation. Consistent with this, the epidermal cell files run almost parallel to the longitudinal axis of the epicotyl (Fig. 6).

Effect of auxin transport inhibitors on the circumnutation of greened adzuki bean epicotyls

Auxin transport inhibitors or de-capping of organs substantially disturbed circumnutation in several plant species (Britz and Galston 1982a; Britz and Galston 1982b; Hatakeda et al. 2003; Kim et al. 2016). To know whether auxin also plays a role in the circumnutation of greened adzuki bean epicotyls, I examined the effect of auxin transport inhibitors. After I confirmed that the epicotyls exhibited normal circumnutation (after the amplitude reached the peak value at around 15–20 h of observation with a constant period of 60 min), those epicotyls were sprayed with test solutions at 24 h (Fig. 2c). While a mock treatment with 0.1% Tween 20 alone did not affect the period of circumnutation, 0.1% Tween 20 plus 20 μM 2,3,5-triiodebenzoic acid (TIBA) persistently increased the period from 60 to 140 min in 18 h treatment (Fig. 7a). These results suggested that the periodic changes in the epicotyl angle could be no longer detected by fast Fourier transform after the TIBA treatment. The movement of the epicotyl tip was slightly disordered in 8 h of the mock treatment, but it recovered to an elliptical trajectory in 12 h of the mock treatment (Figs. 8e′–i′). In contrast, after treatment with TIBA, the epicotyl tip no longer drew a circular trajectory (Figs. 9e′–i′).

The mock treatment decreased the amplitude of circumnutation at a descending rate of 4.3 ± 0.6 °/h in the first 2 h of treatment (Fig. 7b, mock), which was significantly higher than that of non-treated epicotyls (0.1 ± 1.0 °/h) during the same observation period ($P < 0.05$; Tukey's test). The effect of the mock treatment was transient, and the descending rate of amplitude over the next 2–12 h was 0.5 ± 0.4 °/h, indicating recovery of circumnutation. The TIBA treatment also decreased the amplitude of circumnutation at a descending rate of 4.8 ± 0.9 °/h in the first 2 h of

treatment (Fig. 7b, TIBA), similar to that of the mock-treated epicotyls ($P > 0.05$; Tukey's test) and significantly higher than that of the non-treated epicotyls during the same observation period ($P < 0.05$; Tukey's test). However, the descending rate of amplitude over the next 2–12 h of TIBA treatment was 1.6 ± 0.2 °/h, significantly higher than that of the mock-treated epicotyls ($P < 0.05$; Student's *t*-test). The amplitudes never recovered and maintained a level lower than 10° until the end of the observation period. Importantly, the TIBA treatment never affected the elongation rate of epicotyls (Fig. 10a), indicating that it specifically impaired circumnutation. These results strongly suggest the involvement of auxin in the maintenance of circumnutation in adzuki bean epicotyls, as demonstrated in other plant species.

Distribution of phytohormones in greened adzuki bean epicotyls exhibiting circumnutation

In gravitropism and phototropism, the asymmetric distribution of auxin in bending axial organs is thought to be the direct cause of tropic curvature, in which a higher auxin level brings about an enhanced elongation of one side compared with the other side (Went and Thimann 1937, Parker and Briggs 1990, Iino 1991, Li et al. 1991, Haga et al. 2005, Haga and Iino 2006). However, as far as I know, no reports have examined the distribution pattern of auxin associated with circumnutation. Taking advantage of its large size, we investigated the distribution of endogenous hormones in greened adzuki bean epicotyls. Since the plane of curvature rotates along the longitudinal axis of the epicotyl during circumnutation, I attempted to compare hormone levels between not only the convex and concave sides at a certain time point (convex/concave) but also what would be the convex and concave sides at the next time point (pre-convex/pre-concave). After epicotyl sections 0–1, 1–2, and 2–3 cm below the

tip were separated along the longitudinal axis into two halves with respect to the plane of curvature of circumnutation (Fig. 11a), namely, convex/concave or pre-convex/pre-concave pairs, the abundances of five different phytohormones in the sections were quantified by LC–MS (Figs. 11b–f′). In the convex/concave pairs, I could not detect any significant difference in IAA abundance at any region along the longitudinal axis of the epicotyl (Fig. 11b). On the other hand, in the pre-convex/pre-concave pairs, IAA was distributed significantly more in the pre-convex half than in the pre-concave half of sections 1–2 cm below the tip (Fig. 11b′). Furthermore, GA_1, which promotes the elongation of adzuki bean epicotyl sections in concert with auxin (Shibaoka 1972), and ABA, which inhibits the elongation of dwarf pea epicotyls (Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993), were also distributed significantly more in the pre-convex half of sections 1–2 cm below the tip (Figs. 11c, c′, d, d′). These distribution patterns were specific to hormone species, and may not result from the experimental procedures, since JA and SA did not exhibit such characteristic distribution patterns (Figs. 11e, e′, f, f′). Furthermore, when I statistically analyzed the IAA content in epicotyl half section 0–1 cm below the tip, I could not detect any significant differences among any parts of epicotyl ($P > 0.05$, Tukey's test). Therefore, differences in the contents of hormones between the convex/concave pairs and the pre-convex/pre-concave pairs may be in a margin of error.

Effect of phytohormones on the elongation of epicotyl sections

Since three different hormones (IAA, GA_1, and ABA) exhibited similar asymmetric distribution patterns in the same epicotyl region (Figs. 11b–d′), I examined their effects on the elongation of epicotyl sections (Fig. 12). First, epicotyl sections 0–1, 1–2, and 2–3 cm below the tip were floated on solutions containing different IAA

concentrations for 60 min and their elongations were measured (Fig. 12a). A significant enhancement in the elongation of sections 0–1, 1–2, and 2–3 cm below the tip was detected only at 1 mM, over 10 μM, and over 100 μM IAA, respectively, compared to the sections treated with the control solution ($P < 0.05$; Tukey's test). Hence, epicotyl sections 1–2 cm below the tip exhibited the highest responsiveness to IAA. I determined to use 100 μM IAA in the following experiments for two reasons. First, the elongations of sections 1–2 and 2–3 cm below the tip were similar at this concentration. Second, the elongations of sections at 100 μM IAA were smaller than those at 1 mM IAA and this concentration might better detect the effects of gibberellin and ABA.

I then examined the effects of gibberellin A_3 (GA_3), a substitute for GA_1, since GA_1 was unavailable in the market. Since GA_3 affects the elongation of adzuki bean epicotyl sections only when it is applied together with auxin (Shibaoka 1972), I treated epicotyl sections with 100 μM IAA plus different concentrations of GA_3 for 60 min (Figs. 12b–d). GA_3 at any concentration did not show any significant effect on the elongation of sections 0–1 cm below the tip at any time point ($P > 0.05$; Tukey's test) (Fig. 12b). On the other hand, the elongation of sections 1–2 and 2–3 cm below the tip clearly increased in the presence of GA_3 (Figs. 12c, d). Treatment with 100 μM IAA plus 1 mM GA_3 for 60 min increased the amount of elongation of epicotyl sections two-fold compared to the treatment with 100 μM IAA alone, and was five-fold larger than the control treatment. Importantly, epicotyl sections 1–2 cm below the tip exhibited a higher responsiveness to GA_3 than those 2–3 cm below the tip. When treated with 100 μM IAA plus 100 μM or 1 mM GA_3, a significant enhancement in the elongation of sections 1–2 cm below the tip was detected from 15 min and that of sections 2–3 cm below the tip was detected from 20 min, compared to the control treatment ($P < 0.05$; Tukey's test). When treated with 100 μM IAA plus 10 μM GA_3, a significant

enhancement in the elongation of sections 1–2 cm below the tip was detected from 25 min, whereas that of sections 2–3 cm below the tip was detected only from 40 min ($P < 0.05$; Tukey's test).

I also examined the effects of ABA. Although ABA inhibited the elongation of dwarf pea epicotyls (Sakiyama and Shibaoka 1990; Sakiyama-Sogo and Shibaoka 1993), 100 μM ABA did not affect the elongation of adzuki bean epicotyl sections 1–2 cm below the tip in 60 min of the treatments, regardless of the presence of 100 μM IAA alone or together with 100 μM GA_3 (Fig. 13). In summary, my results suggest that the epicotyl region 1–2 cm below the tip has the highest responsiveness to auxin and gibberellin in terms of enhancement of the elongation.

Effect of exogenously applied auxin and gibberellin on the circumnutation of adzuki bean epicotyls

To verify whether the asymmetric distributions of auxin and gibberellin are critical for the maintenance of circumnutation, I applied these hormones exogenously to intact adzuki bean epicotyls by a spray treatment, which should disturb the distribution pattern of endogenous hormones. As expected, the period of circumnutation of the 6-day-old greened epicotyls increased from 60 to 120 min in an 8 h IAA treatment (Fig. 14a). This effect was transient, and the period recovered to 60 min in an 18 h IAA treatment. This result suggested that the periodic changes in the epicotyl angle could not be detected transiently by fast Fourier transform after the IAA treatment. The movement of the epicotyl tip was disordered in a 2 h IAA treatment, but was also restored to the elliptical trajectory in an 18 h IAA treatment (Figs. 15e′–i′). The descending rates of amplitude in the first 2 h and over the next 2–12 h of IAA treatment were 6.3 ± 1.0 °/h and 0.9 ± 0.4 °/h (Fig. 14b), not significantly different from the rate in mock-treated

epicotyls over a respective period of observation ($P > 0.05$; Student's *t*-test). The treatment with 100 μM IAA did not affect the elongation rate of epicotyls (Fig. 10b). On the other hand, I could not detect any remarkable effect of exogenously applied GA_3 at 100 μM on circumnutation (Figs. 14c, d). Neither the period (Fig. 14c) nor the amplitude (Fig. 14d) was significantly different from those of mock-treated epicotyls at any time point ($P > 0.05$; Tukey's test). The elongation rate of epicotyls was also never affected by the GA_3 treatment (Fig. 6c, Fig. 10c, Fig. 16).

Discussion

Circumnutation and asymmetric distributions of auxin and gibberellin

In the case of gravitropism and phototropism, the asymmetric distribution of auxin in the opposing sides of axial organs causes differential growth (Morita 2010; Liscum et al. 2014). Although auxin has long been assumed to play an important role in circumnutation (Britz and Galston 1982b; Hatakeda et al. 2003; Kim et al. 2016), how auxin is distributed in organs exhibiting circumnutation and how it regulates circumnutation have not yet been investigated. In this study, after specifying that the bending zone of circumnutation of greened adzuki bean epicotyls was in the region 1–3 cm below the tip (Fig. 5a), I further revealed that IAA together with GA_1 was asymmetrically distributed there. The pre-convex half of epicotyls 1–2 cm below the tip contained significantly larger amounts of these hormones than the pre-concave half (Figs. 11b, b′). As far as I know, this is the first report on the asymmetric distribution of hormones associated with circumnutation. Furthermore, the epicotyl sections of this region exhibited the highest responsiveness to exogenously applied IAA and GA_3. Compared to epicotyl sections 0–1 and 2–3 cm below the tip, epicotyl sections 1–2 cm

below the tip responded to lower concentrations of IAA and GA_3 (Figs. 12b–d), and besides, when treated with 100 μM IAA plus 10 μM GA_3, a significant enhancement in the elongation of epicotyl sections 1–2 cm below the tip was detected earlier than that of sections from other regions (Figs. 12c, d).

The reason why asymmetric hormone distributions were detected in the pre-convex/pre-concave pairs and not in the convex/concave pairs, as in gravitropism or phototropism, could be explained by the latent period in hormone responses. I found that, in epicotyl sections 1–2 cm below the tip, the latent period between the application of IAA plus GA_3 and the detection of a significant enhancement in elongation was 15 min (Fig. 12c). This is consistent with previous reports, which demonstrated that auxin stimulated elongation growth with a latent period of 10–15 min in various plant species (Evans 1974; Badescu and Napier 2006). For example, Barkley and Evans (1970) precisely monitored the elongation of stem sections of pea and cucumber using a custom-made chamber filled with the growth medium, and showed that 100 μM IAA increased the elongation rate from 10 min after the treatment began. Takahashi et al. (2012) measured the elongation of *A. thaliana* hypocotyl sections put on agar plates containing IAA, and showed that 10 μM IAA increased the elongation rate from around 10 min. Therefore, I can assume that the mode of elongation of the bending zone of adzuki bean epicotyls is determined by the hormonal status 15 min before, and that circumnutation with a 60 min period is maintained by the differential growth reflecting the hormonal status 15 min before, which is shifting sequentially in the circumferential direction.

As reported in pea epicotyls (Britz and Galston 1982a), morning glory shoots (Hatakeda et al. 2003), and pea roots (Kim HJ et al. 2016), auxin-transport inhibitors impaired the circumnutation of adzuki bean epicotyls (Fig. 7). Furthermore,

exogenously applied IAA, which was expected to disturb the distribution pattern of endogenous IAA, transiently increased the period of circumnutation (Fig. 14a). These results strongly suggest that the distribution pattern of endogenous IAA is critical to maintain circumnutation, supporting my hypothesis described above. On the other hand, exogenously applied GA_3 did not affect the circumnutation of greened adzuki bean epicotyls (Figs. 14c, d). I raise two possible explanations for the results. One is that the exogenous GA_3 could not fully permeate into the epicotyl, and the other is that the endogenous level of gibberellin was already saturated in the epicotyl. The latter is suggested from the results that different concentrations of GA_3 applied with IAA produced similar levels of enhancement in the elongation of epicotyl sections 1–2 cm below the tip (Fig. 12c). Although the endogenous concentration of GA_1 estimated from the LC–MS measurements is much lower than 10 μM, the lowest concentration of GA_3 exogenously applied (Fig. 11c; Fig. 12c), I suppose that the endogenous gibberellin might be localized only in the limited part, where the local concentration of gibberellin is expected to be higher than 10 μM. In any case, the significance of asymmetric distribution of gibberellin in the regulation of circumnutation needs to be further investigated.

Possible mechanisms for the asymmetric distribution pattern of hormones

The next important subject is how the circumnutation-specific asymmetric distributions of auxin and gibberellin are maintained. In the case of auxin, auxin carriers involved in polar auxin transport may play important roles. For example, PIN3, the major auxin efflux carrier functioning in lateral auxin transport, is responsible for the asymmetric distribution of auxin in organs exhibiting gravitropism and phototropism in *A. thaliana* (Friml et al. 2002). Rakusová et al. (2011) reported the details of PIN3

relocalization during gravitropism in hypocotyls. In vertically placed hypocotyls, the signals from PIN3-GFP were detected at the plasma membrane in both the outer and inner sides of endodermis cells. After hypocotyls were placed horizontally for gravistimulation, the GFP signals at the outer sides of the endodermis cells in the upper half of the hypocotyl gradually disappeared. Since *DR5rev::GFP* fluorescence was detected in the lower half of the hypocotyl after gravistimulation, this relocalization of PIN3 must be responsible for the asymmetric distribution of auxin. Similarly, Ding et al. (2011) reported PIN3 relocalization during phototropism. When hypocotyls were illuminated unilaterally with white light, the signals from PIN3-GFP at the outer sides of endodermis cells in the illuminated half of the hypocotyl gradually disappeared. Since *DR5rev::GFP* fluorescence was detected in the shaded half of the hypocotyl after unilateral illumination, this relocalization of PIN3 must be responsible for the asymmetric distribution of auxin.

Such polar relocalization of putative auxin carriers could also explain the asymmetric distribution of auxin in adzuki bean epicotyls. In epicotyls exhibiting circumnutation, gravity and/or light from above would stimulate the displacement of auxin carriers from the outer sides of endodermis cells in the convex part, and auxin would start to be asymmetrically distributed to the concave part. Since auxin takes some time to move across the epicotyl and circumnutation continues, auxin would be asymmetrically distributed more in the pre-convex part than in the pre-concave part. To ascertain this possibility, once the auxin carriers that participate in circumnutation are identified, their localization patterns associated with circumnutation should be urgently investigated.

On the other hand, how the gibberellin distribution is maintained is more uncertain since the mechanism of gibberellin transport has not yet been revealed

(Yamaguchi 2008). Although the possibility that unknown gibberellin carriers are involved in the maintenance of gibberellin distribution still remains, another possibility should also be considered. Since GA_1 synthesis is promoted through auxin signaling (Yamaguchi 2008; Ross et al., 2000), a local synthesis of gibberellin might contribute to maintain the distribution pattern. In pea epicotyls, IAA increases the level of GA_1 through upregulating the expression of *PsGA3ox1*, which encodes the enzyme that converts gibberellin A_{20} (GA_{20}) to GA_1 (O′neill and Ross 2002). Therefore, if the IAA level exhibits a periodic change, the gibberellin level may also change periodically.

Driving mechanisms for circumnutation

In greened adzuki bean epicotyls, the basal end of the bending zone was almost always identical to the apical end of the completed region, and as epicotyls elongated, it moved up along the longitudinal axis of the epicotyl (Fig. 5a). These results indicate that epidermal cells constructing the bending zone are regularly turning over with their elongation growth. In the apical region of the bending zone, the elongation of young epidermal cells is sequentially accelerated in a circumferential direction by the asymmetrically distributed hormones, resulting in the initiation of differential growth between the opposing sides of the epicotyl. After being involved in the maximal differential growth in the middle region, older epidermal cells sequentially complete the elongation in the basal region of the bending zone, resulting in the termination of epicotyl differential growth. This driving mechanism of circumnutation seems to be different from that proposed in the French bean. In the twining shoots of French beans, whose epidermal cell file is almost vertical, as in adzuki bean epicotyls, partially reversible length changes in epidermal cells were associated with the revolving movement (Caré et al. 1998). This reversible cell elongation is possibly caused by

alternating changes in turgor pressure, ionic composition, and water permeability (Millet et al. 1988; Badot et al. 1990; Comparot et al. 2000).

On the other hand, it has also been proposed that skewed cell files drive circumnutation. When the stalk of a female flower of aquatic eelgrass (*Vallisneria asiatica*) elongated toward the water surface, it rotated around the longitudinal axis (Kosuge et al. 2013). After the bud reached the water surface, the stalk ceased rotation, and thereafter, exhibited both circumnutation and rotation. When the female flower exhibited circumnutation, the peduncle was strongly twisted and the epidermal cell files were strongly skewed. With experiments using an artificial model, Kosuge et al. (2013) proposed that the helical growth of the peduncle drives circumnutation. These studies indicate that different mechanisms drive circumnutation in different organs depending on their characteristic cellular organizations and modes of cell elongation.

