AUXIN POLAR TRANSPORT IS ESSENTIAL FOR GRAVIRESPONSE OF ETIOLATED PEA SEEDLINGS

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The purpose of this study is to verify the hypothesis that auxin polar transport is essential for graviresponse in plants, focused on the mode of action of auxin polar transport on graviresponse of etiolated Alaska pea seedlings. Automorphogenesis of etiolated Alaska pea (Pisum sativum L.) seedlings together with reduced auxin polar transport in epicotyls was observed in true microgravity conditions in space (BRIC-AUX on STS-95 in 1998). On Earth, a potent inhibitor of auxin polar transport, 2,3,5-triiodobenzoic acid (TIBA), substantially induced automorphogenesis-like growth and development in etiolated Alaska pea seedlings. Similar powerful inhibitors of auxin polar transport, N-(1-naphthyl)phthalamic acid (NPA) and 9-hydroxyfluorene-9-carboxylic acid (HFCA), were found to phenocopy automorphogenesis-like epicotyl bending in etiolated Alaska pea seedlings. However, an inhibitor of auxin action, p-chlorophenoxyisobutylic acid (PCIB), had little effect. Auxin polar transport in the proximal side of the 1st internodes to the cotyledons, but not in the 2nd internodes, in etiolated Alaska pea seedlings was significantly higher than in the distal side. Gene expression of PsPINs but not PsAUX1 is significantly higher than that in the distal one. TIBA did not affect gene expression of PsPIN1, PsPIN2 and PsAUX1 in the proximal and the distal sides of epicotyls to cotyledon. In addition, altered localization of PsPINs proteins in plasma membrane was observed in hook region but not in middle region of epicotyls in etiolated Alaska pea seedlings, suggesting the lateral auxin distribution from the distal to the proximal sides in epicotyls and resulted in normal graviresponse of etiolated Alaska pea seedlings.

INTRODUCTION

Terrestrial plants, which have evolved under a 1 g gravitational force, have acquired the ability to use and/or to resist gravistimuli to regulate their growth and development. Under the 1 g conditions on Earth, stems and roots of plants grow upwards against, and downwards with, respectively, the direction of gravity due to gravitropic responses enabled by differential cell elongation in these organs. A step forward in the understanding of the cellular mechanisms involved in
gravimorphogenesis has already been achieved using microgravity conditions in space, indicating that the morphology of plants is substantially influenced by gravistimulation (Halstead and Dutcher 1987; Brown et al. 1990; Musgrave et al. 1997, 2000; Kiss et al. 1998; Hoson et al. 1999; Takahashi et al. 1999; Kiss 2000). In STS-95 space experiments, we also demonstrated that microgravity conditions substantially affected the growth and development of etiolated pea seedlings (Ueda et al. 1999, 2000). Such morphogenesis observed under microgravity conditions has been designated as automorphogenesis (Hoson et al. 1992; Stankovic et al. 1998). Automorphogenesis-like growth and development of etiolated pea seedlings was demonstrated to be induced when pea plants are constantly rotated three-dimensionally on a three-dimensional (3D) clinostat (Shimazu et al. 2001; Miyamoto et al. 2005). As well as application of the 3D clinostat to simulate microgravity conditions, mutants showing automorphogenesis-like growth and development are valuable in understanding how gravity regulates morphogenesis in plants. One such agravitropic pea mutant, the ageotropum pea (from Pisum sativum L. cv. Weibull’s Weitor), whose roots and shoots lack the ability to orient with respect to gravity (Blixt et al. 1958; Takahashi and Suge 1991), showed automorphogenesis-like epicotyl bending (Hoshino et al. 2007). A possible role for auxin in tropic bending has been proposed by the Cholodny-Went hypothesis, which contends that unequal distribution of auxin between the opposite sides of an organ causes differential cell elongation (Went 1974). Recently, we demonstrated a characteristic asymmetrical polar auxin movement that is gravity-controlled in the early growth stages of etiolated pea epicotyls, and suggested its importance for inducing asymmetrical accumulation of auxin during the negative gravitropic response of the epicotyls, based on the results of transport experiments using radiolabeled auxin (Hoshino et al. 2005). In the present study, to clarify the hypothesis that auxin transport is essential for epicotyl bending for determination of growth direction by gravistimulation in the early growth stage of etiolated pea seedlings, relationships among polar auxin transport in epicotyls, gene expression and the distribution of its product closely related to auxin polar transport were examined.

