Gravistimulation Changes the Accumulation Pattern of the CsPIN1 Auxin Efflux Facilitator in the Endodermis of the Transition Zone in Cucumber Seedlings

Chiaki Watanabe, Nobuharu Fujii*, Kenichi Yanai, Takuya Hotta, Dai-Hee Kim, Motoshi Kamada2, Yuko Sasagawa-Saito, Takeshi Nishimura, Tomokazu Koshiba, Yutaka Miyazawa, Kyung-Min Kim, and Hideyuki Takahashi

Graduate School of Life Sciences, Tohoku University, Aoba-ku, Sendai 980–8577, Japan (C.W., N.F., K.Y., T.H., D.-H.K., M.K., Y.S.-S., Y.M., H.T.); Department of Chemistry, College of Natural Science, Changwon National University, Changwon City 641–773, Korea (D.-H.K.); Division of Plant Biosciences, College of Agriculture and Life Science, Kyungpook National University, Daegu 702–701, Korea (K.-M.K.); and Department of Biological Sciences, Tokyo Metropolitan University, Hachioji-shi, Tokyo 192–0397, Japan (T.N., T.K.)

Cucumber (Cucumis sativus) seedlings grown in a horizontal position develop a specialized protuberance (or peg) on the lower side of the transition zone between the hypocotyl and the root. This occurs by suppressing peg formation on the upper side via a decrease in auxin resulting from a gravitational response. However, the gravity-stimulated mechanism of inducing asymmetric auxin distribution in the transition zone is poorly understood. The gravity-sensing tissue responsible for regulating auxin distribution in the transition zone is thought to be the endodermal cell. To characterize the gravity-stimulated mechanism, the auxin efflux facilitator PIN-FORMED1 (CsPIN1) in the endodermis was identified and the localization of CsPIN1 proteins during the gravimorphogenesis of cucumber seedlings was examined. Immunohistochemical analysis revealed that the accumulation pattern of CsPIN1 protein in the endodermal cells of the transition zone of cucumber seedlings grown horizontally differed from that of plants grown vertically. Gravistimulation for 30 min prompted changes in the accumulation pattern of CsPIN1 protein in the endodermis as well as the asymmetric distribution of auxin in the transition zone. Furthermore, 2,3,5-triiodobenzoic acid inhibited the differential distribution of auxin as well as changes in the accumulation pattern of CsPIN1 in the endodermis of the transition zone during gravistimulation. These results suggest that the altered pattern of CsPIN1 accumulation in the endodermis in response to gravistimulation influences lateral auxin transport through the endoderm, resulting in asymmetric auxin distribution in the transition zone.

1 This work was supported by the Japan Society for the Promotion of Science (Grant-in-Aid for Scientific Research [B] no. 20370017 to H. T., Grant-in-Aid for Scientific Research [C] no. 19570031 to N.F., and Research Fellowship for Young Scientists no. 21–9981 to C.W.), by the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant-in-Aid for Scientific Research on Innovative Areas no. 22120004 to H.T.), by the Funding Program for Next-Generation World-Leading Researchers (grant no. G5002 to Y.M.), and by the Ground-Based Research Announcement for Space Utilization, promoted by the Japan Space Forum and the Global Center of Excellence Program J03 (Ecosystem Management Adapting to Global Changes). This work was also supported by the Cooperative Research Grant of the Gene Research Center, the University of Tsukuba (to N.F.).

2 Present address: Advanced Engineering Services Co., Ltd., 1–6–1 Takezono, Tsukuba 305–0032, Japan.

* Corresponding author; e-mail nobuharu@ige.tohoku.ac.jp.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Nobuharu Fujii (nobuharu@ige.tohoku.ac.jp).

[1] The online version of this article contains Web-only data.

Plants respond to gravity by regulating their growth direction and morphogenesis (Trewavas, 1992; Takahashi, 1997). Peg formation in Cucurbitaceae plants was previously shown to be a unique form of gravimorphogenesis. A peg protuberance is formed in the transition zone between the hypocotyl and the root (for review, see Takahashi, 1997; Fig. 1). When cucumber (Cucumis sativus) seeds germinate in a horizontal position, a peg forms on the lower side of the transition zone (Witztum and Gersani, 1975; for review, see Takahashi, 1997; Fig. 1, A and B). The peg anchors the seed coat so that elongation of the hypocotyl pulls the cotyledons out of the seed coat (Fig. 1C). When cucumber seeds germinate in a vertical position, with the radicle pointing down, or under microgravity conditions in space, the seedlings develop a peg on each side of the transition zone (Takahashi et al., 2000). Thus, cucumber seedlings have the potential to develop a peg on each side, and peg formation on the upper side of the transition zone is suppressed in response to gravity when the seedlings are grown horizontally on the ground (Takahashi et al., 2000).

Auxin plays an important role in gravimorphogenesis by determining the lateral placement of peg formation in the transition zone (Witztum and Gersani, 1975; Takahashi and Suge, 1988; Takahashi, 1997). Both...
endogenous indole-3-acetic acid (IAA) concentration and auxin-inducible gene expression are significantly reduced on the peg-suppressed side (the upper side) of the transition zone (Fujii et al., 2000; Kamada et al., 2000). Application of exogenous auxin induces the development of a peg on the upper side of the transition zone in a horizontal position (Kamada et al., 2000). Furthermore, application of an inhibitor of auxin action, p-chlorophenoxyisobutyric acid, inhibits peg formation (Shimizu et al., 2008). These results indicate that gravity-induced differential auxin-inducible gene expression and suppression of peg development result from a decrease in auxin on the upper side of the transition zone. By contrast, the lower side of the transition zone maintains the auxin levels required for peg formation (Kamada et al., 2000, 2003; Shimizu et al., 2008). Seedlings treated with an auxin transport inhibitor, 2,3,5-triodobenzoic acid (TIBA), or 9-hydroxyfluorene-9-carboxylic acid block the suppression of peg formation on the upper side and develop a peg on each side of the transition zone, regardless of the position of seedling germination (Kamada et al., 2003). In addition, the differential accumulation of the auxin-inducible CsIAA1 mRNA in the gravistimulated transition zone was inhibited by TIBA treatment (Kamada et al., 2003). According to the Cholodny-Went theory, originally proposed in 1937, the lateral transport of auxin across gravity-stimulated organs may cause differential gravitropic growth (Went and Thimann, 1937; Evans, 1991; Trewavas, 1992; Iino, 1995). The results of this study suggest that lateral auxin transport, which is modified by gravity, is required for the differential decrease in auxin level on the upper side of cucumber seedlings grown in a horizontal position.