Angle of curvature of circumnutation and differential growth in epicotyls

I showed that the mean period and amplitude of circumnutation of greened adzuki bean epicotyls was 61.3 min and 24.3° (Table 1), respectively, and the bending zone was located in 1–3 cm below the tip (Fig. 5a). Based on the results obtained in this study, I assumed that circumnutation is maintained by the differential growth sifting sequentially in the circumferential direction among the four parts of epicotyls, namely, convex, pre–concave, concave, and pre-convex. Then, I asked whether the angle of curvature of circumnutation would be explained from the elongation rate of epicotyls. Since the diameter of greened adzuki bean epicotyls is around 1 mm, the whole outer side of the bending zone should be longer than the whole inner side by 0.42 mm when the bending zone is assumed as an arc with a curvature of 24.3°. Furthermore, since the inner side of the bending zone may also elongate, the elongation in the outer side of the

bending zone for 15 min should be larger than 0.42 mm. When treated with 100 μM IAA plus 100 μM GA_3, the epicotyl sections 1–2 cm below the tip, in which the asymmetric distribution of hormones was detected (Fig. 11), and the epicotyl sections 2–3 cm below the tip finally elongated at 0.026 and 0.017 mm/min (Figs. 12c, d), respectively. At these enhanced elongation rates, the epicotyl sections 1–2 and 2–3 cm below the tip can elongate 0.38 and 0.25 mm for 15 min, respectively. Therefore, the differential growth in the region 1–3 cm below the tip can explain most of the curvature of the epicotyl with 24.3°. Although the asymmetric distribution of IAA and GA_1 was not detected in the epicotyl sections 2–3 cm below the tip (Fig. 11), this region may somehow contribute to make the curvature of the epicotyl. To verify these hypotheses, the differential growth in intact epicotyls should be analyzed in real time. Caré et al (1998) observed the epidermal cell elongation in an epicotyl of French bean which exhibits circumnutation, using a horizontally placed microscope with a rotating stage. Such experimental systems enable to follow the differential growth that is shifting sequentially in the circumferential direction in adzuki bean epicotyls.

Characteristics of circumnutation

I showed that the period of circumnutation of greened adzuki bean epicotyls was maintained at approximately 60 min (Fig. 2b). Although circumnutation occurs in various plant species, the period of circumnutation differs from species to species. In etiolated rice coleoptiles, the period of circumnutation slightly increased from 160 to 200 min with the elongation of coleoptiles (Yoshihara and Iino 2005). In *A. thaliana*, hypocotyls grown under white light exhibited two patterns of circumnutation with a shorter period of 30 min (SPN) and a longer period of 90 min (LPN). The period lengths of both SPN and LPN tended to increase during a 3-day observation (Schuster

and Engelmann 1997). One of the possible regulators for the periodicity of circumnutation is a circadian rhythm. The period of circumnutation in *A. thaliana* inflorescence stems under continuous white light fluctuated in a circadian manner, and was disturbed in the *toc1* and *elf3* mutants, which are deficient in circadian clock functions (Niinuma et al. 2005). However, as described above, greened adzuki bean epicotyls did not exhibit such fluctuation in the period of circumnutation (Fig. 2b). This difference is attributable to growth conditions. Niinuma et al. (2005) cultivated *A. thaliana* seedlings under light and dark cycles, but I raised adzuki been seedlings under continuous white light (see Materials and Methods). Someya et al. (2006) raised *A. thaliana* under continuous light and showed that the period of circumnutation of inflorescence stems did not fluctuate under continuous light conditions. Growth conditions might affect circadian clock responsiveness and/or hormone status in plant organs and cause differences in circumnutation characteristics even in the same plant species.

The direction of movement is also an important parameter of circumnutation. I showed that all adzuki bean epicotyls eventually exhibited a clockwise circumnutation (Figs. 2d, e, Fig. 3). On the other hand, in the case of rice coleoptiles, the ratio of counter-clockwise circumnutation to clockwise was approximately 3:1 (Yoshihara and Iino 2005). In *A. thaliana* hypocotyls, the direction of SPN was usually counter-clockwise, while that of LPN was usually clockwise (Schuster and Engelmann 1997). French bean twining shoots always exhibited counter-clockwise movements (Millet et al. 1984). Therefore, the mechanisms that regulate the period of circumnutation and determine the direction of movement are tremendously diverse. Smyth (2016) pointed out that the handedness of some kinds of helical growth is variable, and the direction might reflect an early developmental asymmetry in the shoot

apical meristem. If the distribution pattern of auxin in the bending zone of circumnutation reflects that in the shoot apical meristem, it might explain how the direction of circumnutation is determined. This should be another important subject to be investigated in future.

Chapter II: Induction of circumnutation by light

Materials and methods

Plant materials

Adzuki bean (*Vigna angularis* cv. Erimowase) seeds stored at 4°C were transferred to a growth chamber (LH-60FL12-DT; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan) for 1 h to adapt to 27 ± 1°C. Thereafter, the seeds were sown in 200 ml pots filled with vermiculite and warm water. The pots were covered with plastic wraps to maintain high humidity and kept in darkness. Three days after, the plastic wraps were removed so as not to interfere with epicotyl growth.

Recording and analysis of epicotyl circumnutation and elongation

Vinyl tape 1 mm in width was carefully pasted on each 5-day-old etiolated epicotyl every 3 mm from the tip (Fig. 17a). Thereafter, epicotyls were put in a clear, colorless acrylic box maintained at 27°C with an electric panel heater (MP-916; TRiO Co., Osaka, Japan) under continuous infrared (900 nm, 10 μmol m^{-2} s^{-1}), red (660 nm, 30 μmol m^{-2} s^{-1}), or blue light (470 nm, 30 μmol m^{-2} s^{-1}). Monochromatic lights were obtained from light-emitting diodes light source system (MIL-C1000T for a light source controller and MIL-U200 for a light source frame; SMS, Osaka, Japan). The fluence rate of infrared light was measured using a photodiode (S2387-1010R; Hamamatsu Photonics K.K., Shizuoka, Japan), and that of red and blue light was measured using a spectroradiometer (LI-1400; Li-Cor, Inc., Lincoln, NE, USA). Epicotyls were photographed every 10 min in the plane parallel (x view) or perpendicular (y view) to the first leaves (Figs. 17a, c) by programmable cameras (Ltl-5210B; Little Acorn

Outdoors, Green Bay, WI, USA). Etiolated epicotyls were handled in complete darkness by using an infrared viewer (PVS7; ATN Co., South San Francisco, CA, USA) until the onset of light irradiation. Circumnutation and epicotyl elongation were analyzed from sequentially recorded images using the Tracker Video Analysis and Modeling Tool (http://www.cabrillo.edu/~dbrown/tracker/).

The angles between the tip or the hook and the basal region of the epicotyl (see Fig. 17b for definition) were independently measured in the x and y views and processed with fast Fourier transform to obtain the periods and amplitudes. Since the number of samples processed with fast Fourier transform is restricted to 2^x, I determined to process every 2^5 samples, at an interval of 10 min, for 320 min (5.3 h) in total, to keep balance between the time-resolution and the precision of the analysis.

Recording of epicotyl gravitropism

Etiolated epicotyls of 5 day old were kept in darkness or exposed to continuous red light (30 μmol m^{-2} s^{-1}) from above for 24 or 36 h, and then placed horizontally under infrared or red light, respectively. Epicotyls were photographed every 10 min in the plane parallel to the first leaves (Figs. 27a, b) by programmable cameras (Ltl-5210B; Little Acorn Outdoors, Green Bay, WI, USA). Gravitropic response was analyzed from sequentially recorded images using the Image J software (https://imagej.nih.gov/ij/).

Visualization of endodermal amyloplasts

Tangential sections were carefully separated from the epicotyl sections 1–2 cm below the tip using a razor blade. After fixed with FAA solution [4% folmaldehyde, 50% ethanol, 5% acetic acid] for 24 h at 4°C, the tangential sections were washed in

50% [v/v] ethanol and stained with IKI solution [2% (w/v) iodine, 5% (w/v) potassium iodine, 20% (w/v) chloral hydrate] for 1 min. Samples were de-stained with a clearing solution [chloral hydrate : glycerol : water = 8:1:2 (w:v:v)] and observed with a light microscope (BX50; Olympus, Co., Tokyo, Japan).

To determine the sedimentation speed of amyloplasts, etiolated epicotyls of 5 day old were kept in darkness or exposed to continuous red light (30 μmol m^{-2} s^{-1}) from above for 24 or 36 h, and then placed upside down in darkness or under red light, respectively. At various times after the inversion, tangential sections were separated from the epicotyls and fixed with FAA solution. The positions of amyloplasts in a cell were determined by measuring the center of gravity of each amyloplast, and normalized by the cell length.

Results

Induction of circumnutation in etiolated adzuki bean epicotyls by light

Since greened adzuki bean epicotyls exhibited circumnutation under white light (Fig. 2), I speculated that the onset of circumnutation could be detected by observing etiolated epicotyls (Fig. 17). Figure 18a shows an example of the movement of 5-day-old etiolated epicotyl under infrared light. As etiolated epicotyls have an apical hook, I measured θ1, which is the angle between the epicotyl tip and the epicotyl base, and θ2, which is the angle between the apical hook and the epicotyl base (see Fig. 17b for definition). From hours 20 to 30, the plumular orientation changed due to a twist of the stem around its longitudinal axis. The direction of this torsional movement was clockwise. All the observed etiolated epicotyls under infrared light exhibited a similar movement, occasionally a rotation in a half of a circle. The movements of the epicotyl

tip in the x view and the y view were analyzed by processing θ1 with fast Fourier transform to detect oscillations (Figs. 19a–i). The obtained Fourier spectra were similar to the spectrum obtained from a linear graph. Therefore, no oscillation was detected. The epicotyl tip moved only linearly or in a quite irregular manner in the horizontal projections (Figs. 19a′–i′).

Since the nutation was observed in the region between the apical hook and the base of etiolated pea epicotyls (Galston et al. 1964), the movements of the apical hook were also analyzed by processing θ2 with fast Fourier transform (Fig. 20). In most cases, no oscillation was detected and the apical hook moved only in a quite irregular manner. Even when the oscillation was detected, the apical hook drew a circle only once, but not more than twice (Fig. 20d′). Therefore, etiolated epicotyls did not exhibit circumnutation under infrared light.

To verify that the different behavior of greened and etiolated adzuki bean epicotyls is attributable to light conditions, I exposed etiolated epicotyls to continuous red or blue light. When the 5-day-old adzuki bean seedling grown in darkness was exposed to continuous red light, its epicotyl exhibited hook opening, and then periodic movements in angle (Fig. 18b). By processing θ1 to analyze the movements of the epicotyl tip, I found that the mode of movement of epicotyl changed roughly through three stages. First, during hook opening, the Fourier spectra were similar to the spectrum obtained from a linear graph (Figs. 21a–c). Therefore, no oscillation was detected. Second, after the hook opened, oscillations were detected sometimes but not other times (Figs. 21d–g). Third, after the second stage, clear oscillations with the period of approximately 60 minutes were detected (Figs. 21h, i). The epicotyl tip moved linearly with the hook opening in the first stage (Figs. 21a′–c′), in an irregular manner in the second stage (Figs. 21d′–g′), and finally exhibited pendulum-like movements in the

third stage (Figs. 21h′, i′). To analyze the movements of the apical hook during hook opening, θ2 was processed with fast Fourier transform (Fig. 22). Oscillations were detected sometimes but not other times. The apical hook of epicotyl moved in an irregular manner.

When the 5-day-old etiolated seedling was exposed to continuous blue light, the epicotyls exhibited similar changes in the mode of movement to those exposed to red light, and finally moved circularly in the third stage (Fig. 18c, Fig. 23). Table 1 summarizes the parameters of growth movements determined for greened epicotyls under white light and de-etiolated epicotyls exposed to red or blue light. While the periods of circumnutation were approximately 60 minutes in every case, the amplitudes were significantly different. The amplitudes under white and blue light were similar and approximately 25°, while that under red light was significantly smaller and 8.6°.

Effects of light on the elongation pattern of epicotyls

Since it is supposed that the growth movements are caused by changes in the growth patterns of organs, I investigated the elongation patterns of etiolated adzuki bean epicotyls under different light conditions (Fig. 25). I traced the position of the apical hook (gray diamonds in Fig. 25), in addition to the bending zone of circumnutation (regions intercepted by the open diamond and open circle in Fig. 25). The mean length of 5-day-old etiolated epicotyls was approximately 8.5 cm, and they elongated at a constant rate of approximately 0.19 cm/h under infrared light (Fig. 25a). The distance between the tip and apical hook was kept at an almost constant length of 0.71 cm. Compared with greened epicotyls under white light (Fig. 5a), it took longer time for the completion of elongation in etiolated epicotyls under infrared light. The regions V and IV completed to elongate at around 33 and 42 h, respectively, while the region III did

not complete to elongate in 48 h of observation.

As anticipated, both red and blue light changed the elongation pattern of etiolated epicotyls (Figs. 25b, c). During hook opening, the elongation pattern of epicotyls was similar to that of etiolated epicotyls under infrared light except the position of the apical hook (gray diamonds in Figs. 25b, c). The apical hook moved up to the tip during hook opening. After the hook opened, the elongation pattern of epicotyls shifted to that of greened epicotyls under white light. The elongation of the region IV and III was completed at around 33 and 42 h, respectively. At around 39 h, the epicotyls started circumnutation, with the bending zone being located in the region 2–4 cm below the tip (regions intercepted by the open diamond and open circle in Figs. 25b, c). The basal end of the bending zone was detected in the region II, above the completed region, and moved up to the tip thereafter (open circles in Figs. 25b, c).

To investigate elongation patterns of epicotyls more precisely, I calculated the elongation rate of apical regions separately (Fig. 26). Although the suppression of elongation of the region III in etiolated epicotyls under infrared light started at around 39 h (Fig. 26a), those in the epicotyls exposed to red or blue light started at 24 or 27 h, respectively (Figs. 26b, c). This was similar to that in greened epicotyls under white light (Fig. 5b). Moreover, the suppression of elongation of the region II also started at around 39 h, which was almost identical time point to the onset of circumnutation.

Repression of gravitropism of etiolated adzuki bean epicotyls by red light

Finally, I explored the possible target process of light in the induction of circumnutation. Since it has been implied that the responsiveness to gravity is critical for circumnutation (Israelsson and Johnsson 1967; Kitazawa et al. 2005), I speculated that light affects the responsiveness to gravity of adzuki bean epicotyls, and examined

the difference in gravitropism between epicotyls kept in darkness and those exposed to light (Fig. 27a, b). To eliminate the effect of phototropism, I used red light for the light stimulation, not blue light.

First, 5-day-old etiolated epicotyls were further kept in darkness or exposed to red light for 24 h, and then placed horizontally under infrared or red light, respectively. At the start of the gravitropic stimulation, the tip of the epicotyls kept in darkness for 24 h fell down to -50° (Fig. 27c, D24 h). The epicotyl started to raise their tip from 20 min of the gravitropic stimulation, and the angles of the tip reached 70° at 100 min of the gravitropic stimulation. Thereafter, the epicotyls maintained the angle of the tip around 75° till the end of the observation. On the other hand, in the epicotyls exposed to red light for 24 h, the tip fell down to -30° at the start of the gravitropic stimulation, not as much as the epicotyls kept in darkness for 24 h (Fig. 27c, RL 24 h, $P < 0.05$; Tukey's test). The epicotyls raise their tip from 20 min of the gravitropic stimulation, and after the angle of the tip reached 80° at 100 min, it decreased to 60° in the next 60 min. The epicotyls maintained the angle of the tip around 60° till the end of the observation, and this steady angle was similar to that of the epicotyls kept in darkness for 24 h ($P > 0.05$; Tukey's test).

Second, 5-day-old etiolated epicotyls were further kept in darkness or exposed to red light for 36 h, and then placed horizontally under infrared or red light, respectively. At the start of the gravitropic stimulation, the tip of the epicotyls kept in darkness for 36 h fell down to -50° (Fig. 27c, D36 h), as the epicotyls kept in darkness for 24 h (Fig. 27c, D24 h). The epicotyl started to raise their tip from 20 min of the gravitropic stimulation, and the angles of the tip reached 70° at 130 min of the gravitropic stimulation. Thereafter, the epicotyls maintained the angle of the tip around 75° till the end of the observation. On the other hand, the epicotyls exposed to red light

for 36 h exhibited a reduced gravitropism (Fig. 27c, RL36 h). The tip of the epicotyls fell down to -50° at the onset of gravitropic stimulation, and started to rise from 20 min, as the epicotyls kept in darkness for 36 h (Fig. 27c, D36 h). After the tip of the epicotyls reached 20° at 120 min, it maintained the angle till the end of the observation. However, the maximum value of curvature of 20° was 30% of that in the epicotyls kept in darkness for 24 or 36 h, or exposed to red light for 24h. The angle of the tip from 1.5 h of the gravitropic stimulation was significantly lower than that in the epicotyls kept in darkness for 24 or 36 h, or exposed to red light for 24h ($P < 0.05$, Tukey's test). Furthermore, I compared the initial rate of gravitropic response in those epicotyls, and found that, at 1 h of the gravitropic stimulation, the rate in the epicotyls illuminated with red light for 36 h was significantly slower than that in the epicotyls under the other light conditions (Fig. 27d). Therefore, since it took 39 h for red light illumination to start circumnutation in etiolated epicotyls (Table 1), the gravitropism of adzuki bean epicotyls was repressed immediately before the onset of circumnutation.

Recent study showed that the decrease in the size of amyloplasts in the endodermis of the shoot caused by red light represses the shoot gravitropism in *Arabidopsis thaliana* (Kim et al. 2011; Kim J et al 2016). To know whether this is also the case in adzuki bean epicotyls, I measured the size of amyloplasts in the endodermis of the epicotyls kept in darkness or illuminated with red light. As expected, red light illumination decreased the size of amyloplasts in the endodermis of the epicotyls (Fig. 28). The sizes of amyloplasts of the epicotyls after illumination with red light for 24 h or 36 h were 74% of those of the epicotyls kept in darkness for 24 h or 36 h, and the values were significantly smaller (Fig. 28e, $P < 0.05$; Tukey's test).

I further investigated the sedimentation rate of amyloplasts in the epicotyls by placing epicotyls upside down (Fig. 28f). Although amyloplasts in all epicotyls were at

the original bottom of a cell at the start of inversion, the sedimentation speed was affected by light conditions. In the epicotyls kept in darkness for 24 h or 36 h, amyloplasts sank to the original top of the cell in 5 min after the inversion, and the displacement was 50% of a cell (Fig. 28f, D24 h, D36 h). On the other hand, red light reduced the sedimentation rate of amyloplasts. In the epicotyls exposed to red light for 24 h, although the endodermal cells were shorter than those in the epicotyls kept in darkness, the displacement of the amyloplasts for the initial 5 min after the inversion was 40% of a cell (Fig. 28f, R24 h). The value was significantly smaller than those in the epicotyls kept in darkness 24 h ($P < 0.05$; Tukey's test). In the epicotyls exposed to red light for 36 h, amyloplasts sank much slower. The displacement of the amyloplasts was 20% and 40% of a cell at 5 min and 10 min after the inversion, respectively. The values were significantly smaller than those in the epicotyls under the other light conditions ($P < 0.05$; Tukey's test). At 20 min after the inversion, the amyloplasts in epicotyls exposed to red light to 24 h and 36 h eventually sank to the original top of the cell. To estimate the absolute sedimentation rate of amyloplast, I used the value for an amyloplast that exhibits the mean position after the inversion (Fig. 28f) in an endodermal cell with the mean length (Fig. 28f, inset). In the epicotyls exposed to red light for 36 h, the assumed sedimentation rate of amyloplast was lower than that of the epicotyls under different light conditions (Fig. 28g).