**Materials and Methods**

*Plant materials.* Pea (Pisum sativum L. cv. Alaska) seeds were set in dry rockwool (Nippon Rockwool Co., Tokyo, Japan) in an acrylic chamber mimicking the plant growth chamber in the STS-95 space experiment (Ueda et al. 1999, 2000), then allowed to germinate and grow after watering with 180 ml of distilled water, as described previously (Ueda et al. 1999, 2000). To study a negative gravitropic response of epicotyls in the early growth stage, the axis of the embryo in dry seed was set horizontally to the rockwool surface. The acrylic chamber in a Zip-lock bag was kept at 23.5°C in the dark. Etiolated pea epicotyls were divided longitudinally into proximal and distal halves after appropriate incubation, frozen in liquid nitrogen and stored at -80°C before use for Northern blot analysis.

*Treatment with inhibitors of auxin.* Pea seeds were set in a horizontal or inclined position in dry rockwool used for seedling growth in an acrylic chamber, watered with 180 ml of 2,3,5-triiodobenzoic acid (TIBA, Sigma-Aldrich, St. Louis, MO), N-(1-
naphthylphthalamic acid (NPA, Tokyo Kasei Kogyo, Tokyo, Japan) and 9-hydroxyfluorene-9-carboxylic acid (HFCA, Tokyo Kasei Kogyo, Tokyo, Japan) and p-chlorophenoxyisobutyric acid (PCIB, Sigma-Aldrich, St. Louis, MO) at a concentration of 10 µM and allowed to germinate and grow for 48 and 60 h in the dark. The growth direction of epicotyls was determined, and is expressed as the angle between the line of normal morphogenesis and the orientation of the organs in that direction. Each experiment was carried out using at least 10 seedlings. The experiment was repeated three times.

Measurement of polar auxin transport. Measurement of polar auxin transport was performed according to the method reported previously (Hoshino et al. 2006) with some modifications. Segments (10 mm long) of the 1st internodes prepared from 60-h-old Alaska pea seedlings grown under 1 g conditions were prepared. Agar medium (0.9% w/v, 20 µl) containing 1.75 µM (1 µCi mL⁻¹) 3-indoleacetic acid [1-¹⁴C]-[1-¹⁴C]IAA (American Radiolabeled Chemical, St. Louis, MO) in Eppendorf tube was applied to the apical (inverted position) or basal (normal position) side of the segments. The tubes were incubated in the dark for 6 h at room temperature. At the end of incubation, a 2-mm piece of the opposite side from the donor side was excised, and the radioactivity was measured directly using a liquid scintillation counter (2200CA, Packard Instrument, Meriden, CT). Almost all the radioactivity in the opposite side of the segments donated radiolabeled IAA in the segments resulted from that of [1-¹⁴C]IAA transported within at least 16 h in planta (Oka et al. 1995; Shimazu et al. 2000). Each experiment was carried out using at least ten segments and was repeated three times.

Northern blot analysis. Total RNA was isolated from etiolated pea epicotyls divided into proximal and distal halves of epicotyls to the cotyledons using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer’s instructions with a minor modification as reported previously (Hoshino et al. 2005). Total RNA was size-fractionated on denaturing 1.0% agarose-formaldehyde gels. Bands of total RNA were transferred onto a nylon membrane (Hybond-N+ Amersham Biosciences, Piscataway, NJ) and fixed by baking at 80°C for 2 h after UV cross-linking. Hybridization was achieved with DIG-labeled DNA probes at 58°C using Perfecthyb Plus hybridization buffer (Sigma-Aldrich, St. Louis, MO). DNA probes of PsPINs, PsAUX1 and PsIAA4/5 were prepared as reported previously (Hoshino et al. 2005, 2006). Hybridization membranes were washed with 2 x SSC and 0.1% SDS for 5 min at room temperature, twice with 0.5 x SSC and 0.1% SDS for 20 min at 58°C, and with 0.1 x SSC and 0.1% SDS for 20 min at room temperature. Blots were developed with anti-DIGAP monoclonal antibody (Roche Diagnostics, Penzberg, Germany) with CSPD as the chemilluminescent substrate (Roche Diagnostics) according to the manufacturer’s recommendations, and were exposed to X-ray film (RXU, Fuji Photo Film, Tokyo, Japan). Density of blots was quantified using a CS Analyzer ver 2.0 system (Atto, Tokyo, Japan). The amount of ribosomal RNA was used as a loading control. Each experiment used at least ten segments and was repeated three times. Statistical analyses were carried out using Student’s t-test for equal variance.