In Arabidopsis (Arabidopsis thaliana), the polarized movement of auxin is mediated by plasma membrane-localized efflux proteins of the PIN-FORMED (PIN) and P-glycoprotein families (Bandyopadhyay et al., 2007). PINs are essential for normal organogenesis and/or auxin-dependent tropic responses (Benjamins et al., 2005). In Arabidopsis, auxin is transported from the shoot apex to the root tip through vascular tissues. Subsequently, the auxin is returned from the root tip to the elongation region through the lateral root cap and epidermis (Gälweiler et al., 1998; Müller et al., 1998). The polar localization of the AtPIN1 auxin efflux facilitator in vascular tissues has been shown to mediate basipetal auxin transport in the stem of the inflorescence (Gälweiler et al., 1998). Similarly, AtPIN2 in the root epidermal cells mediates auxin transport from the root cap to the elongation zone (Müller et al., 1998). AtPIN3 is expressed in gravity-sensing tissues of the columella and endodermis (Friml et al., 2002b). In Arabidopsis roots, gravistimulation induces the relocalization of AtPIN3 and AtPIN7 in the plasma membrane of the lower side of the columella cells (Friml et al., 2002b; Kleine-Vehn et al., 2010). This gravity-modulated relocalization of AtPIN3 and AtPIN7 may explain the lateral auxin transport system modified by gravity in the root (Friml et al., 2002b; Kleine-Vehn et al., 2010). These findings prompted an investigation into the cellular localization and gravity responses of cucumber PIN proteins.

The endodermis is known to sense gravity in the shoots (Fukaki et al., 1998). The endodermis in the transition zone of cucumber seedlings has also been suggested to sense gravity (Takahashi and Scott, 1994). Therefore, to reveal the gravity response of the auxin efflux facilitator, the auxin efflux facilitators expressed in the endodermis of the transition zone of cucumber seedlings were identified. CsPIN1, encoding a PIN auxin efflux facilitator from cucumber, was identified previously (Kamada et al., 2003). In this study, five cDNAs encoding PIN family proteins were isolated from cucumber. Upon examination of mRNA accumulation in the transition zone, CsPIN1, -4, and -6 mRNAs were found to accumulate in a tissue-specific manner in the transition zone. In particular, CsPIN1 mRNA accumulated in the endodermal cells of the

![Figure 1. Lateral positioning of the peg in cucumber seedlings grown in a horizontal position.](image_url)
RESULTS

Isolation of cDNAs Encoding PIN Auxin Efflux Facilitators in Cucumber

Previously, the CsPIN1 cDNA encoding a PIN auxin efflux facilitator was identified in cucumber (Kamada et al., 2003). To isolate additional cDNAs encoding PIN proteins from cucumber, several degenerate primers were designed based on an alignment of the amino acid sequences of PIN protein family members. These primers were used to amplify partial cDNAs via reverse transcription-PCR. Ultimately, five partial cDNAs were isolated (CsPIN2-CsPIN6). A cucumber cDNA library was screened to isolate the full-length cDNAs of CsPIN2 and CsPIN3, while RACE was used to identify the 5′ and 3′ regions from each of the remaining partial CsPIN cDNAs (CsPIN4-CsPIN6). The deduced amino acid sequences of these CsPINs and AtPIN1 were aligned (Fig. 2). Each CsPIN contained the highly conserved hydrophobic regions of the PIN protein family in both the N-terminal and C-terminal regions. By contrast, the portion located between the terminal hydrophobic regions was less conserved (Fig. 2). Genomic DNA gel-blot analysis indicated that the genes corresponding to CsPIN2 to -6 are present as single copies in the genome of cucumber (Supplemental Fig. S1).

cDNAs encoding the PIN protein family have been isolated from several plant species (Ni et al., 2002a, 2002b; Schnabel and Frugoli, 2004; Xu et al., 2005). Phylogenetic analysis was conducted using the nucleotide sequences encoding the conserved hydrophobic regions of the PIN protein family (Fig. 3). CsPINs belong to a well-characterized clade containing AtPINs (AtPIN1, AtPIN2, AtPIN3, AtPIN4, and AtPIN7; Gälweiler et al., 1998; Müller et al., 1998; Friml et al., 2002a, 2002b). Within this family of PINs, five major clades diverged: clade I included Poaceae PINs; clade II included AtPIN2 and CsPIN5; clade III and clade IV included Fabaceae, Salicaceae, and Curcurbitaceae PINs (CsPIN1/CsPIN3 and CsPIN4) but not Brassicaceae PINs; and clade V contained PINs from Curcurbitaceae (CsPIN2/CsPIN6), Brassicaceae (AtPIN3/AtPIN4/AtPIN7), Fabaceae, and Salicaceae. It was unclear which clade included AtPIN1 due to a lack of support (bootstrap < 50%) for the AtPIN1-containing clade.

The relationship between the direction of auxin transport and the tissue-specific expression of each PIN in Arabidopsis roots has been well characterized (Blilou et al., 2005). Therefore, to clarify which cucumber PIN corresponded to each Arabidopsis PIN, mRNA accumulation in cucumber roots was analyzed (Supplemental Fig. S2). Using in situ hybridization, CsPIN1 and CsPIN4 mRNAs were detected in the vascular bundle; CsPIN1, CsPIN2, and CsPIN3 mRNAs were detected around the quiescent center; CsPIN6 mRNA signals were present in the columella; and CsPIN5 mRNA was located in the lateral root cap and epidermis.

Accumulation of CsPIN1 mRNA in the Endodermis of the Transition Zone in Cucumber Seedlings

To identify which CsPIN mRNAs accumulated in the transition zone, northern-blot analysis was conducted using total RNAs isolated from different organs. The results suggested that all of the CsPIN mRNAs examined, with the exception of CsPIN2 mRNA, accumulated in the transition zone of 24-h-old cucumber seedlings grown in a horizontal position (Supplemental Fig. S3). In a previous study, in situ hybridization was used to detect CsPIN1 mRNA in the vascular tissue, epidermal cells, and cortex in the transition zone (Kamada et al., 2003). Previously, hybridization was conducted at 50°C and nonhybridized RNA probes were removed by electrophoresis (Kamada et al., 2003). In this study, hybridization was performed at 65°C and nonhybridized RNA probes were removed by incubating the hybridized sections for 2 h in 0.2× SSC at 65°C. Using these conditions for in situ hybridization, the CsPIN1 mRNA was more specifically detected compared with the previous study (Kamada et al., 2003). The transition zone of cucumber seedlings has four vascular strands, and the endodermis occurs around each vascular strand in the transition zone (Fig. 4; Supplemental Fig. S4). The signals for CsPIN1 mRNA were detected in the endodermis and xylem (Fig. 4, A and B). In addition, the signals for CsPIN4 and CsPIN6 were detected primarily in the xylem (Fig. 4, C and D) and in the phloem (Fig. 4, E and F) within the transition zone, respectively. Upon observation of the in situ hybridization using a phase-contrast microscope (Olympus IX71), CsPIN1 signals were found in the inner layer of the cortex and the outer side of the vascular bundles (Supplemental Fig. S4, A–D). Because these cells were referred to as endodermis (Esau, 1965), these results support the conclusion that CsPIN1 mRNA accumulates in endodermal cells.