Discussion

Induction of circumnutation by light

Almost all the previous studies on circumnutation focused on the organs that have already exhibited circumnutation. On the other hand, the induction processes of

circumnutation have not been characterized well. To investigate how adzuki bean epicotyls start circumnutation, I observed etiolated epicotyls transferred to different light conditions for 48 h. Although greened adzuki bean epicotyls continuously exhibited circumnutation under white light, etiolated epicotyls under infrared light did not (Fig. 2, Fig. 18a). On the contrary, the epicotyls started circumnutation after exposure to continuous red or blue light (Figs. 18b, c). In etiolated pea epicotyls, the periodic movement is induced by light. Britz and Galston (1982a) showed that, while etiolated pea epicotyls did not exhibit nutation in darkness, they started the movement in darkness following the red light pre-illumination for 20 h. On the other hand, in etiolated rice coleoptiles, which exhibit circumnutation in darkness, the movement is inhibited by a 3-min pulse illumination with red light (Yoshihara and Iino 2005). Therefore, the modes of regulation of circumnutation by light seem to be different from species to species, and at least in adzuki bean epicotyls, light is essential to start circumnutation.

To date, the photoreceptor involved in the termination of circumnutation is revealed only in rice, and there is no report identifying photoreceptors for the induction of circumnutation. In rice coleoptiles, circumnutation is inhibited by red light through phytochrome A-dependent signaling (Yoshihara and Iino 2005). In pea epicotyls, although red light induces nutation, the photoreceptor mediating the response has not been clarified (Britz and Galston 1982a). Baskin (1986) further showed that, in pea epicotyls grown under continuous dim red light, blue light enhances the amplitude of nutation. However, the photoreceptor mediating this response is neither revealed. In adzuki bean epicotyls, since both red and blue light induces circumnutation, the induction of circumnutation may be mediated by phytochrome. The photoreceptors mediating specific effects on circumnutation in adzuki bean epicotyls will be discussed

below.

Possible target process of light in the circumnutation induction

I investigated the possible target process of light in the circumnutation induction, and found that, after 36 h of the red-light illumination, the gravitropism of the epicotyls was significantly repressed (Fig. 27), preceding the onset of circumnutation. In these endodermis of the epicotyls, the size of amyloplasts was significantly smaller (Fig. 28c), and furthermore, the sedimentation speed of amyloplasts was significantly lower than that in the epicotyls kept in darkness (Fig. 28d). These results were consistent with the previous reports in *A. thaliana* hypocotyls. Kim et al. (2011) revealed that the *A. thaliana* hypocotyls grown in darkness exhibited the negative gravitropism, whereas those grown under continuous red light did not. Furthermore, Kim et al. (2011) showed that this red-light dependent inhibition of gravitropism is mediated by PHYTOCHROME-INTERACTING FACTOR (PIF) transcription factors. PIFs are negative regulators in a phytochrome signaling, which suppress the downstream gene expression in darkness (Castillon et al. 2007). When plants are exposed to light, PIFs are degraded via phytochrome-dependent pathway, resulting in the upregulation of downstream gene expression. Since the negative gravitropism of *PIF* quadruple mutant (*pifQ*) hypocotyls grown in darkness was substantially impaired, like as that of *pifQ* or WT hypocotyls grown under continuous red light, it is indicated that PIFs function to maintain the negative gravitropism of hypocotyls in darkness (Kim et al. 2011). Since the amyloplast size in the *pifQ* hypocotyls was significantly smaller than that in WT hypocotyls, and the sedimentation speed of amyloplasts in the *pifQ* hypocotyls was lower than that in WT hypocotyls, the negative gravitropism of hypcotyls might be regulated through the amyloplast size and

mobility. Kim J et al. (2016) further showed that the amyloplast size in WT hypocotyls grown under continuous red light also reduced, and it was mediated by phytochrome B expressed in the epidermis of hypocotyls. Therefore, in adzuki bean epicotyls, it is possible that the repression of gravitropism under red light is regulated through phytochrome signaling.

The repression of gravitropism by light possibly provides etiolated adzuki bean epicotyls with the cue to start circumnutation. Since the shoot of plants maintains standing straight by negative gravitropism, if the gravitropism is repressed, such shoots may tend to tilt. Since gravitropism is not completely lost even in such epicotyls, auxin is asymmetrically distributed in the lower side of the tilted epicotyls with respect to the gravity direction, through the re-localization of PIN auxin carriers. Although it is not clear at present how the hormone distribution starts to sequentially shift in the circumferential direction, the initial asymmetry in hormone distribution in circumnutation may occur at this time point. In etiolated pea epicotyls, their irregular movements were sometimes detected even in darkness (Britz and Galston 1982a). Based on this observation, Britz and Galston (1982b) proposed that light may enhance the movements of etiolated pea epicotyls to those with higher amplitude and eventually periodic nutations. If the periodic movements in pea epicotyls are inhibited by stronger gravitropism in darkness, and the inhibition is repressed by light, my hypothesis can explain their proposition. Since the onset of circumnutation is clearly detected in adzuki bean epicotyls exposed to light, the causal relationship between the responsiveness to gravity and the induction of circumnutation may be clarified by studying adzuki bean mutants that exhibit a reduced gravitropism, which have not yet been identified though.

Gravitropic response and amyloplast mobility

The epicotyls illuminated with red light for 24 h did not exhibit a reduced gravitropism (Fig. 27c), whereas the amyloplasts in these epicotyls were significantly smaller than those in the epicotyls kept in darkness (Fig. 28e). Since the amyloplasts in these epicotyls did not sink to the bottom of a cell as slow as those in the epicotyls exposed to red light for 36 h (Fig. 28f, g), it is suggested that the effect of the amyloplast degradation on gravitropism is only partial one, and that the effect of the amyloplast mobility on gravitropism is more critical. In the mechanism regulating the amyloplast mobility, one of the possible targets of red light is a development of vacuolar structure. In the endodermis of *A. thaliana* inflorescence stems, amyloplasts are tightly associated with the vacuolar membrane, and the sedimentation of amyloplasts is severely restricted in the agravitropic mutant *zigzag-1*, which harbors the abnormally fragmented vacuoles (Morita et al. 2002; Saito et al. 2005). To understand the detailed mechanism how red light regulates gravitropism, it should be investigated if red light regulates the development of vacuolar structure and the mobility of amyloplasts.

Although the molecular mechanism through which amyloplasts transfer the gravity direction to the endodermis has not been completely characterized, the kinetic energy of amyloplasts is likely to be important to elicit the downstream signals. There is a hypothesis that sedimented amyloplasts give the mechanical stimuli onto the cortical endoplasmic reticulum (ER), and the ER release Ca^{2+} as the messenger of gravity response, especially in root columella cells (Morita 2010). To compare the kinetic energy of amyloplasts, I assumed an amyloplast that is a sphere, of which the area of the cross section passing through the center is equal to the mean area shown in Fig. 28e, and sinks within a cell at the assumed sedimentation rate shown in Fig. 28g. In the epicotyls exposed to red light for 36 h, the assumed kinetic energy of amyloplast at 5

min of the inversion is less than 6% of that in the epicotyls kept in darkness for 36 h, and 20% of that in the epicotyls exposed to red light for 24 h. Such a substantial reduction in kinetic energy is likely to cause a decrease in gravity perception in the epicotyls.

Induction of circumnutation and the change in the elongation pattern of epicotyls by light

At around the onset of circumnutation, the adzuki bean epicotyls exposed to continuous red or blue light exhibited the elongation patterns similar to that of greened epicotyls, not of etiolated epicotyls kept in darkness (Fig. 25, Fig. 26). Such light-dependent changes in the elongation pattern, one of the typical photomorphogenetic responses, may be helpful for the epicotyls to start circumnutation. In seedlings grown under light, the cell size and cell wall plasticity are smaller and lower, respectively, than those in seedlings grown in darkness (Kutschera and Niklas 2013). Therefore, the basal region of the epicotyls exposed to red or blue light may gain the mechanical strength to support the dynamic and periodic movement of the apical elongating region. However, the causal relationship between the change in the elongation pattern and the induction of circumnutation is still unknown at present.

When I specified the bending zone of circumnutation in adzuki bean epicotyls, its basal end in greened epicotyls was almost identical to the apical end of the completed region, while that in the epicotyls exposed to red or blue light was detected with some distance above the apical end of the completed region (Fig. 5a, Figs. 25b, c). Nevertheless, in both cases, the bending zone was always located in the more or less fixed distance from the tip. It was always located in the region 1–3 cm below the tip of greened epicotyls under white light, and in the region 2–4 cm below the tip of epicotyls

exposed to red or blue light (Fig. 5a, Figs. 25b, c). These results suggest that the location of the bending zone is determined by the distance not from the basal completed region but from the apical tip of epicotyls. It probably depends on the localization of the mechanism regulating the asymmetric distribution of hormones.

Possible photoreceptors regulating circumnutation

In adzuki bean epicotyls, the amplitude of circumnutation under red light is significantly smaller than that under white or blue light, but the period is not different (Table 1). There are two possible explanations for these light effects on the amplitude of circumnuation. First one is that red light reduces the amplitude of circumnutation through the reduction of the responsiveness to gravity of the epicotyls. In *A. thaliana* inflorescence stems of the agravitropic mutant *shoot gravitropism 5* (*sgr5*), the amplitude of circumnutation was smaller than that of WT (Tanimoto et al. 2008). In rice coleoptiles of the *lazy* mutant, which has the reduced responsiveness to gravity, circumnutation could not be detected (Yoshihara and Iino 2006). In pea roots of the agravitropic *ageotropum* mutant, the period of circumnutation was longer, and the amplitude was smaller than those of WT (Kim HJ et al. 2016). Furthermore, in *A. thaliana* inflorescence stems of the *scarecrow* (*scr*) mutant, which has neither the endodermis nor the responsiveness to the gravity of the shoot, circumnutation was not detected (Kitazawa et al. 2005). Since the red-light illumination for 36 h reduces gravitropism of adzuki bean epicotyls (Fig. 27), it may cause the suppression of the amplitude of circumnutation other than the induction of circumnutation. In this case, phytochromes are candidates for the photoreceptor mediating the response since the reduction of gravitropism may depend on the phytochrome signaling (Kim et al. 2011; Kim J et al. 2016).

Second possibility is that blue light enhances the amplitude of circumnutaion. In pea epicotyls exposed to blue light, the maximum value of the amplitude of nutation increased although the period and the mean value of the amplitude were not affected (Baskin 1986). Therefore, if adzuki bean also has a mechanism to enhance the amplitude of circumnutation responding specifically to blue light, the small amplitude of circumnutation under red light is attributable to the absence of blue-light signaling. In this case, since phototropism is supposed to enhance the curvature of circumnutation, phototropin is a candidate photoreceptor. To understand the pathway through which light regulates circumnutation, these possibilities should be further investigated.

Significance of circumnutation

Although Darwin and Darwin (1880) proposed that circumnutation helps to grow through the soil and to seek mechanical support for the shoot of plants, and it is believed that circumnutation is helpful for plants to find suitable environment, the significance of circumnutation is still unclear. An experimental approach was conducted only in rice, and it demonstrated that circumnutation plays an important role to establish the seedlings on the flooded soil (Inoue et al. 1999). In this study, I found that adzuki bean epicotyls start circumnutation under red or blue light (Fig. 18). This environmental cue of light implies the purpose of the movement. In other words, circumnutation in adzuki bean epicotyls may play a role after the epicotyls emerge from the soil. For example, circumnutation may help the epicotyls to find mechanical support, or the sunny space in concert with phototropism. Although the cultivar used in this study does not need mechanical support to grow, the nature of the wild species might be conserved. On the other hand, in rice coleoptiles, circumnutation is inhibited by red light (Yoshihara and Iino 2005). In this case, circumnutation may help the coleoptiles to

grow up through the soil. As mentioned above, since the mode of regulation of circumnutation is different in different species, it is implied that the significance of circumnutation is also different. However, each plant species or organ eventually makes similar periodic and circular movements. This fact suggests that circumutation has some conserved significance in plants that we have not yet been able to disclose.

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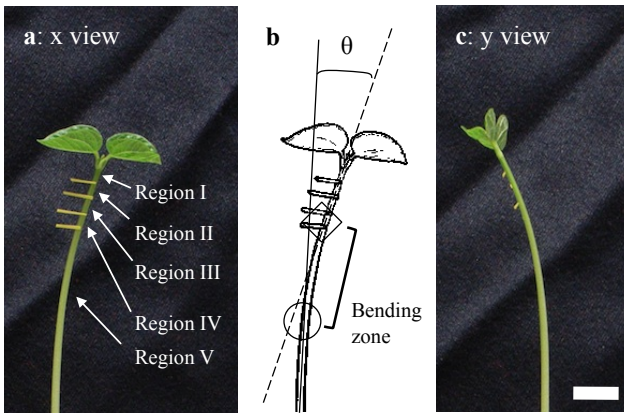

Figure 1. Greened adzuki bean epicotyls used for growth movement analyses. Photographs of 5-day-old greened epicotyls were taken in the plane parallel (a; x view) or perpendicular (c; y view) to the first leaves. Four pieces of tapes 1 mm in width were pasted onto each epicotyl every 3 mm from the tip to trace the elongation pattern. Regions between the tip and the top piece, between each piece, and between the bottom piece and the base, respectively, were defined sequentially as the regions I–V. (b) Scheme to determine the angle of curvature of circumnutation and the bending zone on the epicotyl. The curvature of circumnutation was defined as the angle (θ) between the longitudinal center line of the basal straight region of the epicotyl (solid line) and that of the apical straight region (broken line). The bending zone was defined as the region between two positions where the longitudinal center line of the epicotyl diverges from that of the basal straight region (open circle) and where from that of the apical straight region (open diamond). Bar = 1 cm.

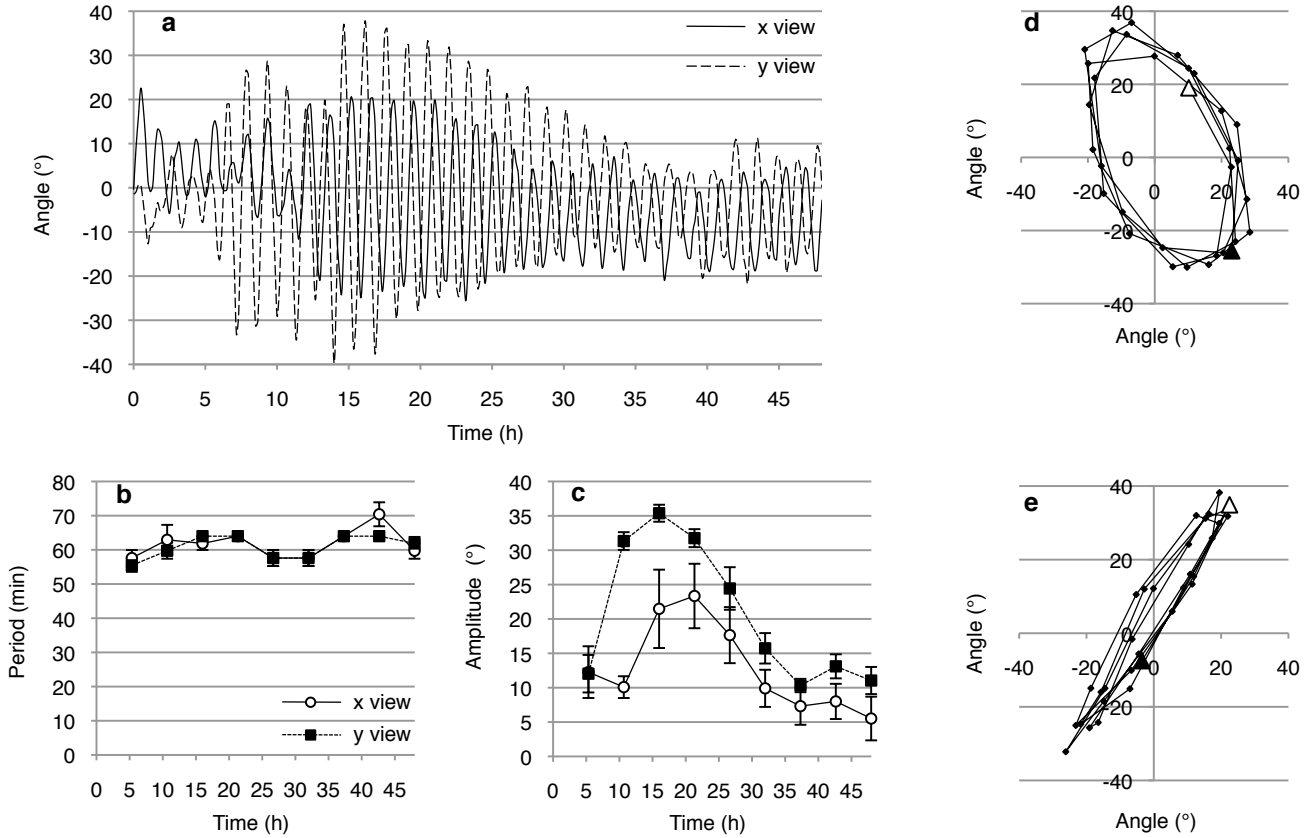

Figure 2. Circumnutation of greened adzuki bean epicotyls under white light. (a) Changes in the angle of circumnutation in a representative 5-day-old epicotyl during a 48 h observation. The *solid line* shows θ in x view and the *broken line* shows θ in y view. (b, c) Parameters of circumnutation detected with fast Fourier transform. The mean period (b) and amplitude (c) were determined every 5.3 h in the x view (*open circles*) and in the y view (*closed squares*), respectively (P_x, P_y, A_x, and A_y in Materials and Methods). The *vertical bar* on each point shows SE (n = 5). (d, e) Horizontal projections of movements of epicotyl tips at 10 min intervals. The tip exhibited elliptical (d) or pendulum-like movements (e). In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively.