Generation of Antibody and determination of immunolocalization. Since ZmPIN1a (Carraro
et al. 2006) showed high homology to PsPINs, ZmPIN1a-specific antibody was decided to introduce for the detection of PsPINs in etiolated Alaska pea seedlings. To generate ZmPIN1a-specific polyclonal antibodies, an oligo-peptide fragment with hydrophilic region was synthesized. The peptide was combined with KLH (keyhole limpet hemocyanin) for the antigen. After immunization of rabbits, the polyclonal antiserum was affinity purified against the ZmPIN1a-specific peptide.

Pea epicotyls were excised from the seedlings and immediately fixed in Carnoy fixative solution (60% Ethanol, 30% Chloroform, 10% Acetic Acid) for 3 h at 4°C maintaining their orientation to gravity. Then samples were extracted from epicotyls and further fixed 90 min. After dehydration through an ethanol and tert-butanol series, the samples were embedded in Paraplast Plus (Sigma-Aldrich). Sections (8–10 µm) were cut using a microtome (RM2135; Leica) and collected on slides. The slides were deparaffinized and treated with detergent solution (10 % DMSO and 3 % Nonidet P-40 in 1-fold MTSB [100 mM piperazine-1,4-bis(2-ethanesulfonic acid) (PIPES) pH 7.2, 10 mM MgSO4, 10 mM EGTA] for) for 30 min and washed twice in 0.5-fold MTSB for 10 min. Then, the slides were treated with blocking buffer (1-fold MTSB with 1.5 % skim milk) for 30 min and incubated overnight at 4°C with an anti-ZmPIN1a antibody at a 150-fold dilution in blocking buffer. The slides were rinsed three times for 10 min with 0.5-fold MTSB containing 0.1 % Tween-20, and were then incubated with Alexa488-conjugated goat anti-rabbit IgG (Molecular Probes) at a 300-fold dilution for 3 h at room temperature. After washing three times for 10 min, the sample sections were sealed with ProLong Gold Anti slow fade reagent (Molecular Probes). The prepared samples were observed with an epi-fluorescence microscope (model BX51; Olympus, Tokyo, Japan).

**Results and Discussion**

Automorphogenesis of etiolated Alaska pea seedlings together with reduced auxin polar transport in epicotyls was observed not only in true microgravity conditions in space (Ueda et al. 1999, 2000) but also in 3D clinostat conditions (Miyamoto et al. 2005) and an agavitropic mutant of ageotropum pea seedlings (Hoshino et al. 2007). Significantly higher auxin polar transport in the proximal side of the 1st internodes to the cotyledons compared to that in the distal side, but not in the 2nd internodes, in etiolated Alaska pea seedlings has also been reported (Hoshino et al. 2006). To clarify close relationships between gravimorphogenesis and auxin polar transport, the effects of inhibitors of auxin polar transport on auxin polar transport, the expression of PsPINs and PsAUX1genes (Chawla and DeMason 2004; Hoshino et al. 2005) related to auxin polar transport in etiolated Alaska pea seedlings were investigated. Together with the expression of PsPINs genes, the localization of PsPIN1 regulating auxin polar transport in epicotyls of etiolated Alaska pea seedlings was also determined. As shown in Table 1, a potent inhibitor of auxin polar transport, TIBA, substantially induced automorphogenesis-like growth and development in etiolated Alaska pea seedlings. Similar powerful inhibitors of auxin polar transport, NPA and HFCA, were found to phenocopy automorphogenesis-like epicotyl bending in etiolated Alaska pea seedlings. However, an inhibitor of auxin action, PCIB, had little effect.
Gene expression detected by Northern blot analysis in the proximal and the distal sides of the 1st internodes of etiolated Alaska pea epicotyls grown on 1 g conditions was investigated. As shown in Fig. 1, gene expression of PsPINs but not PsAUX1 is significantly higher than that in the distal one as well as significant higher auxin polar transport in the proximal side of the 1st internode in etiolated Alaska pea epicotyls (Hoshino et al. 2006).