Effects of Gravitostimulation on the Accumulation of CsPIN1 in the Endodermis of the Transition Zone

The in situ hybridization results indicated that CsPIN1 mRNA accumulates in the endodermis of the transition zone (Fig. 4). In the cucumber transition zone. Accordingly, the localization of the CsPIN1 proteins was characterized. Immunohistochemical analysis revealed that gravistimulation induces changes in CsPIN1 localization patterns in the endodermal cells of the transition zone. Furthermore, the change in CsPIN1 localization in the transition zone after gravistimulation is accompanied by the asymmetric distribution of auxin. These results provide novel insight into the mechanism of lateral auxin transport through the endodermis upon gravistimulation.

Gravity Response of CsPIN1
zone, the endodermis is thought to include gravity-sensing tissues due to the presence of sedimentable amyloplasts (Takahashi and Scott, 1994). Upon gravistimulation of Arabidopsis, the AtPIN3 expressed in gravity-sensing columella cells is rapidly relocalized (Friml et al., 2002b; Kleine-Vehn et al., 2010). Thus, the PIN proteins expressed in gravity-sensing tissues may play important roles in the induction of asymmetric auxin distribution after gravistimulation. However, the effects of gravistimulation on the localization of PIN proteins in the endodermal cells are still unknown. For this reason, the CsPIN1 localization in the endodermis of the transition zone of cucumber seedlings was studied. To produce polyclonal antibodies of CsPIN1, a recombinant protein containing a hydrophilic internal region of CsPIN1, located between the hydrophobic N-terminal and C-terminal regions, was expressed in *Escherichia coli*. The hydrophilic internal regions of the PIN proteins are not conserved, while the hydrophobic regions are conserved (Fig. 2). To examine the specificity of the polyclonal antibodies of CsPIN1, a recombinant protein containing a hydrophilic internal region of CsPIN1, located between the hydrophobic N-terminal and C-terminal regions, was expressed in *Escherichia coli*. The hydrophilic internal regions of the PIN proteins are not conserved, while the hydrophobic regions are conserved (Fig. 2). To examine the specificity of the polyclonal antibodies of CsPIN1, a recombinant protein containing a hydrophilic internal region of CsPIN1, located between the hydrophobic N-terminal and C-terminal regions, was expressed in *Escherichia coli*. The hydrophilic internal regions of the PIN proteins are not conserved, while the hydrophobic regions are conserved (Fig. 2).

When the cell wall was stained with Fluorescent Brightener 28 (Nagata and Takebe, 1970), the cells of the endodermis and cortex were easily distinguished from those of the vascular tissues. This was because the cells of the endodermis and cortex were noticeably larger than those of the vascular tissues (Supplemental Fig. S4, E–H). The endodermis is the inner layer of the cortex and the outer side of the vascular bundles (Supplemental Fig. S4, E–H; Esau, 1965). In the cross-section of the transition zone, immunohistochemical

---

**Figure 2.** Alignment of the deduced amino acid sequences of the cucumber CsPINs and Arabidopsis PIN1. The black background indicates identical amino acid residues among all sequences in the alignment. The red background indicates the putative Tyr motif, which signals for recruitment into the clathrin-coated vesicles (Supplemental Fig. S6; Mravec et al., 2009). Bars above the alignment indicate the hydrophobic regions that are highly conserved in the PIN protein family. Amino acid position numbers are indicated to the right.
staining with anti-CsPIN1 antibodies produced signals in the endodermal cells (Fig. 5, B and C; Supplemental Fig. S4, I–L). When the immunohistochemical staining was conducted using the preimmune antiserum, no signals were detected (data not shown). Therefore, the anti-CsPIN1 antibodies did not produce any background signals in the endodermis. In contrast to the endodermal signals, the CsPIN1 signals in the vascular bundles were not clear (Fig. 5, B and C). The transition zone contains two endodermal layers on the upper side and two layers on the lower side. When seedlings were grown in a vertical position, the CsPIN1 signal intensities were almost equal in the endodermis between the sides of the transition zone. When cucumber seedlings were grown in a horizontal position, much stronger CsPIN1 signals were detected on the lateral inner side of the endodermal cells on the upper side of the transition zone compared with the endodermal cells on the lower side (Fig. 5, B and C).

Figure 5, D to K, shows the results of the immunohistochemical staining of the longitudinal section of the transition zone with anti-CsPIN1 antibodies. In seedlings grown in a vertical position, the CsPIN1 signals were primarily detected on the lateral inner side and the apical side of the endodermal cells, and CsPIN1 signal intensities in the longitudinal section were nearly equal in the endodermis between the sides of the transition zone (Fig. 5, D–F). On the other hand, in seedlings grown in a horizontal position, the CsPIN1 signals on the lateral inner side of the endodermal cells on the upper side of the transition zone were much stronger than those on the lower side (Fig. 5, G–J). In the vascular bundle, the CsPIN1 signals were detected on the basal side of the cells (Fig. 5K). The CsPIN1 signals in the vascular bundle of horizontally grown seedlings were not significantly different from those of vertically grown seedlings (Fig. 5, D, G, and H).

These results suggested that the CsPIN1 signals in the endodermal cells of the transition zone of cucumber seedlings differed depending on the orientation of gravity response of CsPIN1.
seedling growth (Fig. 5). To clarify differences in CsPIN1 localization, the fluorescence of CsPIN1 signals in the endodermal cells was quantified (Fig. 6A). In the transition zone of cucumber seedlings grown vertically, the CsPIN1 signals on the lateral inner side of the endodermal cells were similar on both sides of the transition zone (represented by bars B and C, respectively, in Fig. 6B). On the other hand, when seedlings were grown in a horizontal position, the CsPIN1 signals on the lateral inner side of the endodermal cells of the upper side of the transition zone (represented by bar B in Fig. 6B) were significantly stronger than the others (represented by bars A, C, and D in Fig. 6B). Fluorescence quantification revealed that the CsPIN1 signals on the lateral inner side of the endodermal cells in the upper endodermis were 2.3 times the level of those in the lower endodermis (Fig. 6B). These results suggest that the pattern of CsPIN1 accumulation in the endodermis is affected by the orientation of gravity.