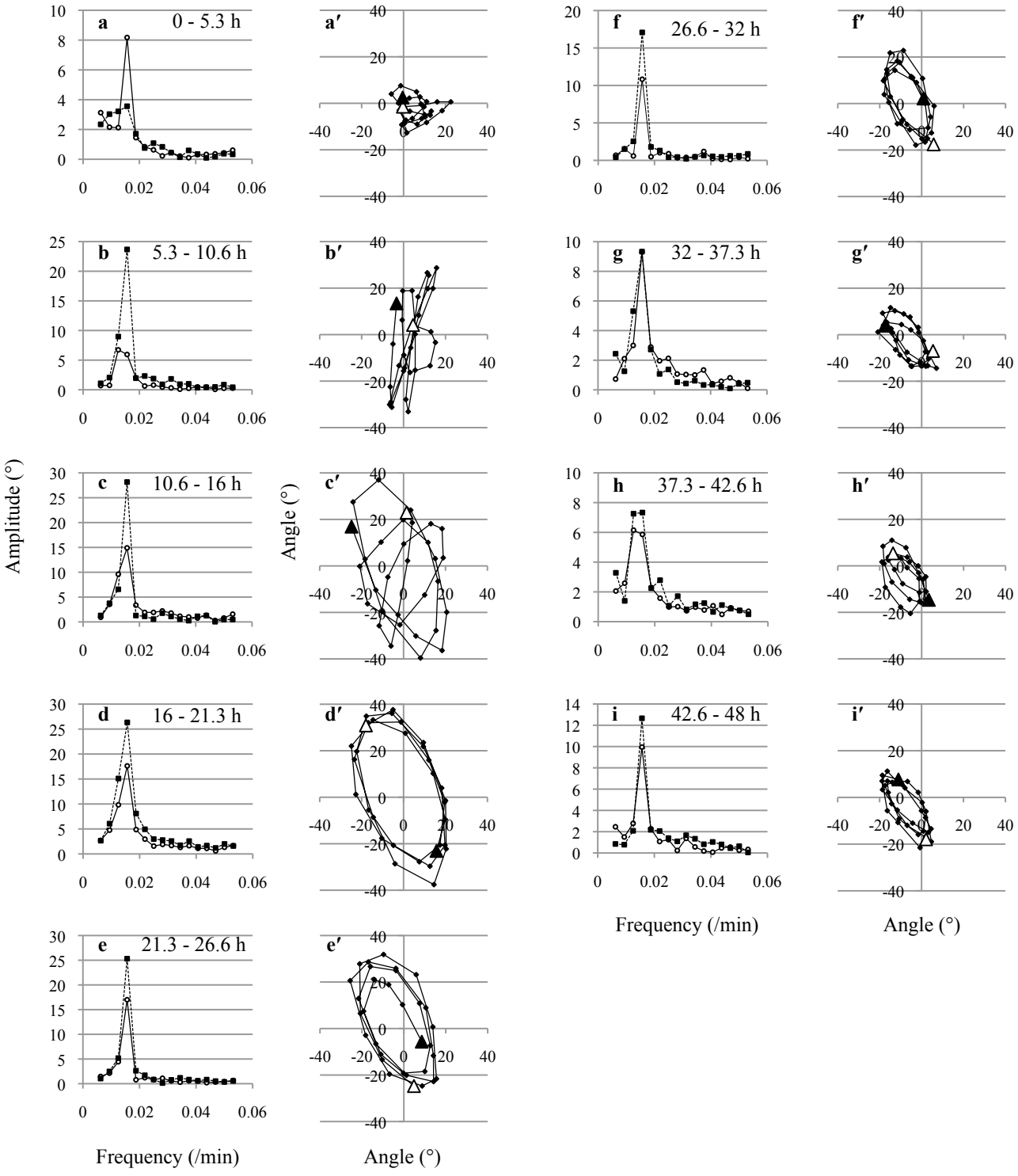

Figure 3. Movements of a greened adzuki bean epicotyl tip under white light. (a– i) Fourier spectra obtained from θ in the x view (*open circles*) and in the y view (*closed squares*). (a′– i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the same epicotyl shown in Fig. 2a were analyzed.

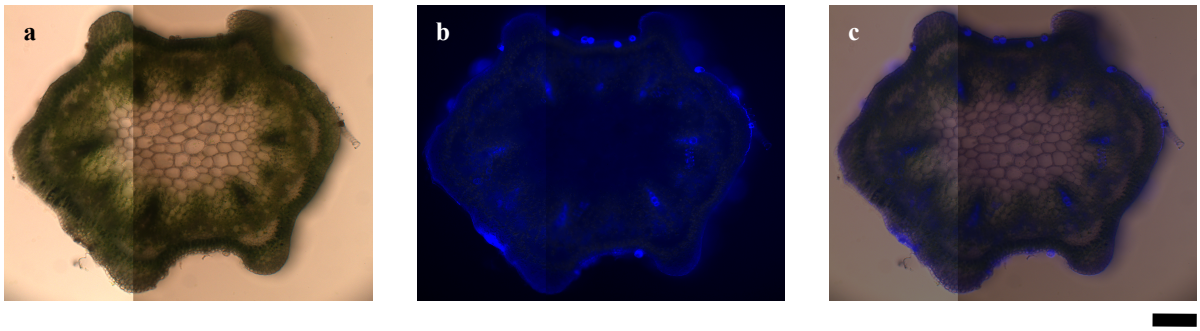

Figure 4. Cross sections at 2 cm below the tip of an adzuki bean epicotyl. (a) Bright field image, (b) lignin autofluorescent, and (c) merged image. The X-axis and the Y-axis in the photographs are parallel to the plane in the x-view and the y-view (see Fig. 1 for the definition), respectively. *Bar* = 100 μm.

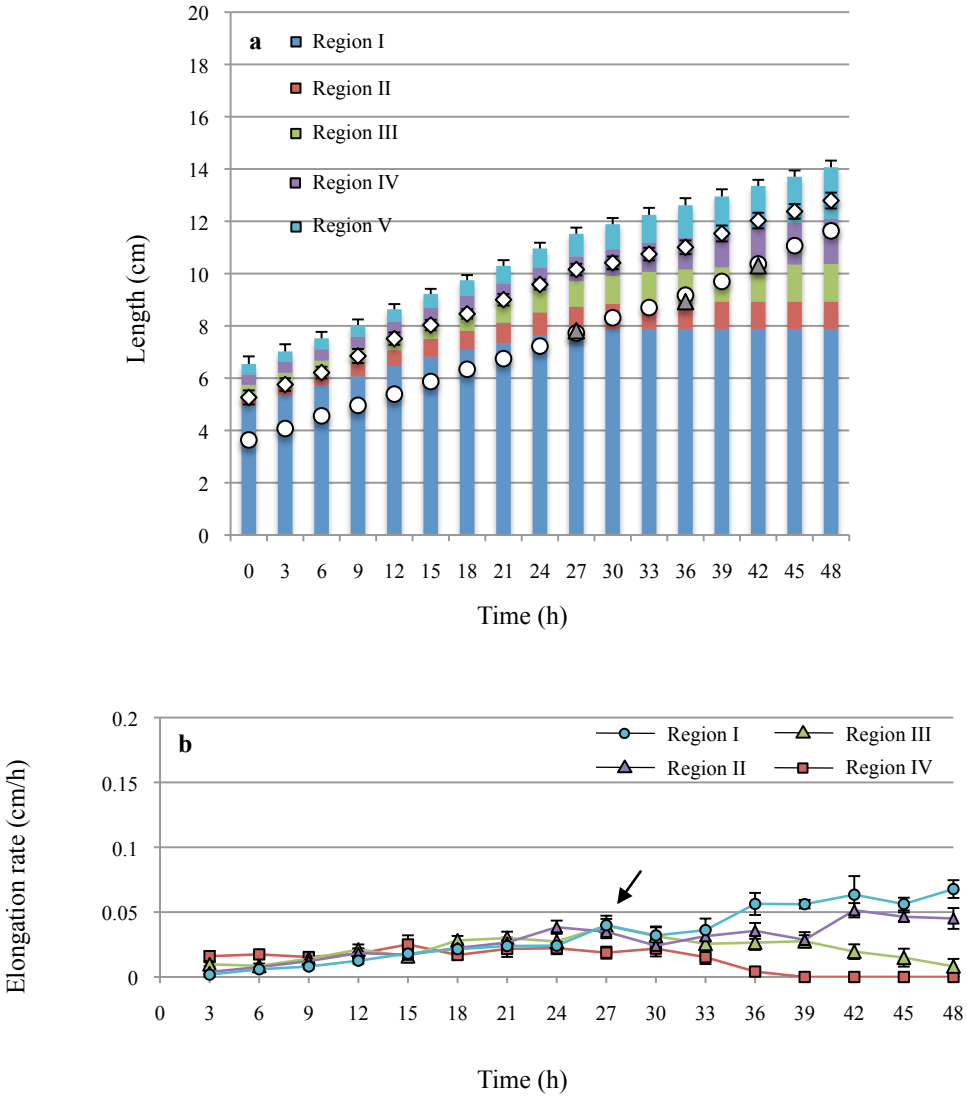

Figure 5. Elongation pattern of greened adzuki bean epicotyls under white light. (a) The elongation pattern of 5-day-old greened epicotyls under white light was examined for 48 h. See Fig. 1 for definition of the regions I–V. *Open diamonds*, *open circles*, and *gray triangles* show the positions of the apical end of the bending zone, of the basal end of the bending zone, and where the elongation of a defined region was completed, respectively. (b) The elongation rate of the regions I–IV of epicotyls shown in (a) was separately examined every 3 h. An *Arrow* indicates the time points when the suppression of elongation of the region III starts. The *vertical bar* on each point shows SE (n = 5).

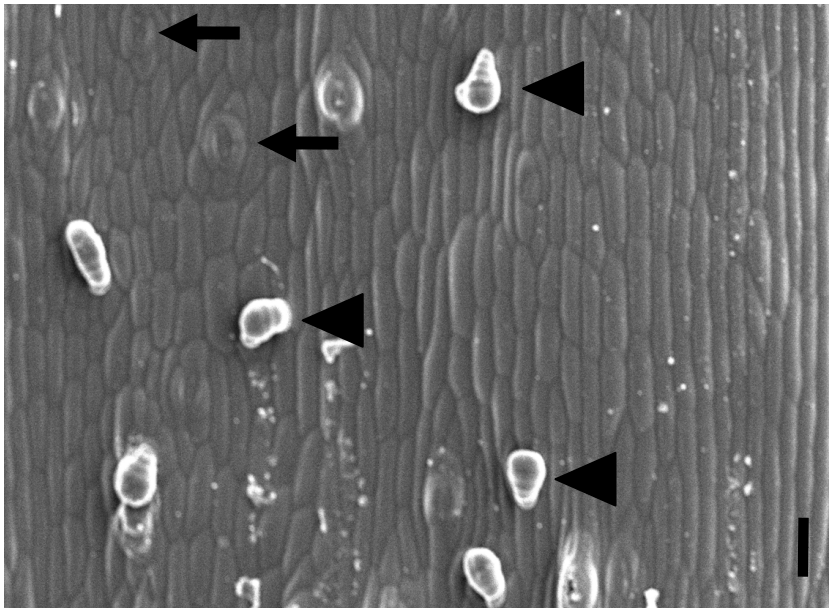

Figure 6. Epidermis of an adzuki bean epicotyl. The SEM image of the epidermis 3 cm below the tip of a vertically oriented greened epicotyl. *Arrows* indicate stomata and *arrowheads* indicate trichomes. *Bar* = 60 μm.

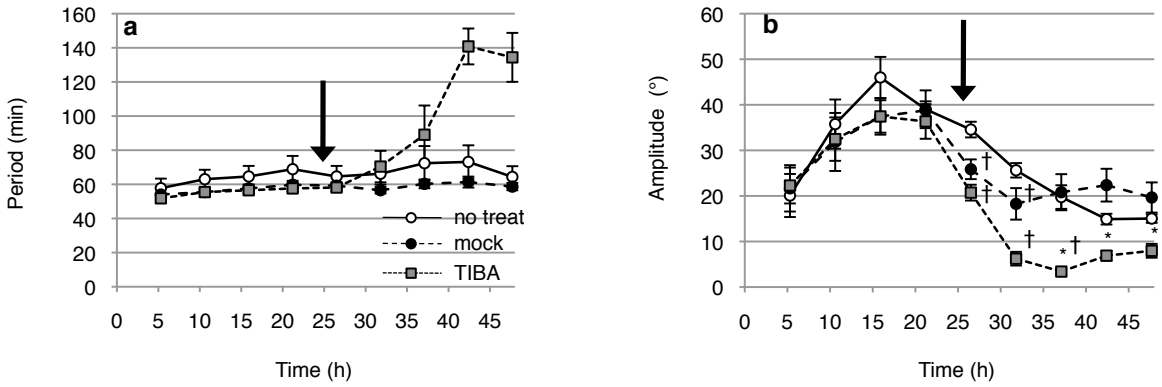

Figure 7. Effect of 2,3,5-triiodebenzoic acid (TIBA) on the period and amplitude of circumnutation of greened adzuki bean epicotyls. Greened epicotyls exhibiting circumnutation were treated either with 0.1% Tween 20 alone (*mock*) or together with 20 μM TIBA (*TIBA*) at 24 h of observation (indicated by *arrows*). The period (a) and amplitude (b) of circumnutation were determined every 5.3 h by integrating the values calculated in the x view and the y view (see Materials and Methods). The *asterisks* and *daggers* indicate significant differences between "*mock*" and "*TIBA*" and between "*no treat*" and "*mock*" or "*TIBA*," respectively, by a Tukey's test ($P < 0.05$). The *vertical bar* on each point shows SE (n = 5).

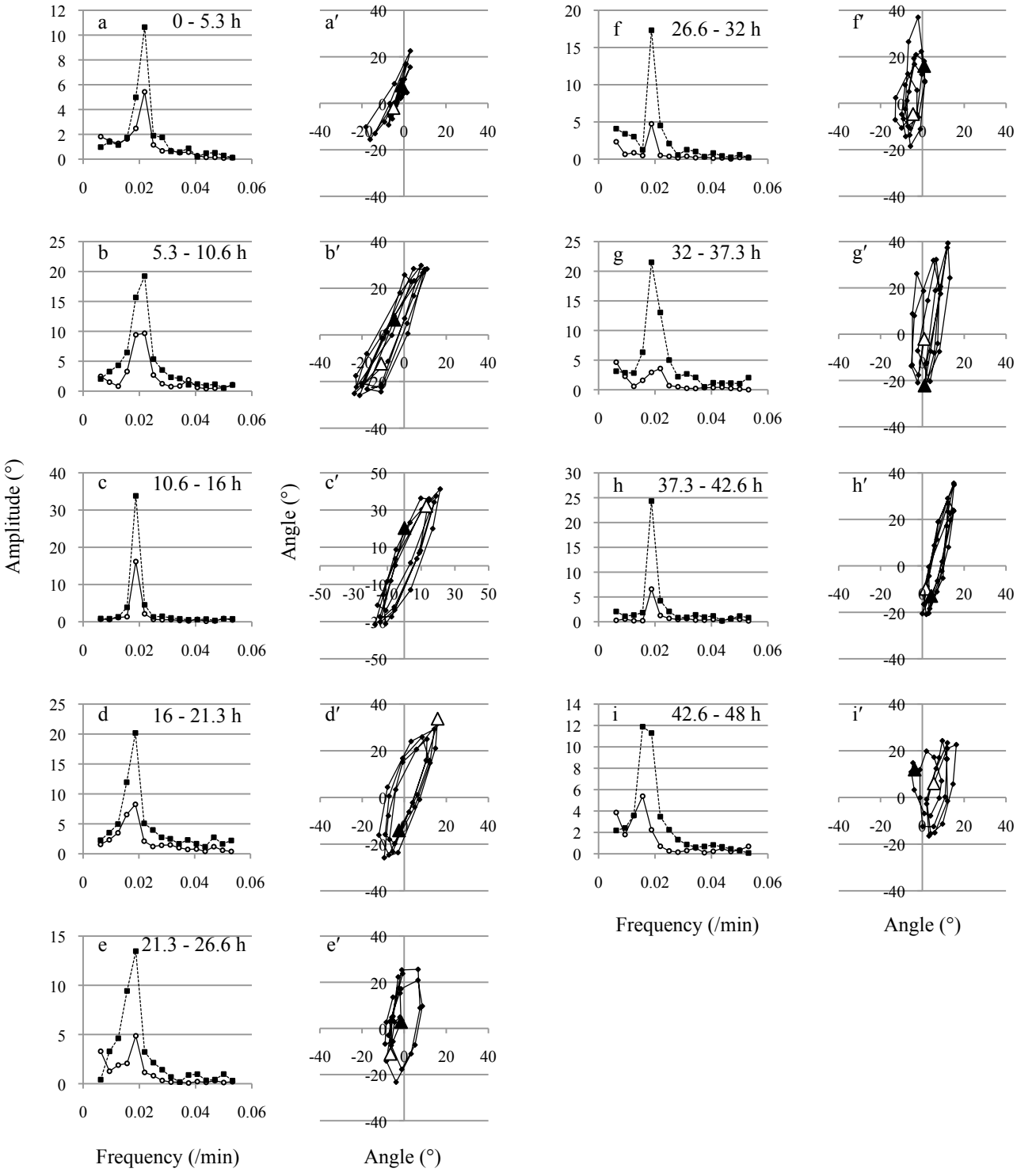

Figure 8. Movements of a greened adzuki bean epicotyl tip treated with Tween 20 at 24 h of observation. (a– i) Fourier spectra obtained from θ in the x view (*open circles*) and in the y view (*closed squares*). (a′– i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation.

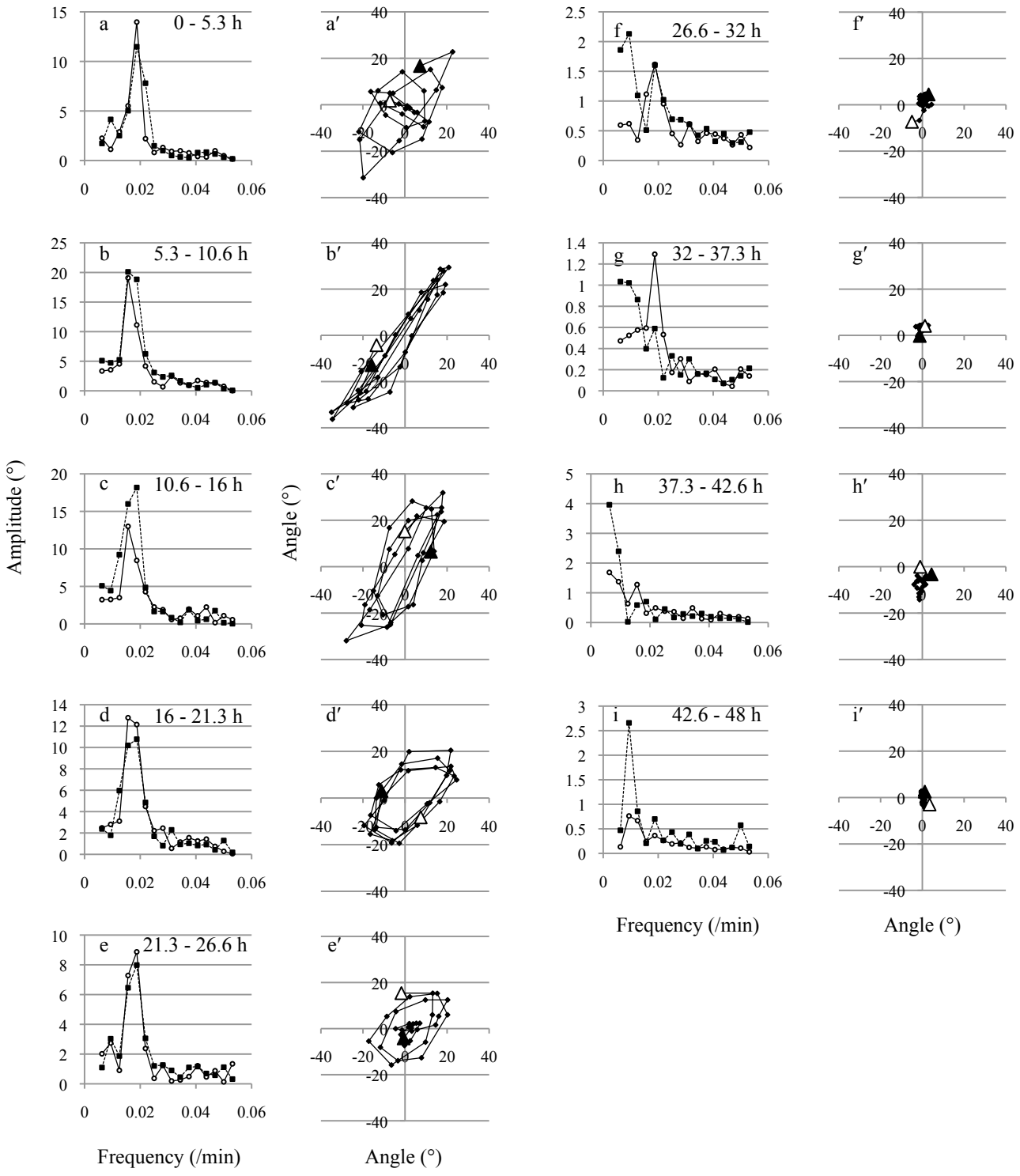

Figure 9. Movements of a greened adzuki bean epicotyl tip treated with 2,3,5-triiodebenzoic acid (TIBA) at 24 h of observation. (a– i) Fourier spectra obtained from θ in the x view (*open circles*) and in the y view (*closed squares*). (a′– i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation.