Fig. 2 shows the effect of TIBA on IAA transport and endogenous levels of auxin detected by PsIAA4/5 gene expression in the 1st internode of etiolated Alaska pea epicotyls. TIBA exogenously applied substantially inhibited auxin polar transport and endogenous levels of IAA detected by gene expression of PsIAA4/5 which is an auxin inducible gene in its concentration dependent manner. Surprisingly TIBA did not affect gene expression of PsPIN1, PsPIN2 and PsAUX1 in the proximal and the distal sides of epicotyls to cotyledon although gene expression of PsPIN1 in the proximal side of epicotyls was higher than that in the distal one (Fig. 3).

Localization of PsPINs was immunohistchemically determined with ZmPIN1a antibody, indicating that altered localization of PsPINs indicated by green fluorescence was observed in the proximal and the distal side of epicotyl cells in hook region but not in those of the middle part of the 1st internode in etiolated Alaska pea seedlings (data not shown).

These results described above strongly suggest that auxin polar transport is essential factor for graviresponse of etiolated Alaska pea seedlings. This is also supported by the fact that an accumulation of PsPIN1 mRNA encoding a putative auxin efflux facilitator, which was observed in vascular tissue, cortex and epidermis in the proximal and distal sides of etiolated epicotyls, was markedly influenced by gravistimulation (Hoshino et al 2006). Judging from the fact described above together with the result in this study that altered localization of PsPINs proteins in plasma membrane was observed in hook region but not in middle region of epicotyls in etiolated Alaska pea seedlings, the lateral auxin distribution from the distal to the proximal sides in epicotyls is required for normal graviresponse of etiolated Alaska pea seedlings. A step forward in the understanding of cellular and molecular mechanisms involved in gravimorphogenesis of plants will be achieved in space. The findings of this study substantially lead us to clarify close relationships between auxin polar transport and gravimorphogenesis of plants in cellular and molecular levels using artificial 1 g and true microgravity conditions on the Japanese Experiment Module "Kibo" in the International Space Station in near future.

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LITERATURE CITED
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Table 1. Effect of inhibitors of auxin polar transport or auxin action, 10 μM of TIBA (2,3,5-triiodobenzoic acid), NPA (N-(1-naphthyl)phthalamic acid), HFCA (9-hydroxyfluorene-9-carboxylic acid), and PCIB (p-chlorophenoxyisobutylic acid), respectively, on gravimorphogenesis of epicotyls in etiolated Alaska pea seedlings.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Angle, degree ± error</th>
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<tbody>
<tr>
<td>Control</td>
<td>12.8±1.4</td>
</tr>
<tr>
<td>TIBA</td>
<td>43.5±1.7*</td>
</tr>
<tr>
<td>NPA</td>
<td>55.6±3.1*</td>
</tr>
<tr>
<td>HFCA</td>
<td>49.1±2.1*</td>
</tr>
<tr>
<td>PCIB</td>
<td>13.7±1.3</td>
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* The growth direction of epicotyls was expressed as the angle between a vertical line and the orientation of the organs in the direction. Values are the mean of at least 20 seedlings with standard errors.

Statistical analyses were carried out using Student’s t-test (P < 0.01). Values marked with an asterisk are significantly higher or lower than DMSO treatment control.
Figure 1. Gene expression of *PsPINs* and *PsAUX1* in the 1st internode of etiolated Alaska pea epicotyls detected by Northern blot analysis.
Figure 2. Effect of TIBA on auxin polar transport (left) and endogenous levels of IAA detected by gene expression of *PsIAA4/5* (right) in etiolated Alaska pea epicotyls. Radiolabeled IAA was applied to the apical (inverted) or basal (normal) side of the segments. Results are expressed as averages of three independent experiments with standard errors of the mean.
Figure 3. Effect of TIBA on gene expression of *PsPINs* and *PsAUX1* detected by Northern blot analysis in etiolated Alaska pea epicotyls. Segments (10 mm long) of the 1st internodes excised from 48 or 60-h-old Alaska pea seedlings grown under 1 g conditions were divided into proximal (P) and distal (D) halves of epicotyls to cotyledons.