The Effect of Gravistimulation on the CsPIN1 Accumulation Pattern and Differential Auxin Distribution

To investigate whether the CsPIN1 accumulation pattern in the endodermal cells of the transition zone is correlated with auxin distribution in the transition zone, vertically grown seedlings were reoriented to a horizontal position. A time-course study of the changes in CsPIN1 accumulation in the endodermis and the auxin content of the transition zone was conducted (Fig. 6C). After gravistimulation for 10 min, the CsPIN1 signals on the lateral outer side of the endodermal cells on the lower side of the transition zone (represented by bar D in Fig. 6C) increased slightly more than those on the upper side of the transition zone (represented by bar A in Fig. 6C). This difference disappeared after gravistimulation for 30 min or longer. When gravistimulation was conducted for 30 min or longer, the CsPIN1 signals on the lateral inner side of the upper endodermis of the transition zone (represented by bar B in Fig. 6C) became significantly stronger than the others, similar to horizontally grown seedlings (Fig. 6C). The CsPIN1 signals on the lateral inner side of the endodermal cells on the lower side of the transition zone (represented by bar C in Fig. 6C) did not decrease completely, as was the case with the CsPIN1 signals in cucumber seedlings grown in a horizontal position. These results suggested that CsPIN1 accumulation in the upper endodermis of cucumber seedlings changes from a vertical pattern to a horizontal pattern within 30 min after gravistimulation. To perform a time-course study of auxin distribution in the transition zone, the endogenous IAA contents of the upper and lower sides of the transition zone were compared after 24-h-old seedlings grown in a vertical position were gravistimulated by reorientation to a horizontal position for various lengths of time. After gravistimulation for 30 to 120 min, the free IAA on the lower side of the transition zone was greater than that on the upper side. By contrast, when 24-h-old seedlings grown in a vertical position were not gravistimulated, free IAA between the left and right sides was maintained at similar levels (Fig. 7).

These results suggest that CsPIN1 may affect the gravimorphogenesis of cucumber seedlings by regulating lateral auxin transport in the transition zone after gravistimulation (Figs. 5–7). To obtain insight into the mechanism of CsPIN1 accumulation on the upper side of the transition zone after gravistimulation, pharmacological experiments were conducted using the auxin transport inhibitor TIBA. Previously, TIBA was shown to inhibit the suppression of peg formation on the upper side, resulting in the development of a peg on each side of the transition zone regardless of the orientation of seedling germination (Kamada et al., 2003). Thus, the effect of TIBA on CsPIN1 relocalization and on auxin redistribution in the gravistimulated transition zone of cucumber seedlings was analyzed. In this study, 24-h-old vertically grown seedlings were treated with 10^{-3} to 10^{-7} M TIBA or 0.1% dimethyl sulfoxide (DMSO; as a mock control) followed by growth for 48 h in a horizontal position (Table I). Cucumber seedlings treated with 10^{-4} and 10^{-3} M TIBA developed two pegs, while those treated with less than 10^{-4} M TIBA did not. In cucumber seed-
lings treated with $10^{-5}$ M TIBA or DMSO, CsPIN1 was
relocalized and accumulated on the lower side of the
upper endodermal cells after 1 h of gravistimulation
(Fig. 8, D–F and J) in a manner similar to the CsPIN1
time-course study (Fig. 6). By contrast, when cucum-
ber seedlings were treated with $10^{-4}$ or $10^{-3}$ M TIBA,
the relocation of CsPIN1 was suppressed after
seedlings were gravistimulated for 1 h (Fig. 8, G–J).
To clarify the correlation between the CsPIN1 pattern
and auxin distribution in the TIBA-treated seedlings,
the transition zone was divided in half and the en-
dogenous IAA content was measured. After gravisti-
mulation of the mock control and $10^{-5}$ M TIBA-treated
seedlings for 1 h, the auxin content increased on the
lower side of the transition zone. By contrast, when
cucumber seedlings were treated with greater than
$10^{-4}$ M TIBA, the auxin content was maintained in a
manner similar to the nongravistimulated samples
(Fig. 8K). These results suggest that TIBA inhibited not
only the relocation of CsPIN1 in the upper endo-
dermal cells of the transition zone but also the increase
of auxin on the lower side of the transition zone.

**DISCUSSION**

**Phylogenetic Diversity of PIN Family Proteins**

In this study, five cDNAs of cucumber CsPIN genes,
in addition to CsPIN1, were isolated. Phylogenetic
analysis indicated that these cucumber CsPINs belong
to a clade containing AtPIN1, AtPIN2, AtPIN3, AtPIN4,
and AtPIN7 that has been well characterized. AtPIN1 facilitates the polar transport of auxin from the shoot apex to the root tip (Galweiler et al., 1998). AtPIN2 transports auxin from the root tip to the root elongation region (Müller et al., 1998). AtPIN3 and AtPIN7 play roles in the asymmetric distribution of auxin in response to gravistimulation in root columella cells (Friml et al., 2002b; Kleine-Vehn et al., 2010). AtPIN4 is responsible for the auxin gradient involved in root patterning (Friml et al., 2002a). CsPIN5 shows an exclusive relationship with an Arabidopsis PIN; that is, clade II contains only CsPIN5 from cucumber and only AtPIN2 from Arabidopsis (Fig. 3). This suggests that cucumber CsPIN5 is an ortholog of Arabidopsis AtPIN2. In Arabidopsis, the PIN proteins are divided into two classes: one class contains plasma membrane-localized PINs such as AtPIN1, and the other class includes endoplasmic reticulum-localized PINs such as AtPIN5 (Mravec et al., 2009). The amino acid sequence around the putative Tyr motif (NPNTY), which is a signal for recruitment into the clathrin-coated vesicles, determines the intracellular localization (Mravec et al., 2009). The sequence around the putative Tyr motif in the cucumber PINs identified in this study is similar to those found in plasma membrane-localized PINs (Fig. 2; Supplemental Fig. S6), suggesting that these cucumber PINs localize in the plasma membrane.

The phylogenetic analysis does not clearly indicate which clade contains AtPIN1, due to a poor bootstrap value (44%) of the clade consisting of AtPIN1 and clade I (Fig. 3). Clades III and IV do not contain Arabidopsis AtPINs, although clades II and V include AtPIN2 and AtPIN3/AtPIN4/AtPIN7, respectively. Therefore, it is likely that CsPINs such as CsPIN1/CsPIN3 and CsPIN4, belonging to clades III and IV, respectively, play a role that requires AtPIN1 in Arabidopsis. This inference is consistent with the accumulation of CsPIN1 and CsPIN4 mRNAs in the xylem tissues of the transition zone, since AtPIN1