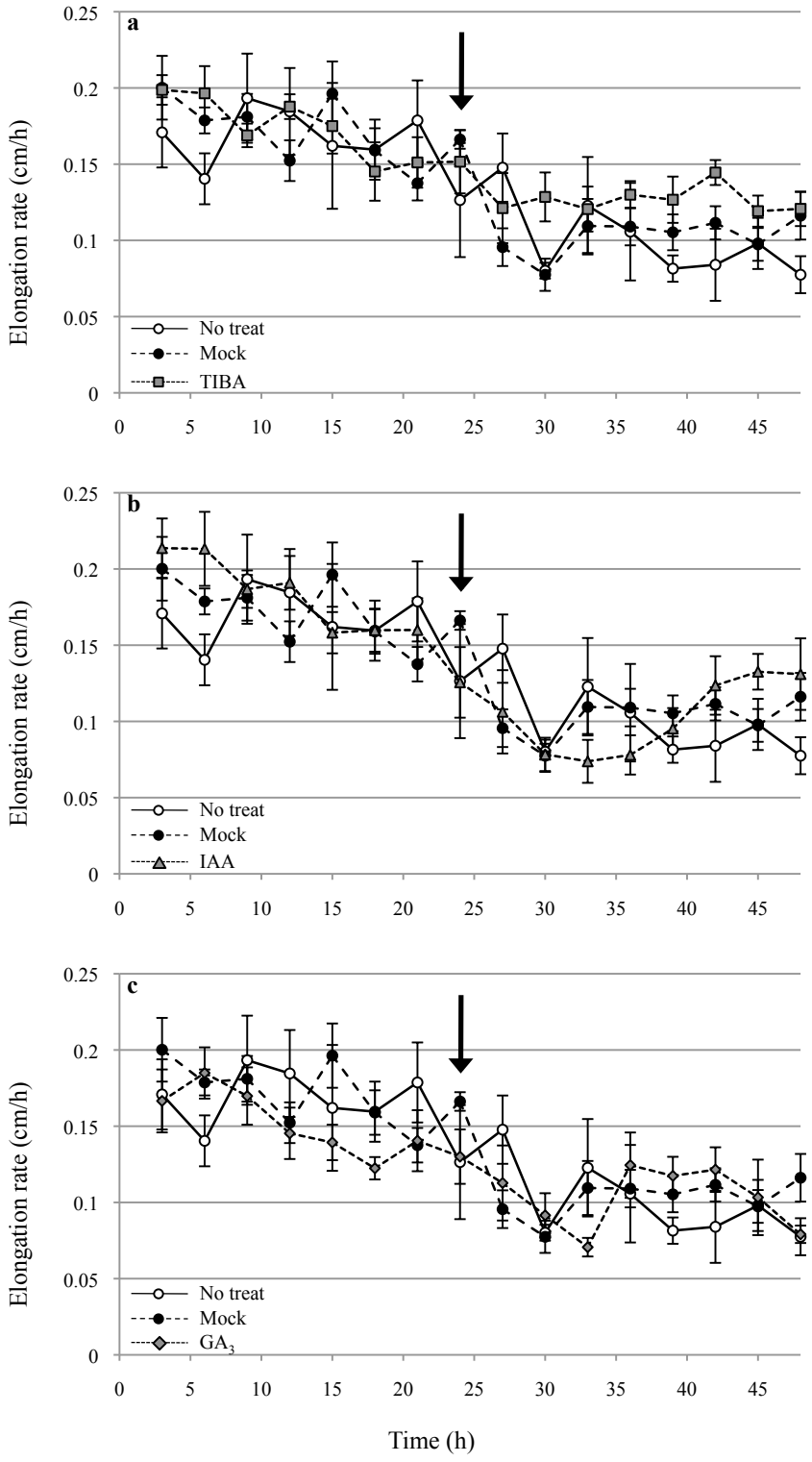

Figure 10. Effects of spray treatments on the elongation rate of greened adzuki bean epicotyls. Greened epicotyls exhibiting circumnutation were treated either with 0.1% Tween 20 alone (*mock*) or with 0.1% Tween 20 plus 20 μM 2,3,5-triiodebenzoic acid (*TIBA*) (a), 100 μM indole-3-acetic acid (*IAA*) (b), or 100 μM gibberellin A_3 (*GA_3*) (c) at 24 h of observation (indicated by an *arrow*). The elongation rates of epicotyls were determined every 3 h. No significant differences among treatments were detected by a Tukey's test. The *vertical bar* on each point shows SE (n = 5).

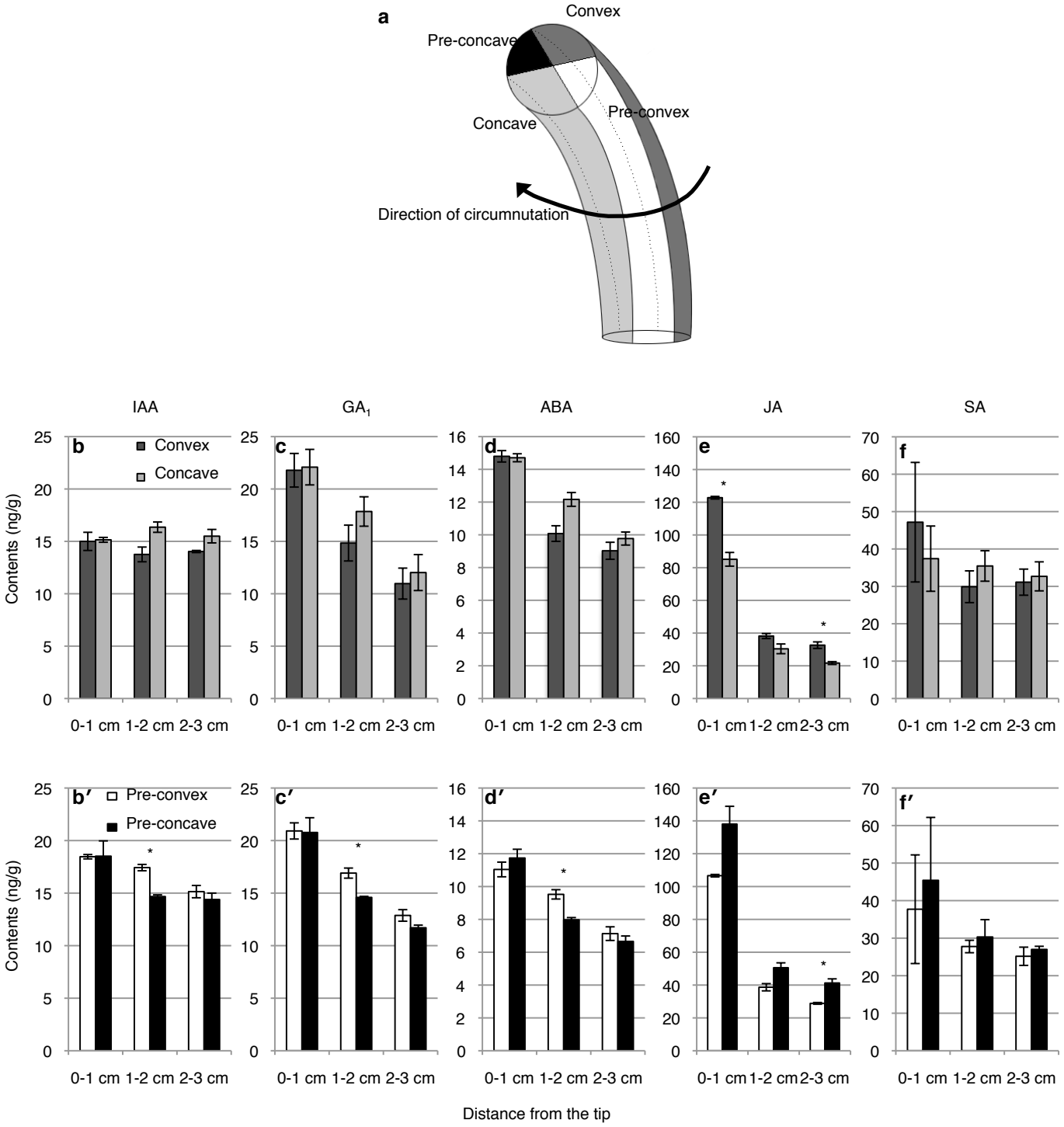

Figure 11. Distribution patterns of endogenous hormones in greened adzuki bean epicotyls exhibiting circumnutation.

(a) Scheme demonstrating the four epicotyl parts along the longitudinal axis with respect to the plane of curvature of circumnutation. The convex and concave parts are in the plane of curvature, while the pre-convex and pre-concave parts are perpendicular to the plane. (b–f, b′–f′) The contents of indole-3-acetic acid (*IAA*) (b, b′), gibberellin A_1 (GA_1) (c, c′), abscisic acid (*ABA*) (d, d′), jasmonic acid (*JA*) (e, e′), and salicylic acid (*SA*) (f, f′) in the epicotyl sections are separated into a convex/concave (b–f) or pre-convex/pre-concave pair (b′–f′). Half sections were taken from 16 seedlings in each measurement, and three independent measurements were conducted. The *asterisks* indicate significant differences between the pairs by a Student's *t*-test ($P < 0.05$). The *vertical bar* on each point shows SE.

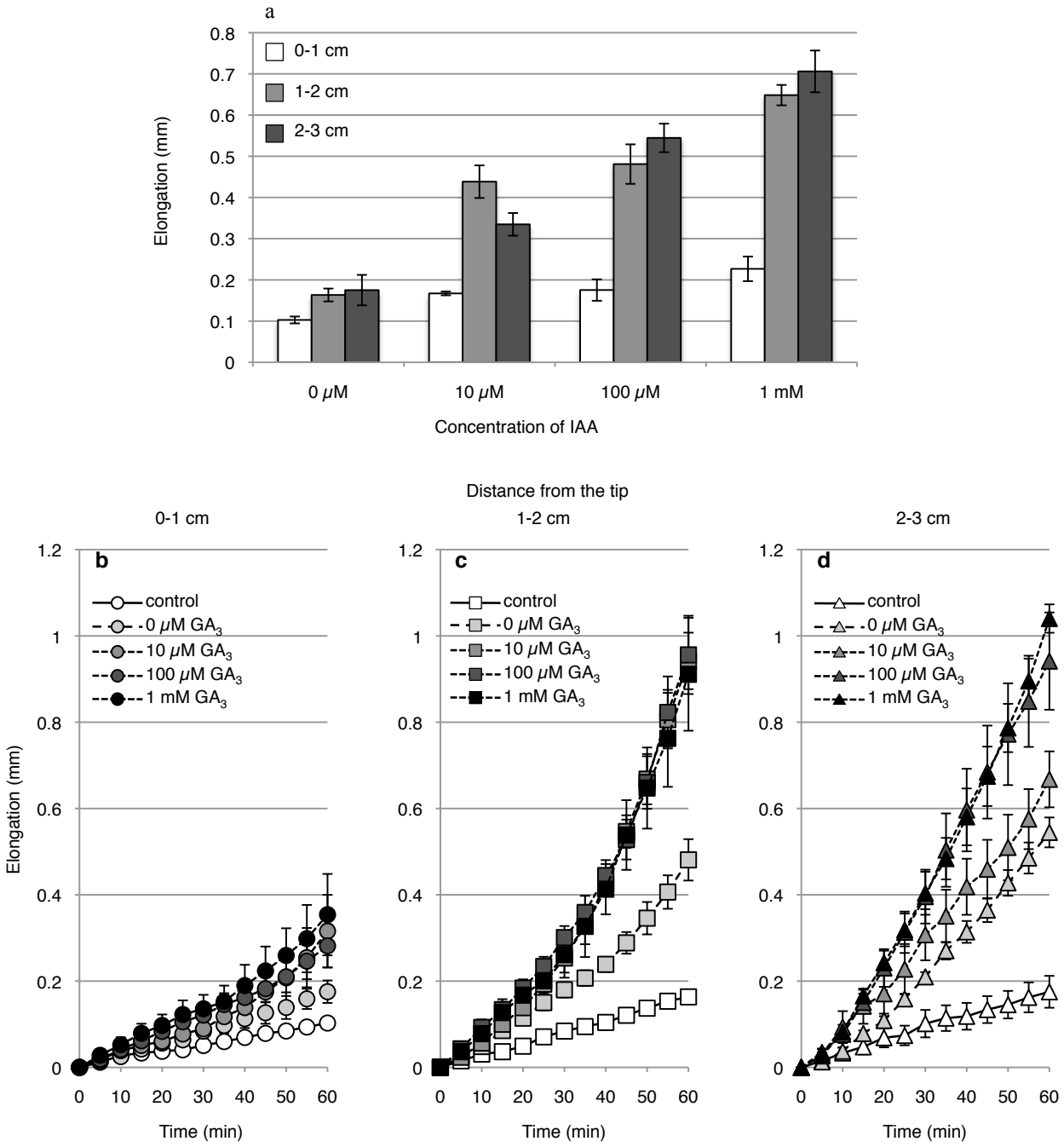

Figure 12. Effect of indole-3-acetic acid (IAA) and gibberellin A_3 (GA_3) on the elongation of sections of adzuki bean epicotyls. Epicotyl sections 0–1, 1–2, and 2–3 cm from the tip were treated with different concentrations of IAA (a), or 100 μM IAA plus different concentrations of GA_3 (b–d). Elongation of each section was monitored for 60 min at 5 min intervals. See text for statistical analyses. The *vertical bar* on each point shows SE (n = 5).

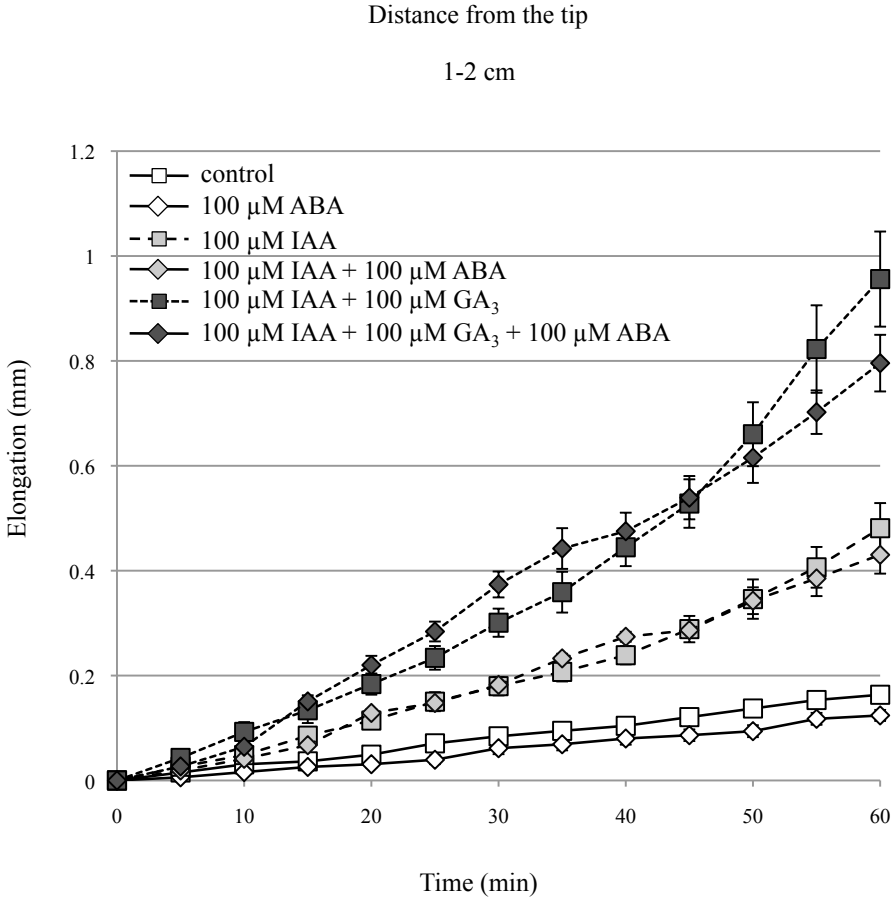

Figure 13. Effects of indole-3-acetic acid (IAA), gibberellin A_3 (GA_3), and abscisic acid (ABA) on the elongation of sections of adzuki bean epicotyls. Epicotyl sections 1–2 cm from the tip were treated with hormone solutions of different combinations. Elongation of each section was monitored for 60 min at 5 min intervals. The *vertical bar* on each point shows SE (n = 4– 5).