Figure 6. Quantification of the intensity of CsPIN1 fluorescence in the endodermis of the transition zone. A, Schematic representation of the longitudinal section of the transition zone and quantification of the intensity of CsPIN1 fluorescence on the lateral sides of the endodermal cells. The red lines that are perpendicular drawn to the lateral sides of the cell indicate the positions that were used to quantify the fluorescence signal intensities. When the localization of CsPIN1 in cucumber seedlings grown in a horizontal position was analyzed, the intensity of CsPIN1 fluorescence on the outer and the inner sides of the endodermal cells of the upper side of the transition zone were measured as A and B, respectively. In the endodermal cells of the lower side of the transition zone, those on the inner and the outer sides were also measured as C and D, respectively. When cucumber seedlings that had been grown in a vertical position were used for analysis, those on the outer and inner sides of the transition zone were measured as A and B on the left side and as C and D on the right side, respectively. B, A comparison of the intensity of CsPIN1 fluorescence on the lateral sides of the endodermal cells at each side (the upper and lower sides or the left and right sides) of the transition zone. Twenty-four-hour-old cucumber seedlings that had been grown in either a horizontal or a vertical position were used in the experiments. C, Time course of the changes in the pattern of CsPIN1 accumulation in the endodermis of the transition zone of cucumber seedlings after gravistimulation. Quantification of the intensity of CsPIN1 fluorescence on the lateral sides of the endodermal cells on each side (the upper and lower sides or the left and right sides) of the transition zone after gravistimulation for 0, 10, 30, 60, 90, and 120 min is shown. The intensity of CsPIN1 fluorescence was quantified on the lateral sides in 10 endodermal cells on each side (the upper and lower sides or the left and right sides) of the transition zone per section. This was repeated in three sections prepared from one seedling, and the average of 30 values on each lateral side in the endodermal cells of each side of the transition zone in each seedling was calculated. Each datum represents the mean ± SD for at least five seedlings. Different letters above the bars indicate statistically significant differences at \( P < 0.05 \) by the Tukey method.

Figure 7. Free IAA levels in the transition zone after gravistimulation. Vertically grown 24-h-old intact seedlings were further maintained in a vertical position (A) or were reoriented 90° (B). After incubation for the indicated time, the right and left halves (A) or the upper and lower halves (B) of the transition zone sections were excised and extracted. Free IAA was determined by gas chromatography-selected ion monitoring-mass spectrometry. The free IAA amount per g fresh weight (FW) is indicated. Each datum represents the mean ± SD from three independent experiments. Single and double asterisks indicate statistically significant differences between the upper side and the lower side at \( P < 0.05 \) and \( P < 0.01 \), respectively, by Student’s t test.
mRNA accumulates in the cambial and xylem tissues of the stem (Gälweiler et al., 1998). AtPIN7 has been shown to accumulate in the vascular tissue of roots, similar to AtPIN1 (Blilou et al., 2005). Furthermore, genetic experiments with Arabidopsis have revealed that mutation of AtPIN1 together with mutations in AtPIN3, AtPIN4, and/or AtPIN7 confer additive mutant phenotypes. This suggests functional redundancy between AtPIN1, AtPIN3, AtPIN4, and AtPIN7 (Friml et al., 2003; Blilou et al., 2005). AtPIN3 mRNA is expressed in the endodermis of the hypocotyl and stem (Friml et al., 2002b). In the transition zone of cucumber, CsPIN1 accumulates an abundance of mRNA in the endodermis, similar to AtPIN3 (Fig. 4). This is not the case for CsPIN2 and CsPIN6, which, along with AtPIN3, belong to clade V. These results indicate that the tissue-specific mRNA accumulation and the physiological and/or developmental function(s) of clade III, clade IV, clade V, and AtPIN1 overlap. Therefore, the role of CsPIN1, expressed in the endodermis of the transition zone of cucumber seedlings, is equivalent to that of Arabidopsis AtPIN3. By contrast, the role of CsPIN1 and CsPIN4, expressed in the xylem, is equivalent to that of Arabidopsis AtPIN1. The results of the immunohistochemical analysis of CsPIN1 in the vascular bundle cells of the transition zone are also consistent with this conclusion, because AtPIN1 localizes at the basal end of cells in the vascular bundle within stems, and CsPIN1 localizes at the basal end of cells in the vascular bundle in the transition zone (Gälweiler et al., 1998; Fig. 5).

In Arabidopsis roots, the direction of auxin transport has been well characterized (Blilou et al., 2005). AtPIN1 and AtPIN7 transport auxin through the vascular bundle from the aerial organs to the root tip. AtPIN4 functions near the quiescent center of the root tip to transport auxin to the columella cells. AtPIN3 and AtPIN7 act in columella cells to transport auxin to the lateral root cap cells. AtPIN2 transports auxin through the lateral root cap cells and epidermal cells to the elongation zone of roots. The results of the in situ hybridization experiments using cucumber root sections indicated that CsPIN1 and CsPIN4 mRNAs accumulate in the vascular bundle; CsPIN1, CsPIN2, and CsPIN3 mRNAs accumulate around the quiescent center of root tips; CsPIN6 mRNA accumulates in the columella cells; and CsPIN5 mRNA accumulates in the lateral root cap cells and epidermal cells (Supplemental Fig. S2). These results, as well as those of the phylogenetic analysis, indicated that CsPIN5 corresponds to AtPIN2, while the other CsPINs do not clearly correspond to specific AtPINs.

### Table 1. Effect of TIBA on peg formation in cucumber seedlings

Cucumber seedlings that were grown for 24 h were treated with DMSO or $10^{-5}$ to $10^{-3} \times$ TIBA for 1 h in a vertical position. The seedlings were reoriented into a horizontal position and grown until 72 h old. The values indicate the percentage of seedlings ($n = 14–19$) that formed one peg, two pegs, or no peg. The experiments were repeated three times, and the mean ± SD was calculated. Asterisks indicate statistically significant differences in the percentage of seedlings that were treated with TIBA compared with the mock control seedlings at $P < 0.05$ by Student’s $t$ test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>One Peg</th>
<th>Two Pegs</th>
<th>No Peg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock</td>
<td>92.6 ± 6.5</td>
<td>7.4 ± 6.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>$10^{-5}$ M TIBA</td>
<td>92.6 ± 6.5</td>
<td>7.4 ± 6.5</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>$10^{-4}$ M TIBA</td>
<td>3.5 ± 3.0*</td>
<td>96.5 ± 3.0*</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>$10^{-3}$ M TIBA</td>
<td>2.4 ± 4.1*</td>
<td>88.4 ± 4.8*</td>
<td>9.3 ± 8.5*</td>
</tr>
</tbody>
</table>

### Auxin Transport in the Transition Zone of Cucumber Seedlings

Similar to other plant species, auxin transport in cucumber seedlings is thought to occur via polar transport from the cotyledons and/or apical meristems to the transition zone (Witztum and Gersani, 1975). In Arabidopsis, AtPIN1 localizes at the basal plasma membrane in xylem parenchyma cells and is responsible for the polar transport of auxin in stems (Gälweiler et al., 1998). The results of this study indicate that mRNAs of CsPIN1, CsPIN4, and CsPIN6 accumulate abundantly in the endodermis/xylem, xylem, and phloem cells of the transition zone, respectively (Fig. 5). Therefore, it is possible that CsPIN1 and CsPIN4 have mRNAs that accumulate in the xylem and are responsible for the polar transport of auxin in cucumber seedlings. Furthermore, the immunohistochemical analysis indicated that CsPIN1 in vascular bundle cells is localized at the basal side of the cells, similar to AtPIN1 in xylem cells (Gälweiler et al., 1998). Experiments using radiolabeled IAA in the internodes of Coleus blumei suggested that auxin is transported polarly and accumulates slightly in the xylem but primarily in the phloem (Wangermann, 1970). In this study, the results indicated that the mRNA of CsPIN6 accumulates in the phloem of the transition zone (Fig. 5, E and F). Thus, it is possible that the phloem is also involved in the transport of auxin. In the endodermis of the transition zone of cucumber seedlings, CsPIN1 is located primarily at the lateral inner side of the endodermal cells. For this reason, CsPIN1 in the endodermis may play a role in lateral auxin transport.

### Change in CsPIN1 Accumulation in the Endodermis of the Transition Zone in Cucumber Seedlings by Gravistimulation

When cucumber seedlings are grown in a horizontal position, a peg forms on the lower side of the transition zone between the hypocotyl and the root (for review, see Takahashi, 1997). Previous spaceflight experiments suggested that the upper side of the transition zone responds to gravistimulation, which results in the suppression of peg formation (Takahashi et al., 2000). In this study, gravistimulation increased CsPIN1 on the lateral inner side (the lower side or the vascular side) in the endodermal cells on the upper side of the transition zone. This gravity response of
CsPIN1 localization occurred on the upper side of the transition zone and was consistent with the findings of spaceflight experiments (Takahashi et al., 2000). Gravistimulation is suspected to induce asymmetric auxin distribution in the transition zone and influence the location of peg formation (Fujii et al., 2000; Kamada et al., 2000, 2003; Saito et al., 2004, 2005). The application of TIBA, an inhibitor of auxin efflux, induces peg formation on the upper side of the transition zone of cucumber seedlings grown horizontally (Kamada et al., 2000). This result suggests that the activation of auxin efflux on the upper side of the transition zone suppresses peg formation. This is consistent with the increase in CsPIN1 in the endodermal cells on the upper side of the transition zone (Figs. 5 and 6). These results also indicated that TIBA treatment inhibited CsPIN1 relocalization in the upper endodermal cells of the transition zone and also inhibited the auxin increase on the lower side of the transition zone (Fig. 8). Furthermore, induction of asymmetric CsPIN1 accumulation in the transition zone after gravistimulation was accompanied by asymmetric auxin distribution (Figs. 6 and 7). Taken together, the accumulation pattern of CsPIN1 in the endodermis is altered in response to gravistimulation, and this alteration induces asymmetrical lateral auxin transport, which suppresses peg formation on the upper side of the transition zone in cucumber seedlings grown horizontally.

To demonstrate that the CsPIN1 protein is involved in the gravistimulation response of cucumber seedlings, reverse genetic studies should be conducted. Unfortunately, a suitable transformation protocol for cucumber plants has not been established. Recently, a TILLING (for targeting induced local lesions in genomes) strategy was developed. This strategy is sufficient for use as a reverse genetics strategy (McCallum et al., 2000). In other Cucurbitaceae species such as melon (Cucumis melo), a TILLING mutant population was harvested (Boualem et al., 2008). However, a cucumber TILLING mutant population has not been harvested. Therefore, to functionally characterize CsPIN1 during gravimorphogenesis, either the construction of a cucumber TILLING mutant population or the isolation of a CsPIN1 ortholog gene in melon plants is useful.

**Figure 8.** Effects of TIBA on CsPIN1 localization and auxin redistribution in the transition zone of cucumber seedlings. A to C, Yellow triangles indicate a peg. Bars = 5 mm. D to I, CsPIN1 immunolocalization in the longitudinal section of the transition zone treated with TIBA. The images represent the transition zone of cucumber seedlings grown in a vertical position for 24 h and treated with 0.1% DMSO (mock control; D) or $10^{-5}$ M TIBA (G) for 1 h and then gravistimulated for 1 h. The top and bottom boxes drawn by white lines in D correspond to E and F, while the boxes drawn in G correspond to H and I, respectively. co, Cortex; g, direction of gravitational force; en, endodermis; vb, vascular bundle. The yellow arrowheads indicate CsPIN1. Bars = 200 μm in D and G and 50 μm in E, F, H, and I. J, Quantification of the CsPIN1 fluorescence intensity in the endodermis of the transition zone. The intensity of CsPIN1 fluorescence was quantified in the same manner as in Figure 6. Each datum represents the mean ± SD for at least five seedlings. Different letters above the bars indicate statistically significant differences at P < 0.05 by the Tukey method. K, Free IAA levels in the transition zone after 1 h of TIBA treatment followed by 1 h of gravistimulation. Each datum represents the mean ± SD from three independent experiments. Different letters above the bars indicate statistically significant differences at P < 0.05 by the Tukey method. FW, Fresh weight.
Cortical cells enlarge perpendicularly against the hypocotyl-root axis, causing the formation of a peg. This result suggests that auxin levels in the cortex of the transition zone determine the side of the transition zone where the peg forms. In addition, gravistimulation did not decrease the auxin content on the upper side of the transition zone but did increase the auxin content on the lower side (Fig. 7). Based on the immunohistochemical results (Figs. 5 and 6), CsPIN1 in the endodermal cells on the upper side of the transition zone is proposed to inhibit auxin transport from the vascular bundle to the cortex on the upper side of the transition zone of cucumber seedlings grown horizontally (Supplemental Fig. S7). CsPIN1 in the vascular bundle facilitates polar auxin transport through the vascular bundle from the apical portion of the seedlings to the root tip. If auxin in cortical and epidermal cells in the transition zone is derived mainly from apical parts, then the CsPIN1 in the endodermal cells should have a large impact on auxin distribution. Endodermal cells that accumulate CsPIN1 on the lateral inner side should prevent auxin transport from the vascular bundle to the cortical cells on the peg-suppressed side (the upper side of the transition zone) in cucumber seedlings grown horizontally (Supplemental Fig. S7). By contrast, endodermal cells that do not accumulate much CsPIN1 on the lateral inner side might diffusely move auxin from the vascular bundle to the cortical and epidermal cells on the peg-forming side of the transition zone in cucumber seedlings grown in either the horizontal or vertical position (Supplemental Fig. S7). In addition, when cucumber seedlings grown vertically were reoriented to the horizontal position, CsPIN1 levels on the outer side of endodermal cells on the lower side of the transition zone transiently increased after gravistimulation for 10 min (Fig. 6C). This transient increase in CsPIN1 appears to contribute to the increase in auxin on the lower side of the transition zone. Other possibilities that auxin facilitator(s) other than CsPIN1 are responsible for the auxin transfer to the cortex/epidermis in the transition zone of cucumber seedlings cannot be excluded. The Cholodny-Went theory holds that plants respond to gravity by altering lateral auxin transport to induce the asymmetric distribution of auxin (Went and Thimann, 1937; Evans, 1991; Trewavas, 1992; Iino, 1995). The gravity responses of CsPIN1 accumulation in the endodermal cells may be involved in regulating the endodermal cell responses to gravity by changing lateral auxin transport, as postulated by the Cholodny-Went theory.