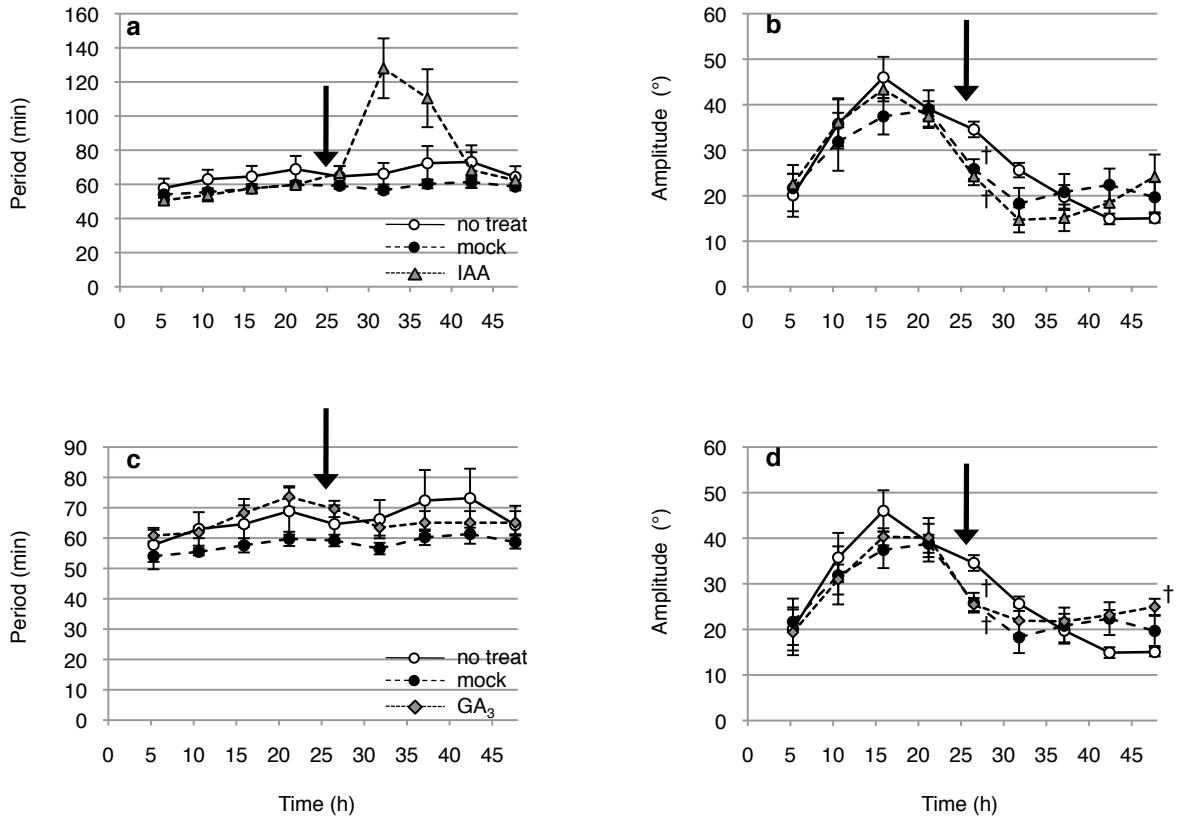

Figure 14. Effect of indole-3-acetic acid (IAA) and gibberellin A_3 (GA_3) on the period and amplitude of circumnutation of greened adzuki bean epicotyls. Greened epicotyls exhibiting circumnutation were treated with 0.1% Tween 20 alone (*mock*) or together with 100 μM IAA (*IAA*) (a, b) or 100 μM GA_3 (*GA_3*) (c, d) at 24 h of observation (indicated by *arrows*). The period (a, c) and amplitude (b, d) of circumnutation were determined every 5.3 h by integrating the values calculated in the x view and the y view (see Materials and Methods). The *daggers* indicate significant difference between "*no treat*" and "*mock*," "*IAA*," or "*GA_3*," by a Tukey's test ($P < 0.05$). The *vertical bar o*n each point shows SE (n = 5).

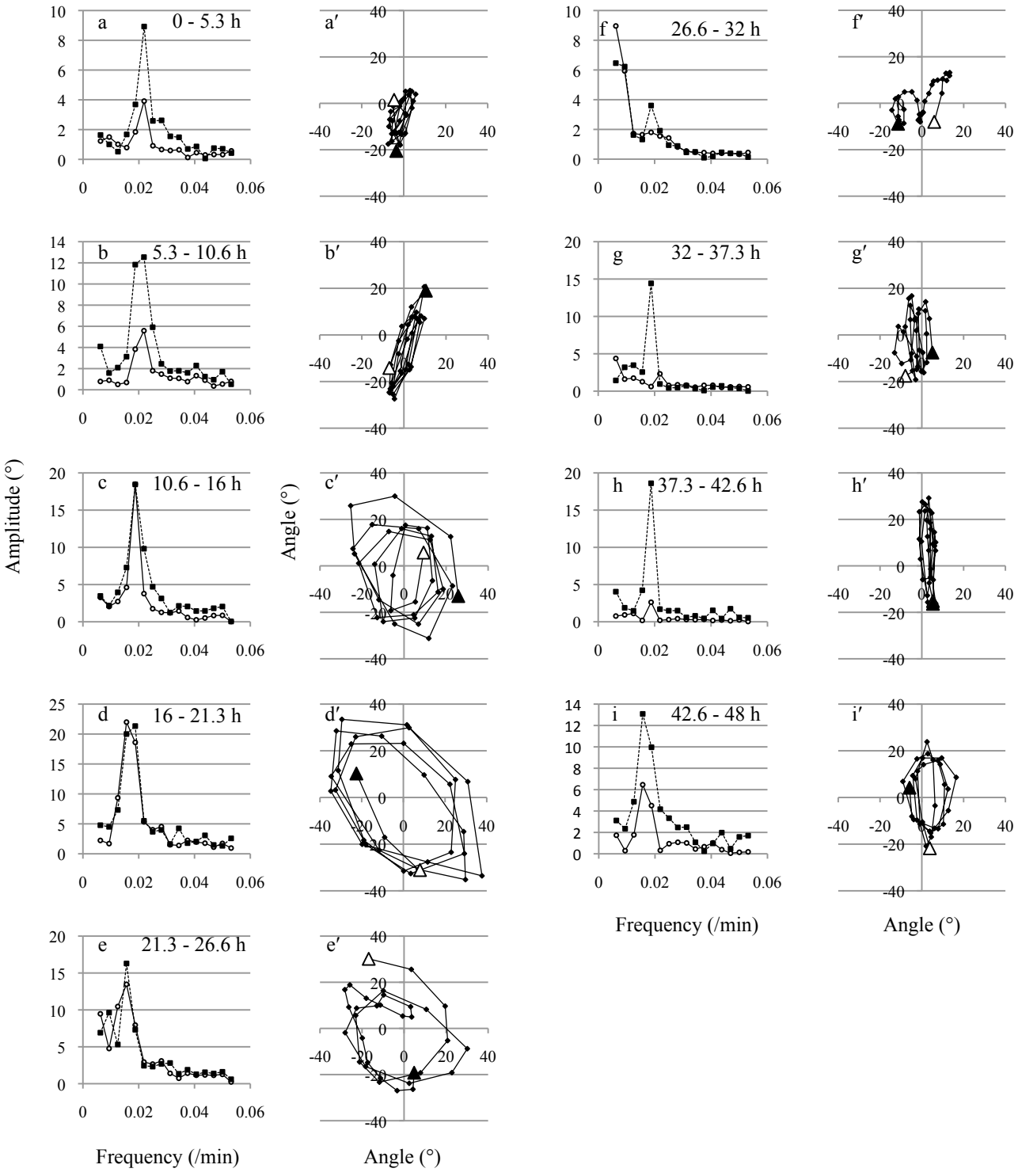

Figure 15. Movements of a greened adzuki bean epicotyl tip treated with indole-3-acetic acid (IAA) at 24 h of observation. (a– i) Fourier spectra obtained from θ in the x view (*open circles*) and in the y view (*closed squares*). (a′– i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation.

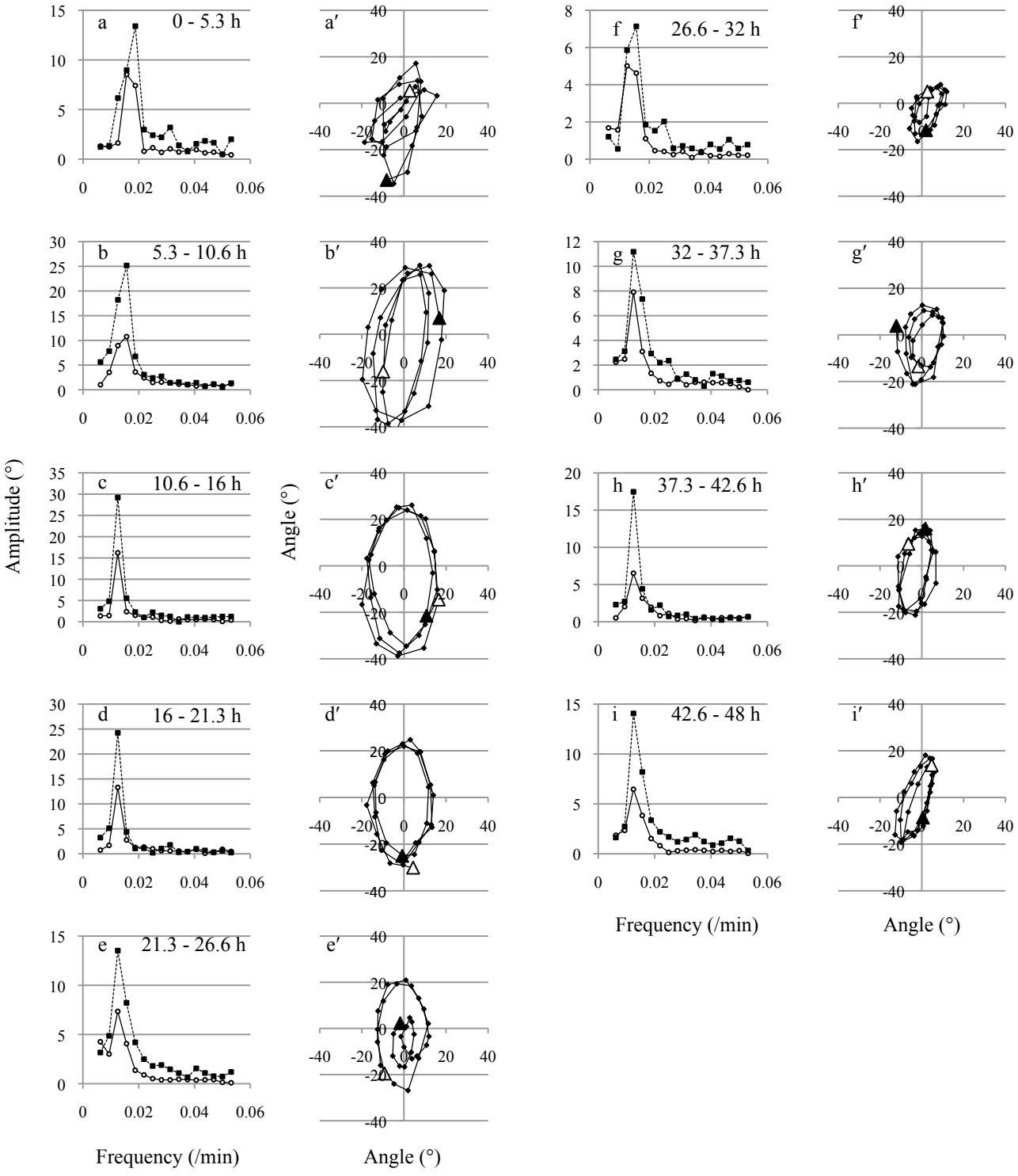

Figure 16. Movements of a greened adzuki bean epicotyl tip treated with gibberellin A_3 (GA_3) at 24h of observation.

(a– i) Fourier spectra obtained from θ in the x view (*open circles*) and in the y view (*closed squares*). (a′– i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation.

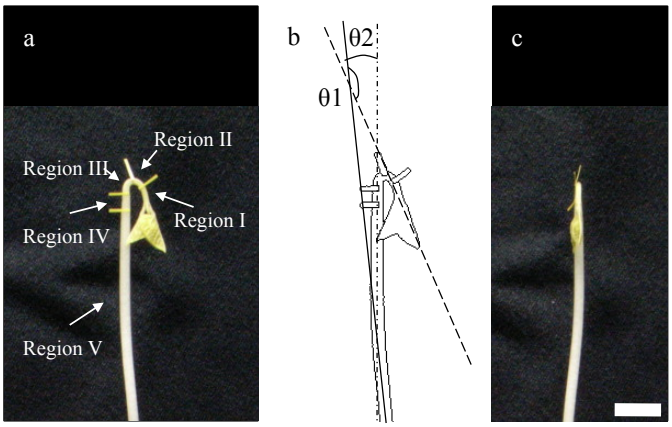

Figure 17. Etiolated adzuki bean epicotyls used for growth movement analyses. Photographs of 5-day-old etiolated epicotyls were taken in the plane parallel (a; X view) or perpendicular (c; Y view) to the apical hook. Four pieces of tapes 1 mm in width were pasted onto each epicotyl every 3 mm from the tip to trace the growth movements. Regions between the tip and the top piece, between each pieces, and between the bottom piece and the base, respectively, were defined sequentially as the regions I–V. (b) Scheme to determine the angle of curvature of the etiolated epicotyls. The angle between the tip and the base of the epicotyl was defined as the angle (*θ1*) between the longitudinal center line of the basal straight region (*solid line*) and that of the most apical straight region of the epicotyl (*broken line*). The angle between the apical hook and the base of the epicotyl was defined as the angle (*θ2*) between the longitudinal center line of the basal straight region of the epicotyl (*solid line*) and that of the basal part of the apical hook (dashed line). Bar = 1 cm.

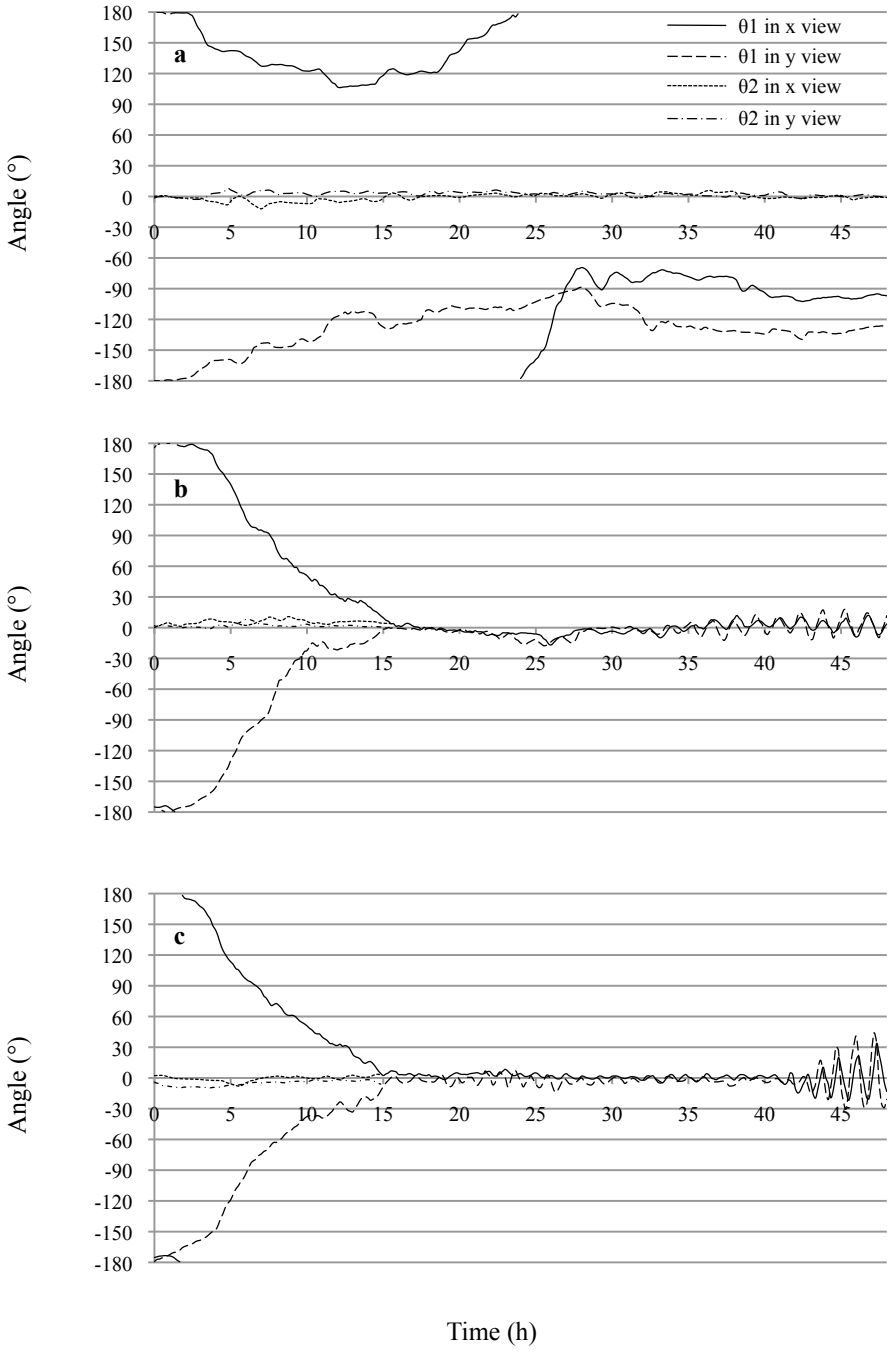

Figure 18. Movements of apical part of etiolated adzuki bean epicotyls transferred to different light conditions.

(a, b, c) Changes in the angle in representative 5-day-old epicotyls transferred to infrared (a), red (b), and blue light (c) during a 48 h observation period. The *solid*, *broken*, *dotted*, and *dashed* line show θ1 in the x view, θ1 in the y view, θ2 in the x view, and θ2 in the y view, respectively. In (b) and (c), θ2 was finally equal to θ1 because of the completion of apical hook opening at around 15 h.