Gravistimulation causes AtPIN3 to relocalize in the columella cells of Arabidopsis roots (Friml et al., 2002b). All columella cells do not uniformly relocalize AtPIN3. Instead, approximately 50% of columella cells relocalize AtPIN3 to the lower side by responding to gravistimulation (Harrison and Masson, 2008; Kleine-Vehn et al., 2010). In contrast to the root tip, which contains only one cluster of gravity-sensing columella cells, the transition zone of cucumber seedlings contains four clusters of gravity-sensing endodermal cells. The results presented in this study indicate that the accumulation pattern of CsPIN1 in the endodermal cells of the upper side of the transition zone of cucumber seedlings is different from that of the lower side (Figs. 5 and 6). The position of the endodermal cells in relation to the vascular bundle cells or to the cortical cells of the upper side of the transition zone is different from that of the lower side. Therefore, it is more probable that the positional information of the endodermal cells would play a role in changing CsPIN1 accumulation by responding to gravistimulation rather than each endodermal cell in the transition zone of cucumber seedlings responding independently to gravistimulation. In addition, TIBA functions as an actin stabilizer and inhibits several actin-based vesicle-trafficking processes, including auxin transport-dependent processes in plants (Dhonukshe et al., 2008). Therefore, it is likely that the actin-based vesicle-trafficking process is involved in the relocalization of CsPIN1 in the endodermal cells during gravity responses in the transition zone of cucumber seedlings.

CONCLUSION

In this study, CsPIN1 was shown to be localized not only in the vascular bundle but also in the endodermis, the supposed gravity-sensing tissue in the transition zone of cucumber seedlings. Immunohistochemical analysis revealed that CsPIN1 is localized at the basal end in vascular cells and at the apical and lateral inner sides in endodermal cells. When cucumber seedlings were placed in a horizontal position, CsPIN1 increased significantly on the lateral inner side in the endodermal cells of the upper side of the transition zone. This increase in CsPIN1 on the lateral inner side in the endodermal cells might be able to prevent the upper side of the transition zone from transporting auxin to cortical cells. As a result, the upper side of the transition zone induced asymmetric auxin distribution in the transition zone and suppressed peg formation.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

Cucumber seeds (Cucumis sativus ‘Shinkasyu-jibai’) were purchased from Watanabe Seed Co. Fourteen cucumber seeds were placed vertically in a petri dish (60 mm × 30 mm × 10 mm; AION) attached to the inner surface of a plastic petri dish (60 mm × 60 mm × 60 mm) cap. After supplying the block with 10 mL of distilled water, this plastic cap was placed in a petri dish to ensure that the seedlings would be suspended in the air space of the container after germination and would be grown aeroponically. This petri dish was placed in a horizontal position or in a vertical position under darkness at 25°C.

Cloning of the CsPIN cDNAs

To isolate cucumber PIN cDNA using reverse transcription-PCR, forward degenerate primers were designed based on the amino acid sequences of IMEQFPE and WWKRI5/7/TP, respectively: CsPIN3 dg-hydrophilic region, 5′-AT(T/A/C)ATGGA/G/A(C/A/G)AT(T/C)ACC(T/C/A/G)GAG-3′, and PIN-dg-F1, 5′-TGTTGAAA/A/G(AT(T/C)TT(T/C/T/A/C)G(G/T/C/A/G)-3′. Degenerate reverse primers were designed based on the amino acid sequences of IMWVRKL and GMAMFSI, respectively: CsPIN3 dg-hydrophilic region, 5′-A(A/G/T/C)CT(A/T/G/C/T/GCA/A/T/G/C)ACCAT(T/GA)AT-3′.
and PIN-dg-R1, 5′-A/G/TG(T/G)(T/A)AAACAT/C/A/GGCAAT/C/G(A) CC-3′. The PCR involved 35 cycles of 94°C for 30 s, 50°C for 30 s, and 70°C for 1 min. The PCR products were ligated into the pGEM-T vector (Promega) according to the manufacturer’s protocol (Promega). The nucleotide sequences of the cloned products were determined using Big Dye Terminator version 3.1 (Applied Biosystems) with an ABI sequencer (model 310; Applied Biosystems).

The α-ZAPf1 cDNA library of cucumber (cv Shinkasyu-jibai), derived from auxin-treated hypocotyls of 72-h-old seedlings (Fujii et al., 2000), was screened using a digoxigenin-labeled RNA probe according to the manufacturer’s protocols (Roche). The probe was derived from the cloned cDNA and used to isolate the full-length cDNAs of CsPIN2 and CsPIN3. To determine the nucleotide sequences of the 5′ end of the full-length cDNAs of CsPIN4, CsPIN5, and CsPIN6, 3′ RACE was performed. cDNA was synthesized from mRNA purified using an mRNA purification kit (Amersham), oligo(dT)-adaptor primer of 5′-ACACCTGGTCGATCATGGC-3′; and ReverTra Ace reverse transcriptase (ToyoBo). The 3′ region of the cDNA was amplified using 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min with the adaptor primer (5′-GACTCCAGTGCCATGATCG-3′) and each gene-specific primer. The gene-specific primers were as follows: CsPIN4-SPF1, 5′-AGAGGGAATGGTGTTGATGGGCCC-3′; CsPIN5-SPF1, 5′-CACCCGCTAAATTAGACCCGCCG-3′; and CsPIN6-SPF1, 5′-AACCCGAACCTCGGAGAGGC-3′. PCR was performed using the adaptor primer and internal gene-specific primers as follows: CsPIN4-SPF2, 5′-TGTGTTGACAGTGCACTGGGCCC-3′; CsPIN5-SPF2, 5′-CAGGAGAACCTTACGTCG-3′; and CsPIN6-SPF2, 5′-GACTCCCACTGTACTCACCG-3′. The 5′ region of the full-length cDNAs of CsPIN4, CsPIN5, and CsPIN6, the 5′ region of cDNA was amplified via 5′ RACE with the SMART RACE cDNA Amplification kit (BD Bioscience) according to the manufacturer’s protocols using gene-specific primers as follows: CsPIN4-SPF1, 5′-ATCTCCCCAAAGAATAACCCG-3′; CsPIN5-SPF1, 5′-TTGTTTGGAGTTCGAGTGCG-3′; and CsPIN6-SPF1, 5′-GACTCCCACTGTACTCACCG-3′. The PCR products were ligated into the pGEM-T plasmid by TA cloning (Promega). Nucleotide sequences were determined using Big Dye Terminator version 3.1 (Applied Biosystems) with an ABI sequencer (model 310; Applied Biosystems).

For genomic DNA gel blot analysis, see Supplemental Materials and Methods S1.