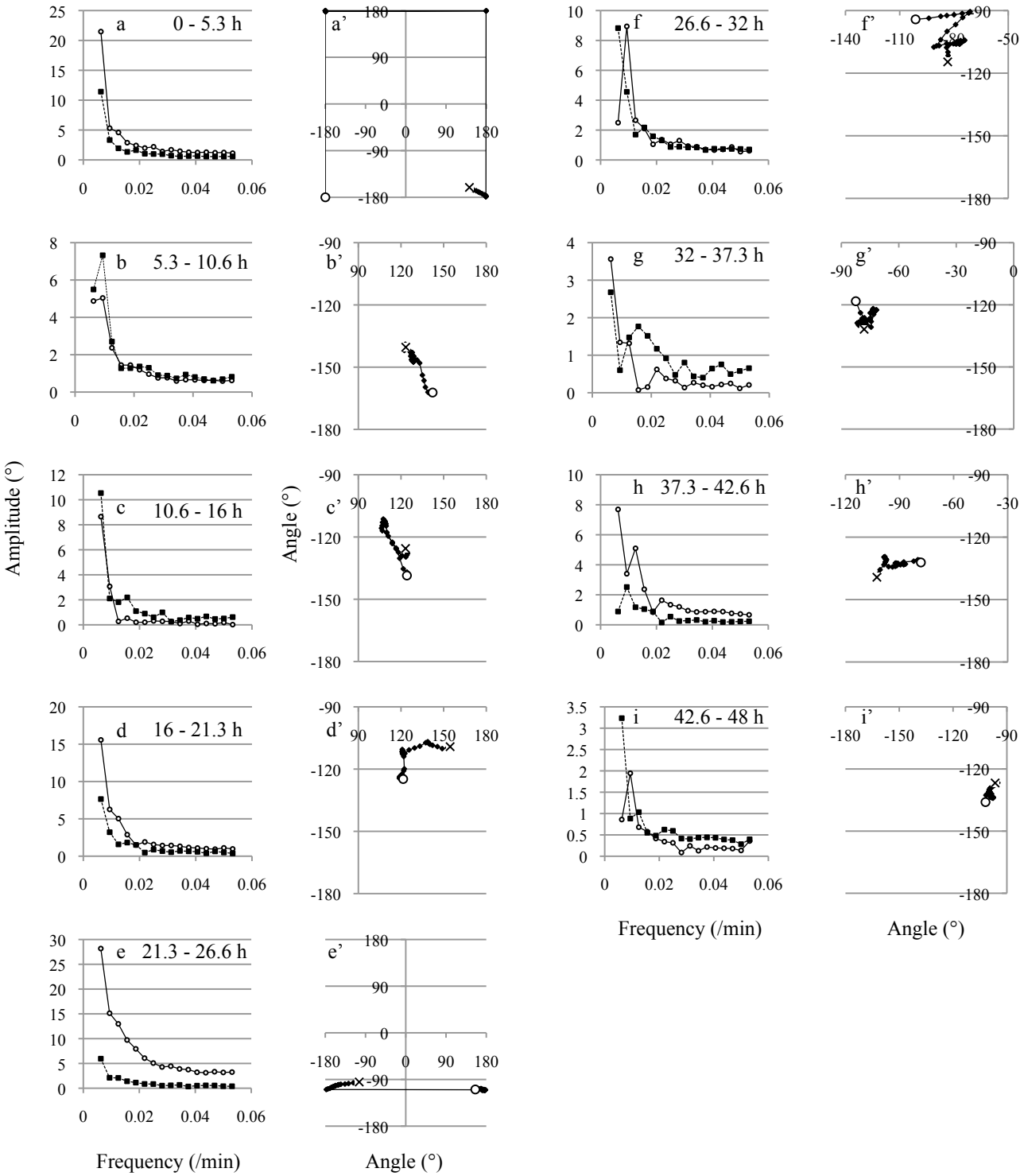

Figure 19. Movements of an etiolated adzuki bean epicotyl tip transferred to infrared light analyzed on θ1. (a–i) Fourier spectra obtained from θ1 in the x view (*open circles*) and in the y view (*closed squares*). (a′–i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18a were analyzed.

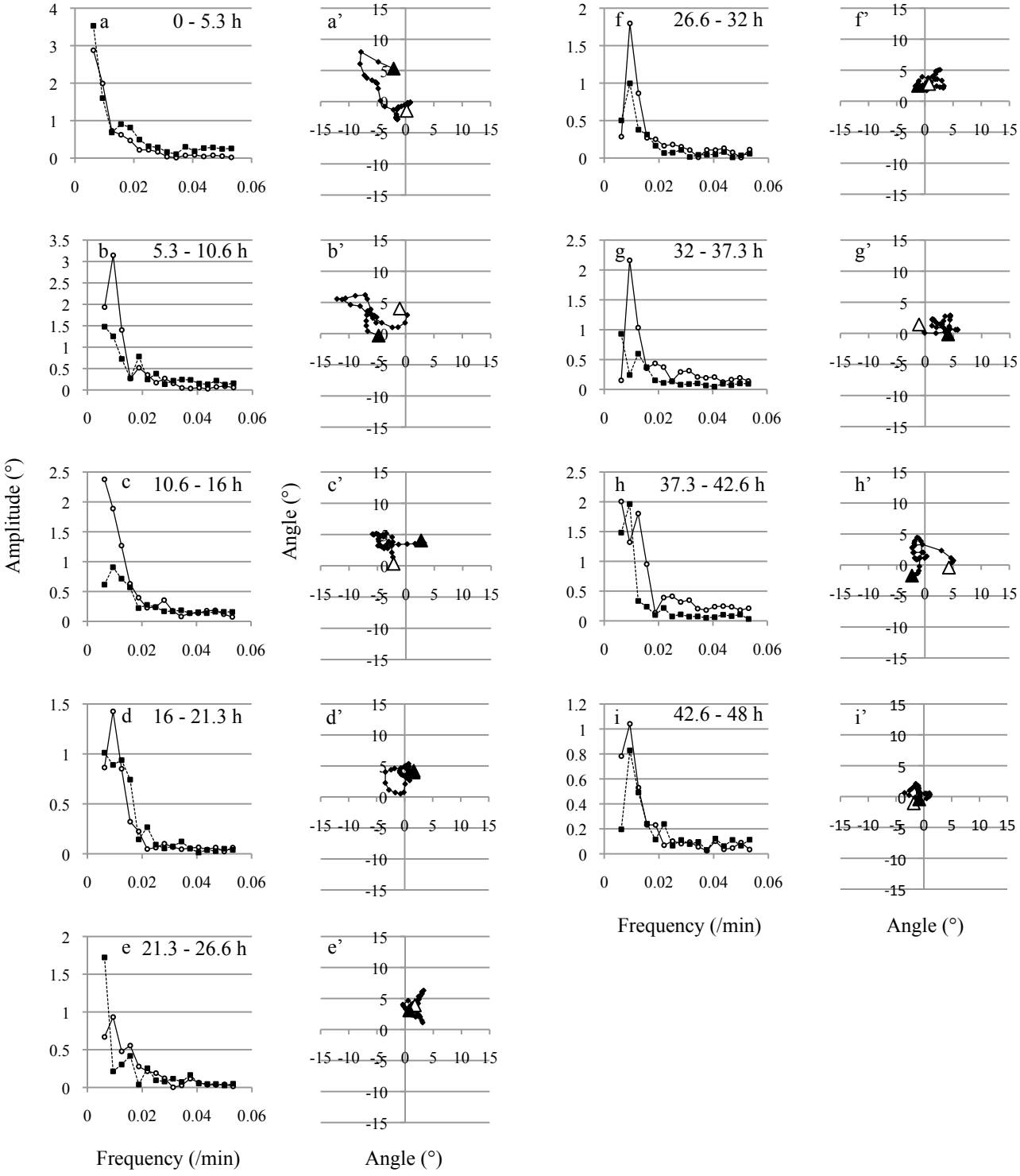

Figure 20. Movements of an apical hook of the etiolated adzuki bean epicotyl transferred to infrared light analyzed on θ2. (a–i) Fourier spectra obtained from θ2 in the x view (*open circles*) and in the y view (*closed squares*). (a′–i′) Horizontal projections of movements of the apical hook at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the apical hook, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18a were analyzed.

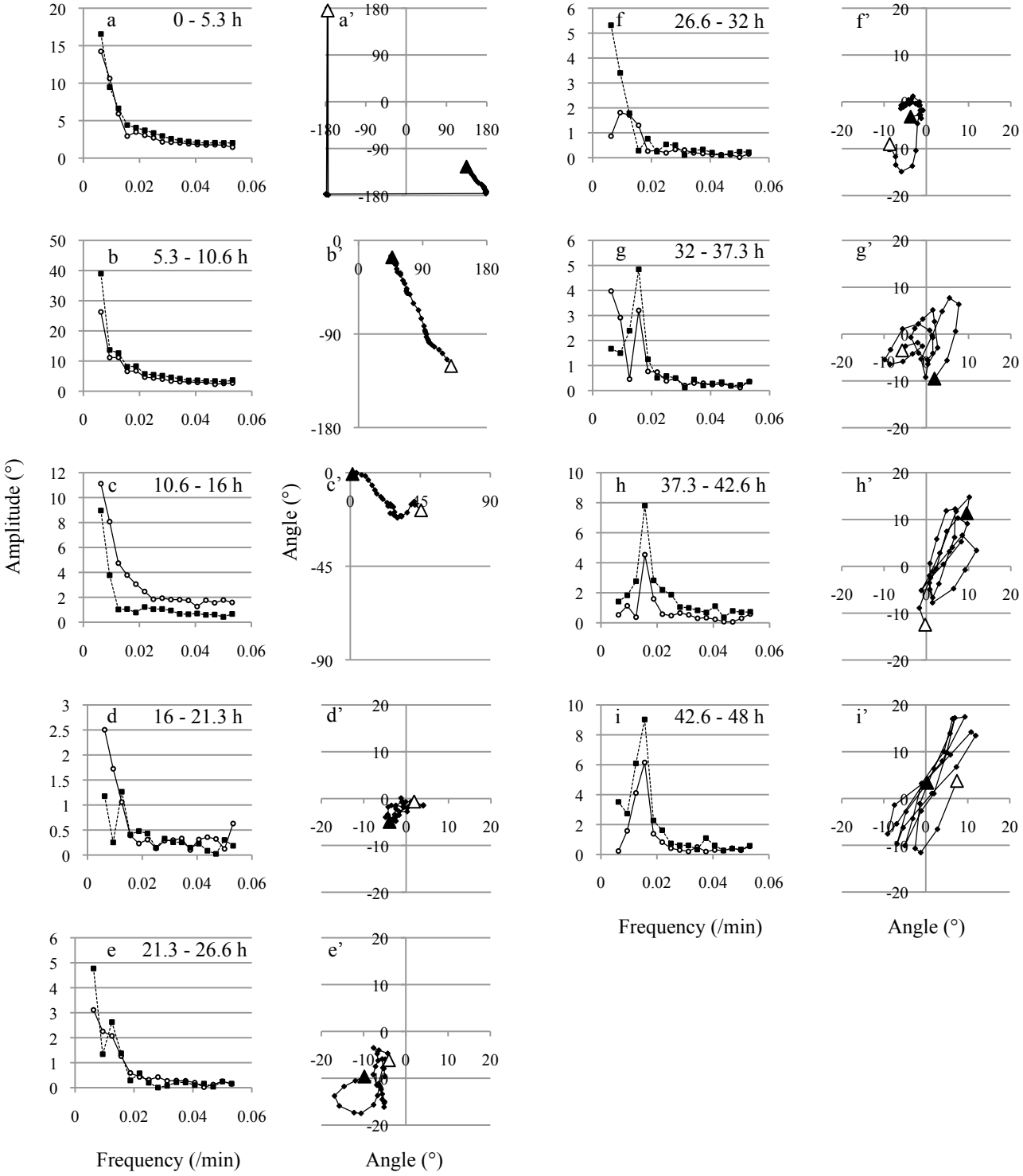

Figure 21. Movements of an etiolated adzuki bean epicotyl tip transferred to red light analyzed on θ1. (a–i) Fourier spectra obtained from θ1 in x view (*open circles*) and in y view (*closed squares*). (a′–i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18b were analyzed.

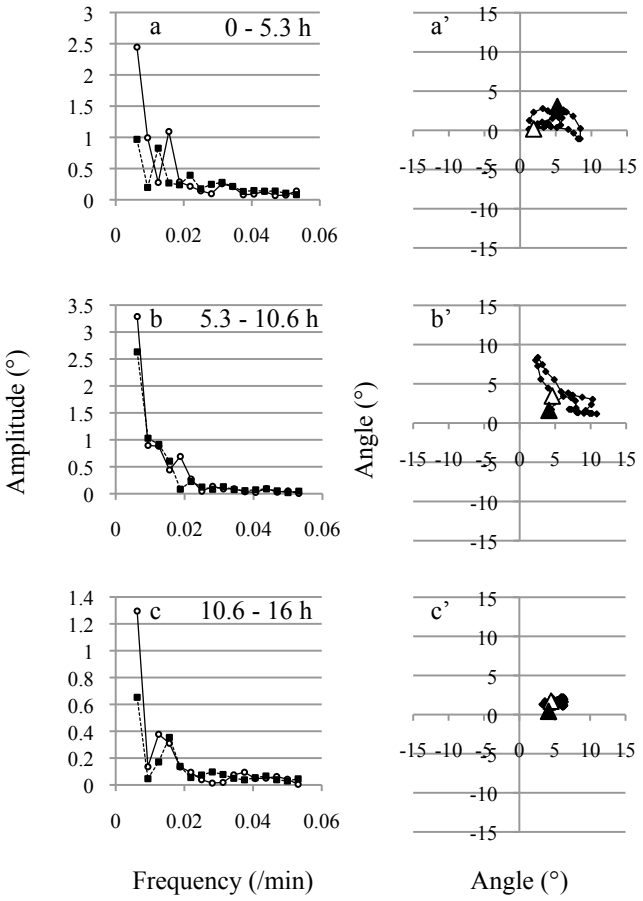

Figure 22. Movements of an apical hook of the etiolated adzuki bean epicotyl transferred to red light analyzed on θ2. (a–c) Fourier spectra obtained from θ2 in x view (*open circles*) and in y view (*closed squares*). (a′–c′) Horizontal projections of movements of the apical hook at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the apical hook, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18b were analyzed.

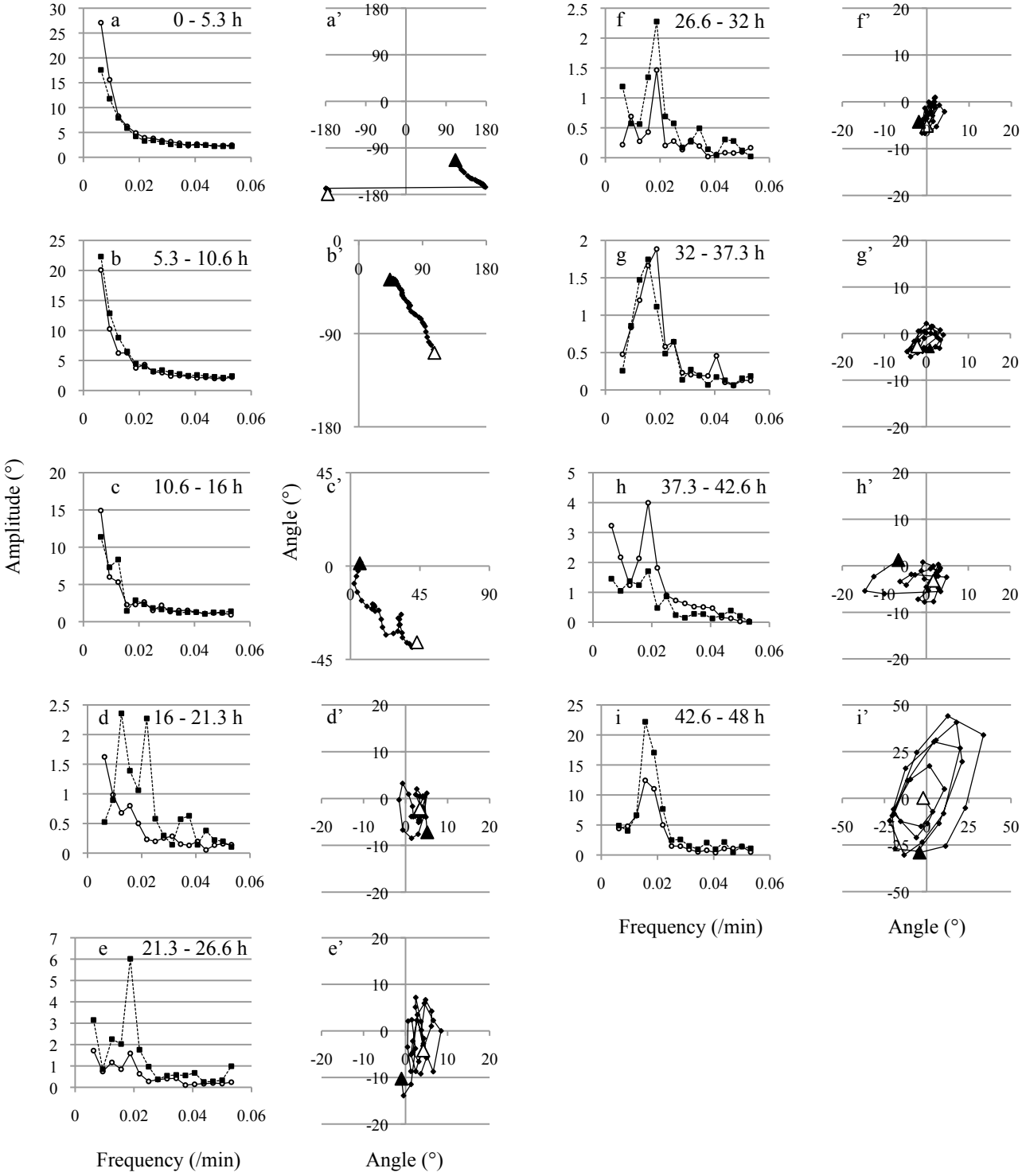

Figure 23. Movements of an etiolated adzuki bean epicotyl tip transferred to blue light analyzed on θ1. (a–i) Fourier spectra obtained from θ1 in x view (*open circles*) and in y view (*closed squares*). (a′–i′) Horizontal projections of movements of the epicotyl tip at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the epicotyl tip, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18c were analyzed.

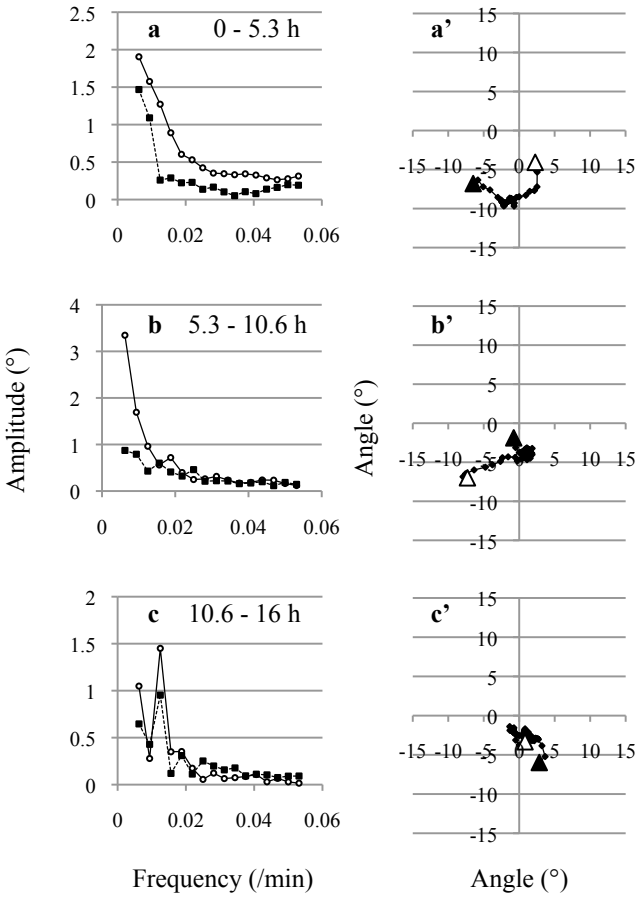

Figure 24. Movements of an apical hook of the etiolated adzuki bean epicotyl transferred to blue light analyzed on θ2. (a–c) Fourier spectra obtained from θ2 in x view (*open circles*) and in y view (*closed squares*). (a′–c′) Horizontal projections of movements of the apical hook at 10 min intervals. In each panel, an *open* and a *closed triangle* indicate the starting and final positions of the apical hook, respectively. Each graph shows the results obtained every 5.3 h of observation. Movements of the epicotyl shown in Figure 18c were analyzed.