Phylogenetic Analysis

Alignment of amino acid sequences was generated using ClustalX (Thompson et al., 1997). The alignment corresponding to the amino acid sequences (indicated by the bars in Fig. 2) was converted into an alignment of nucleotide sequences using CodonAlign 2.0 software (http://sinauer.com/hall/). Insertions and deletions were ignored in the analyses. A neighbor-joining tree was obtained using PAUP software version 4.0b10 (Sinauer Associates). Bootstrapping of 1,000 replicates for neighbor-joining analysis was processed to estimate the confidence probabilities on each branch of the phylogenetic trees that were constructed.

In Situ Hybridization

In situ hybridization was performed following the method of Kamada et al. (2003) with modifications (Sugiyama et al., 2006). Before hybridization, 100 μL of prehybridization solution [50% formamide, 300 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0), 1× Denhardt’s solution, 10% dextran sulfate, 60 μM dithiothreitol, 1 mg mL−1 Escherichia coli RNA, and 500 μg mL−1 poly(A)] was applied to the cDNA-microarray slides. After hybridization for 2 h at 50°C, 50 μL of prehybridization solution was removed, and 100 μL of prehybridization solution containing 1 μL μL−1 digoxigenin-labeled antisense RNA probe was added. The slides were incubated in a humid box at 65°C overnight. An additional incubation was conducted for 2 h in 0.2× SSC at 65°C to remove any nonhybridized probe.

Immunohistochemical Analysis

To generate CsPIN1-specific polyclonal antibodies, a fragment of the CsPIN1 cDNA was amplified using CsPIN1-Nid-forward primer (5′-CTC-TGATGGCTGCTCGGTGAAAG-3′) and CsPIN1-Inv-reverse primer (5′-AGTGGGCTGTCAGGTTTCCG-3′). The fragment amplified encodes the antigenic peptide of CsPIN1 from amino acids 263 to 450. The fragment was ligated into the bacterial expression vector pET18b (Novagen) to produce an expression construct encoding a recombinant fusion protein with an N-terminal His tag. After expression in E. coli BL21 (DE3), the recombinant protein was purified on a His-Trap HP column (GE Healthcare) and examined by SDS-PAGE. After immunization of rabbits, the polyclonal antiserum was affinity purified against the recombinant CsPIN1 peptide. Cucumber seedlings were fixed in ethanol:chloroform:acetic acid solution (6:3:1) and cooled at 4°C (Ruzin, 1999). The seedlings maintained their orientation to gravity for 4 h. The transition zones of cucumber seedlings were dissected and further fixed overnight at 4°C. Fixed segments were embedded in Paraplast Plus (Oxford Labware) and sectioned into 10-μm-thick slices. Immunohistochemical analysis was performed according to Reinhardt et al. (2003). After the removal of Paraplast Plus and rehydration, immunofluorescence detection of CsPIN1 was performed using primary rabbit anti-CsPIN1 antibody (1:100) and Alexa Fluor 488-conjugated goat secondary antibody IgG (Molecular Probes). After immunostaining, the cell wall was stained with 0.08% Fluorescent Brightener 28 (Sigma) in water for 5 min (Nagata and Takebe, 1979) and washed three times in phosphate-buffered saline (pH 7.4) containing 0.1% (v/v) Tween 20. Fluorescence was detected with a laser-scanning confocal microscope (Fluoview FV1000 Olympus). For expression of recombinant proteins in E. coli, preparation of microsomal membrane fractions, and protein gel blot analysis, see Supplemental Materials and Methods S1.

Extraction and Determination of Free IAA

IAA was purified and measured following the methods of Nishimura et al. (2006) with slight modifications. To extract free IAA, the halves of the cucumber seedling transition zones (10–15 mg fresh weight) were placed in 2.0-mL tubes with 180 μL of cold 80% methanol containing 0.1 mg mL−1 2,6-di-tert-butyl-4-methylphenol, 2.0 to 4.0 ng of [13C6]IAA (Cambridge Isotope Laboratories), and a small amount of glass beads. The tubes were sealed and shaken vigorously for 4 min using a Tissue Lyser (Qiagen). After filtration at 15,000g for 10 min at 4°C, IAA was purified directly from the supernatant using an HPLC (LC-10AD, C-RSA; Shimadzu) connected to a fluorescence detector (RF-10A; Shimadzu). Ion-charged HPLC was carried out on a Nucleosil N(C18), 2 μm i.d. × 100 mm; GL Sciences) at a flow rate of 0.8 mL min−1 with 0.3% acetic acid in methanol. IAA was eluted at a retention time of about 14 min by monitoring at 235 nm and excitation at 290 nm. From the crude extracts, IAA was quantified by a gas chromatography-selected ion monitoring-mass spectrometry device (QP2010; Shimadzu) coupled to a mass chromatograph (GC-2010; Shimadzu) equipped with a capillary column (HR-1701; 0.25 mm i.d. × 30 m, film thickness of 0.32 μm; Shinwa Chemical Industries). The injector temperature was set at 290°C; the flow rate of the helium carrier gas was 1 mL min−1. The column temperature was maintained at 80°C for 1 min and increased to 280°C at a rate of 30°C min−1. Dried samples were trimethylsilylated with N-(methyl-N-trimethylsilyl)-trifluoroacetamide at 60°C for 1 min. Ions with mass-to-charge ratios of 202 and 208 (ions from native IAA and [13C6]IAA internal standard, respectively) and 319 and 325 (molecular ion and m/z +6, respectively) were monitored.

Statistical Analysis

To compare the intensity of CsPIN1 fluorescence on the lateral side of the endodermal cells and the auxin content, the Tukey method was used. Watanabe et al. (2012) used the same methods to compare the CsPIN1 fluorescence on the lateral side of the endodermal cells and the auxin content.
Gravity Response of CsPIN1

Zoa mazes; and SePIN (CP000029.1) from Staphylococcus epidermidis as an outgroup.

Supplemental Data

The following materials are available in the online version of this article.
Supplemental Figure S1. Southern-blot analysis of CsPIN2–CsPIN6.
Supplemental Figure S2. Accumulation of CsPIN mRNA in cucumber roots.
Supplemental Figure S3. Northern-blot analysis of CsPIN mRNAs.
Supplemental Figure S4. The magnified cross section of the transition zone.
Supplemental Figure S5. Western-blot analysis by anti-CsPIN1 antibody.
Supplemental Figure S6. Alignment of the potential signal motif for recruitment into the clathrin-coated vesicles of PIN proteins.
Supplemental Figure S7. Schematic representation of a model to explain how gravistimulation induces the asymmetric distribution of auxin in the transition zone of cucumber seedlings.

Supplemental Materials and Methods S1.

Received October 12, 2011; accepted November 2, 2011; published November 7, 2011.

LITERATURE CITED


Witztum A, Gersani M (1975) The role of IAA in the development of the peg in Cucumis sativus L. Bot Gaz 136: 5–16


Downloaded from on November 11, 2017 - Published by www.plantphysiol.org
Copyright © 2012 American Society of Plant Biologists. All rights reserved.