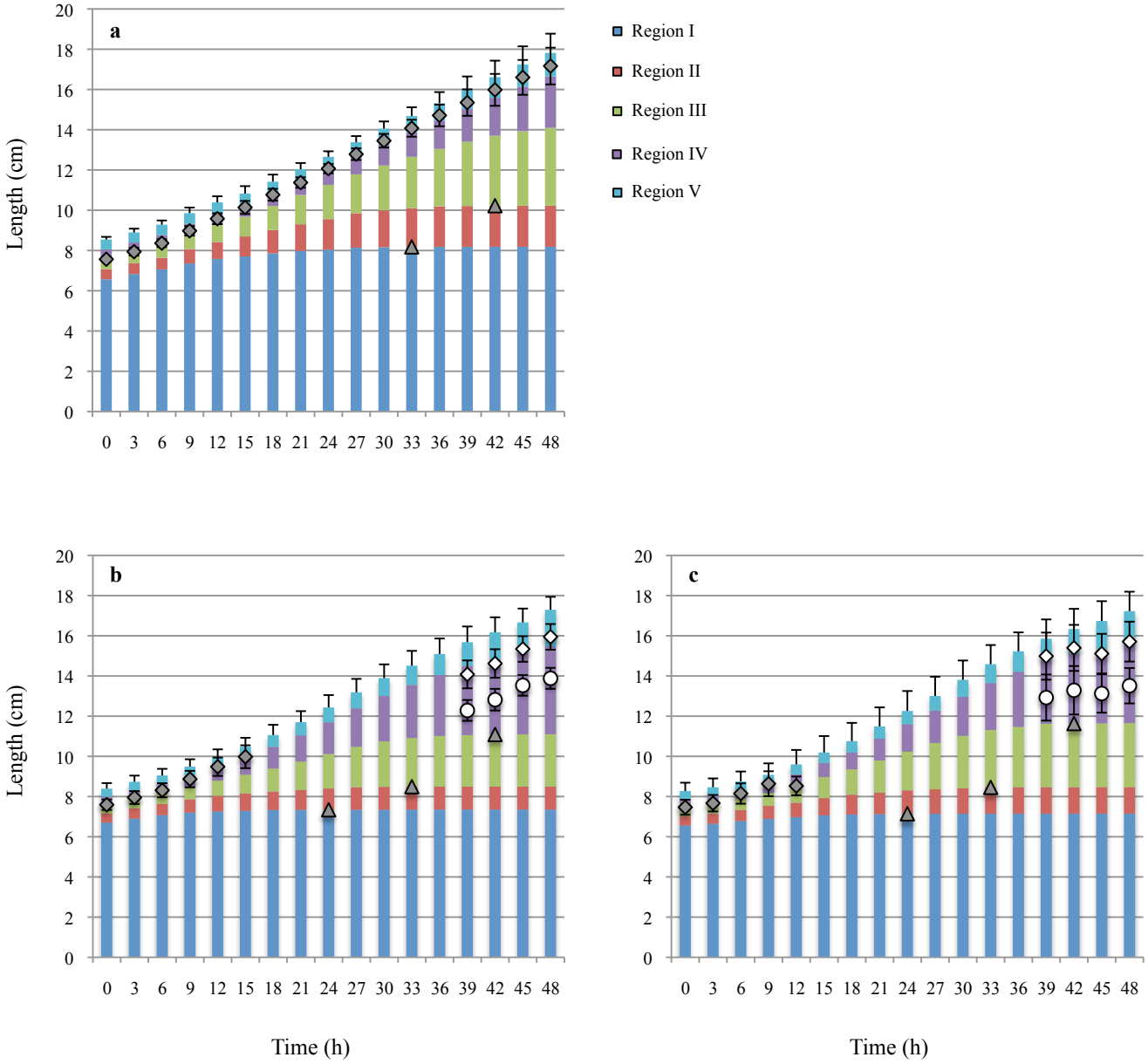

Figure 25. Elongation pattern of etiolated adzuki bean epicotyls transferred to different light conditions. The elongation pattern of 5-day-old etiolated epicotyls transferred to infrared (a), red (b), and blue light (c) was examined for 48 h. See Fig. 17 for definition of the regions I–V. *Gray diamonds*, *gray triangle*, *open diamonds*, and *open circles* show the position of the apical hook, where the elongation of a defined region was completed, of the apical end of the bending zone, and of the basal end of the bending zone, respectively. The *vertical bar* on each point shows SE (n = 5).

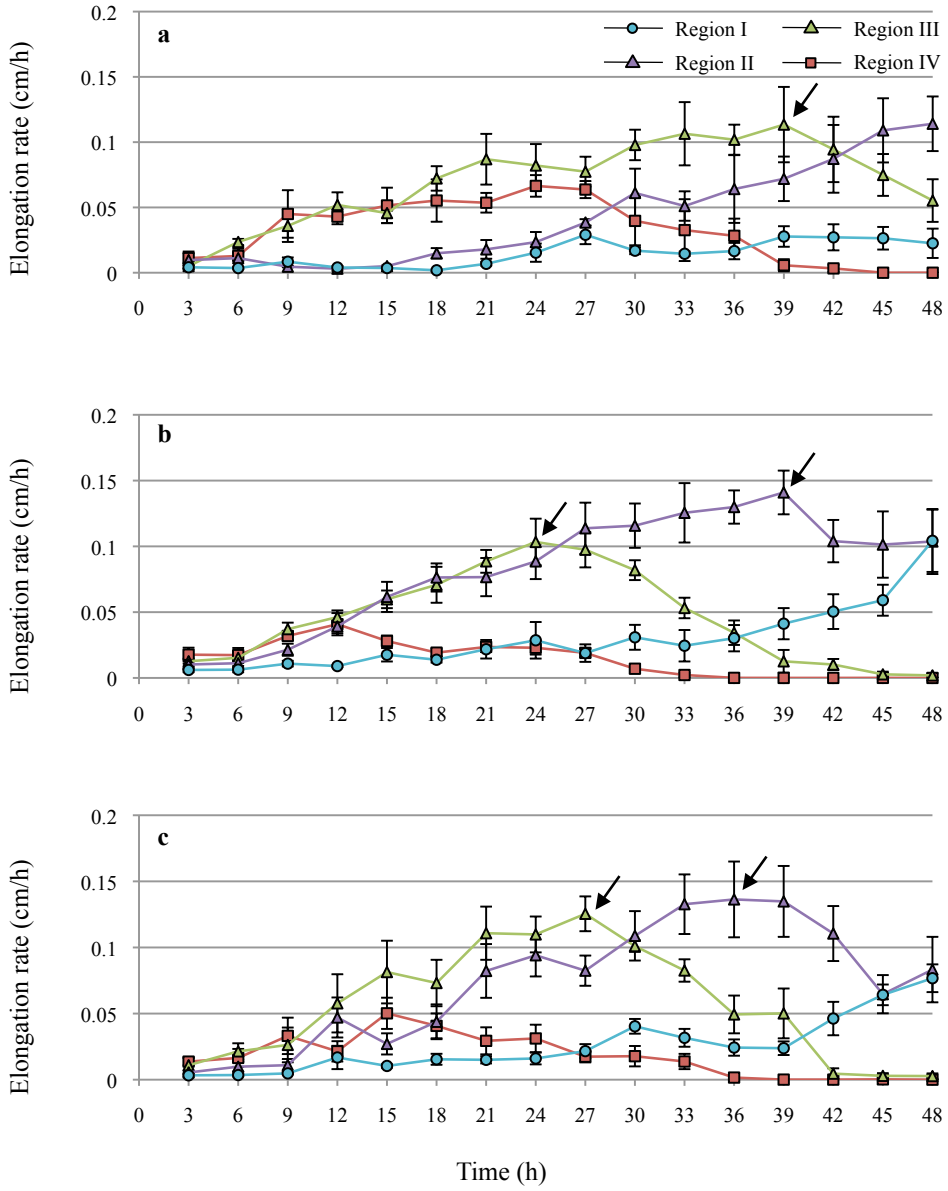

Figure 26. Elongation rate of the elongating regions of etiolated adzuki bean epicotyls transferred to different light conditions. Etiolated epicotyls of 5 day old were transferred to infrared (a), red (b), and blue light (c). The elongation rate of the regions I–IV of epicotyls shown in Fig. 25 was separately examined every 3 h. *Arrows* indicate the time points when the suppression of elongation of the region II or III starts. The *vertical bar* on each point show SE (n = 5).

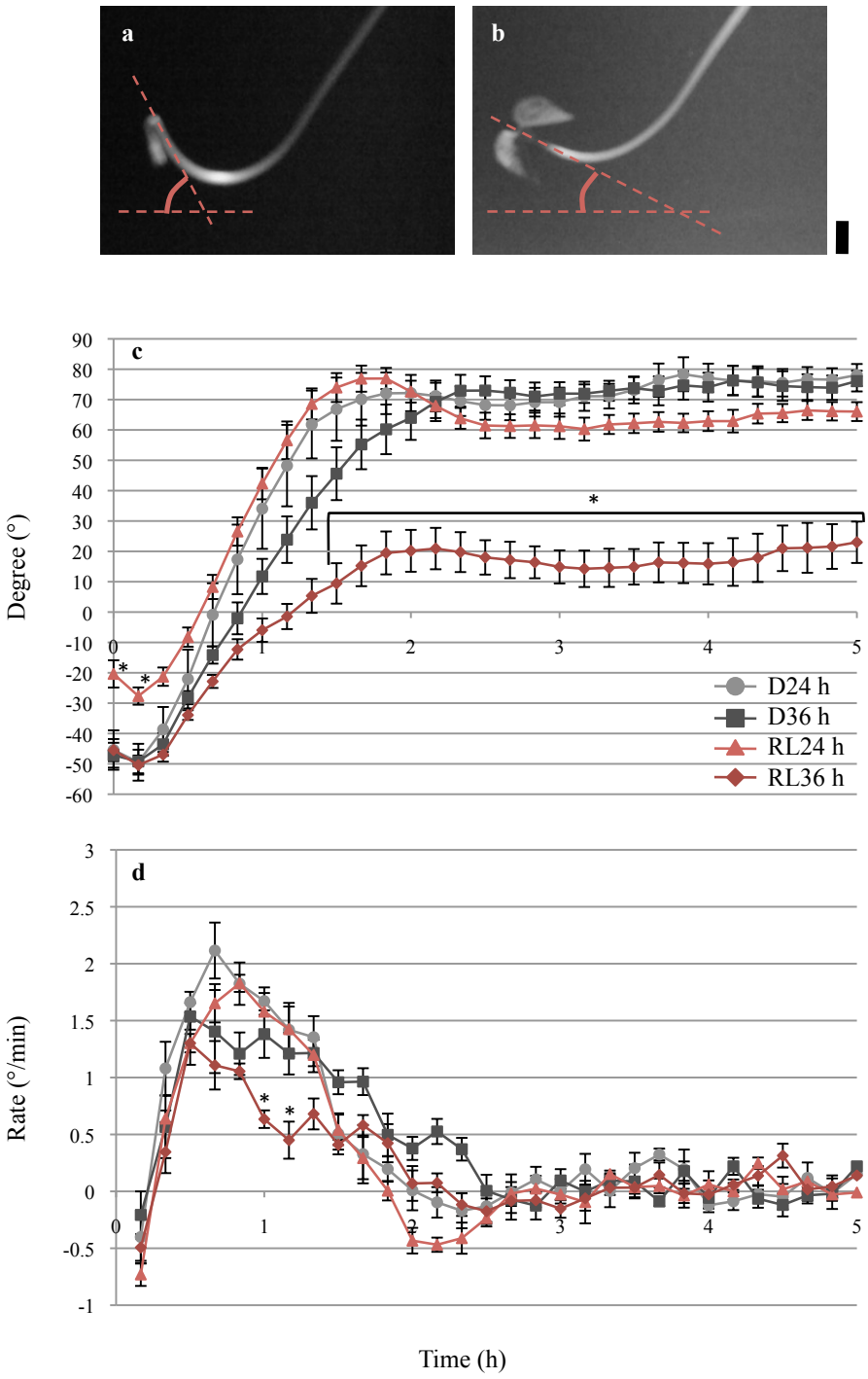

Figure 27. Gravitropic responses of adzuki bean epicotyls grown under different light conditions. Etiolated epicotyls of 5 day old were kept in darkness or exposed to continuous red light for 24 or 36 h (D24 h; D36 h; RL24 h; RL36 h), and then placed horizontally under continuous infrared or red light, respectively. (a, b) Photographs of epicotyls of D24 h (a) and RL36 h (b) at 2 h of the gravistimulation. The angle of curvature of gravitropism was defined as the angle between the horizontal line and the longitudinal center line of the apical region of the epicotyl, below the hook (a; D24 and 36 h) or below the tip (b; RL24 and 36 h). Bar = 1 cm. (c, d) Time course (c) and rate (d) of gravitropic response in the epicotyls after the start of gravistimulation. The *asterisks* indicate that significant differences were detected by Tukey's test ($P < 0.05$). The *vertical bar* on each point shows SE (n = 5).

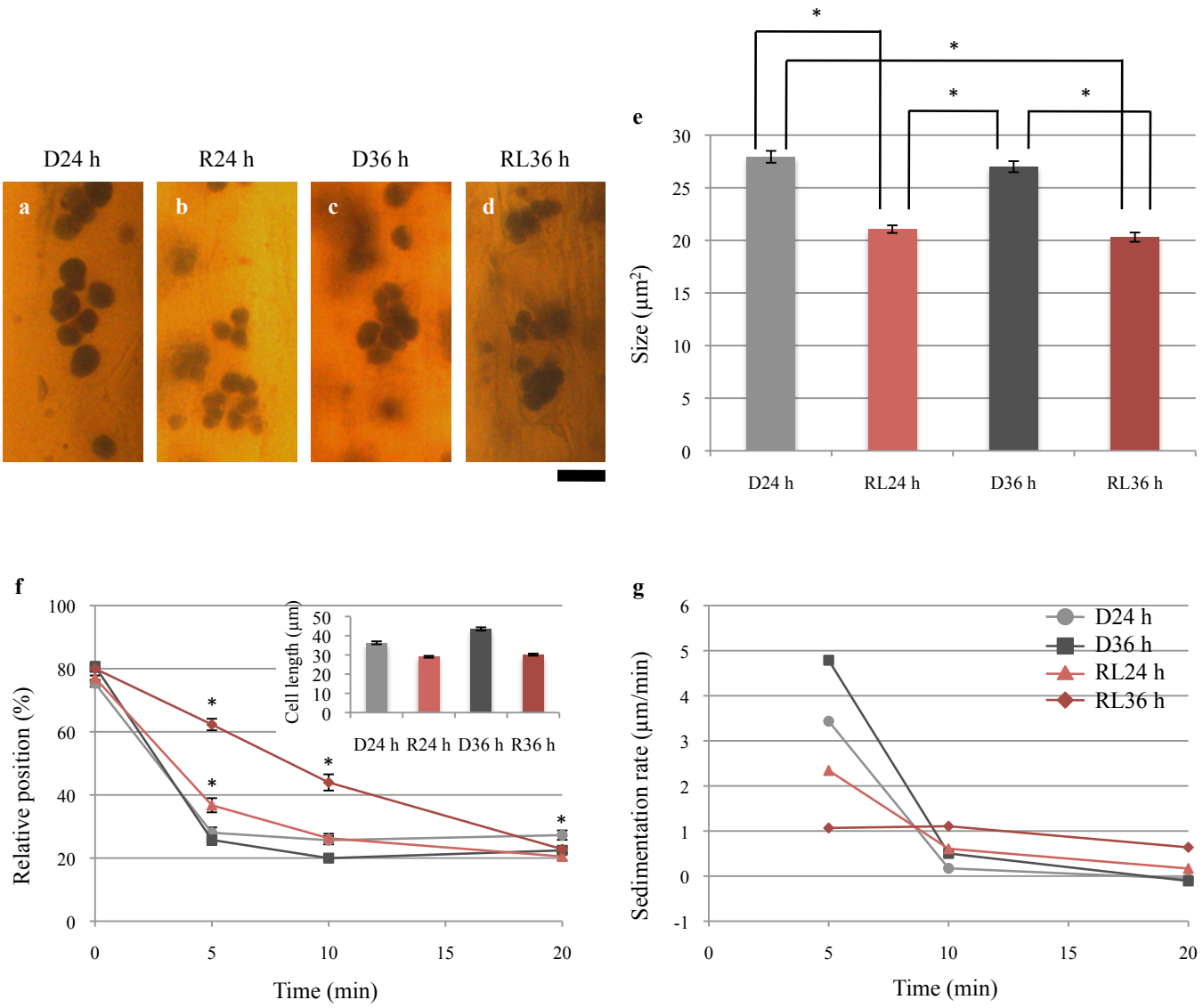

Figure 28. Amyloplasts in the endodermis of adzuki bean epicotyls grown under different light conditions.

(a–d) Photographs of amyloplasts in the endodermis of epicotyls of D24 h (a), D36 h (b), RL24 h (c), or RL36 h (d). *Bar* = 10 μm. (e) Quantification of amyloplast size in the epicotyls grown under different light conditions. The *asterisks* indicate that significant differences were detected by Tukey's test ($P < 0.05$). The *vertical bar* on each point shows SE (n = 221–244). (f) Quantification of amyloplast sedimentation in response to the changing gravity vector. The relative positions of amyloplast were determined by assigning the original bottom of a cell to 100% and the original top of the cell to 0%. The *asterisks* indicate that significant differences were detected by Tukey's test ($P < 0.05$). The *vertical bar* on each point shows SE (n > 100). (inset) The mean endodermal cell length in epicotyls under different light conditions. (g) Assumed sedimentation rate of amyloplasts. The values were determined from the mean position of amyloplasts in the endodermal cell with the mean length (f).

Table 1. Parameters of growth movements in adzuki bean epicotyls grown under different light conditions.

	Completion of hook opening (h)	Onset of circumnutation (h)	Period of circumnutation (min)	Amplitude of circumnutation (°)
Greened epicotyls under white light	-	-	61.3 ± 1.3	24.3 ± 1.2
Etiolated epicotyls exposed to red light	15.4 ± 0.2	38.4 ± 1.1	65.6 ± 1.4	8.6 ± 1.4 *
Etiolated epicotyls exposed to blue light	13.4 ± 0.9	38.0 ± 2.8	60.3 ± 2.1	27.4 ± 4.5

The asterisk means that the value is significantly different from those obtained under other conditions ($P < 0.05$ with Tukey's test). Values are means ± SE ($n = 5$).