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Requirement of the Auxin Polar Transport System in Early Stages of *Arabidopsis* Floral Bud Formation

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The *pin-formed* mutant *pin*1-1, one of the *Arabidopsis* flower mutants, has several structural abnormalities in inflorescence axes, flowers, and leaves. In some cases, *pin*1-1 forms a flower with abnormal structure (wide petals, no stamens, pistil-like structure with no ovules in the ovary) at the top of inflorescence axes. In other cases, no floral buds are formed on the axes. An independently isolated allelic mutant (*pin*1-2) shows similar phenotypes. These mutant phenotypes are exactly the same in wild-type plants cultured in the presence of chemical compounds known as auxin polar transport inhibitors: 9-hydroxyfluorene-9-carboxylic acid or *N*-(1-naphthyl)phthalamic acid. We tested the polar transport activity of indole-3-acetic acid and the endogenous amount of free indole-3-acetic acid in the tissue of inflorescence axes of the *pin*1 mutants and wild type. The polar transport activity in the *pin*1-1 mutant and in the *pin*1-2 mutant was decreased to 14% and 7% of wild type, respectively. These observations strongly suggest that the normal level of polar transport activity in the inflorescence axes is required in early developmental stages of floral bud formation in *Arabidopsis* and that the primary function of the *pin*1 gene is auxin polar transport in the inflorescence axis.

INTRODUCTION

Developmental processes of flower formation and morphogenesis can be dissected genetically by the isolation and characterization of mutants with flowers of immature or abnormal structure. Recently, this approach was taken using *Arabidopsis* because this plant has several features suited for molecular genetic analyses: small genome size, short generation time, ease of cultivation and genetic analyses, accumulated genetic information, and the recent development of experimental procedures such as transformation and gene tagging (for review, see Estelle and Somerville, 1986; Meyerowitz, 1987, 1989). Many kinds of flower mutants have been isolated and characterized (Haughn and Somerville, 1988; Komaki et al., 1988; Bowman et al., 1989; Meyerowitz et al., 1989; Okada et al., 1989). The *pin-formed* mutant *pin*1-1 shows a unique structure in the inflorescence axis. The mutant has either no floral buds or one flower with a heavily deformed structure at inflorescence (Goto et al., 1987; Haughn and Somerville, 1988). The genetic defect of the mutation appears to reside in some early stage(s) of floral bud formation. Genetic analyses showed that the mutant carries a recessive, nuclear mutation.

To complement the physiological abnormalities of the *pin*1-1 mutant, gibberellins were supplied to the plant. However, the mutant phenotype was not changed by the treatment (Goto et al., 1987). We have found recently a striking structural resemblance between the phenotype of the mutant and that of wild-type plants that were cultured in the presence of auxin polar transport inhibitors. In this paper, we describe the phenotypic resemblance of the mutant and wild-type plants treated with auxin polar transport inhibitors and the reduced activity of polar transport in the mutant tissue. The results strongly suggest that the auxin polar transport system is required in the early stage(s) of floral bud formation.

RESULTS

Phenotype of the *pin-formed* Mutant

Because the *pin*1 mutation is recessive, about 75% of the progeny plants generated from a heterozygous parent (*pin*1/+) have a normal morphology and the rest have abnormal inflorescence axes, flowers, and leaves. Two types of structural abnormalities were observed in the

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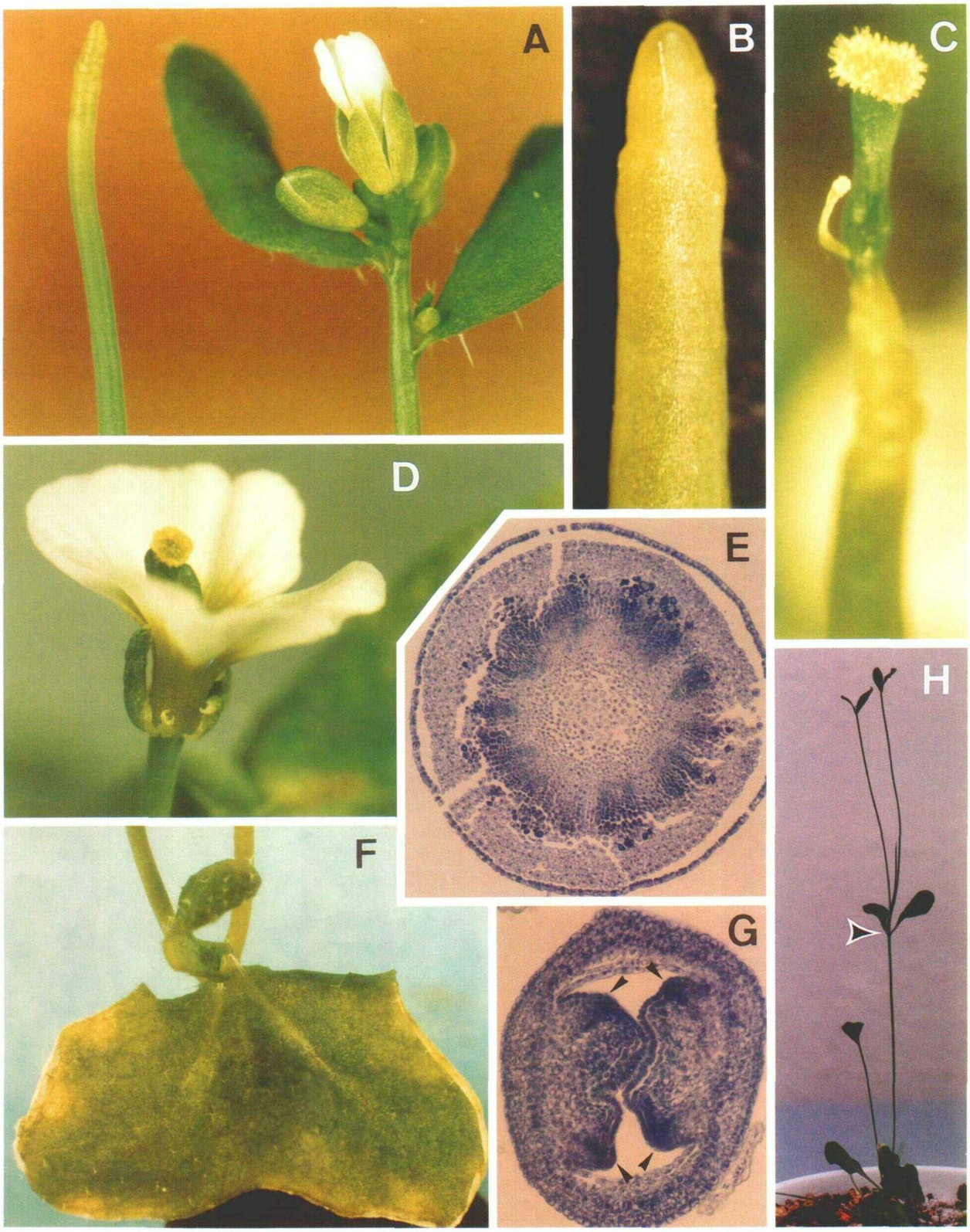


Figure 1. Phenotype of the *pin*1 Mutant.

inflorescence axes and flowers. Figures 1A and 1B show that the type A inflorescence has no flowers or flower-related structures. There are no obvious traces of pedicels or floral buds at or near the top of the axes. The transverse section at the top of the axis has no floral meristems (Figure 1E). Type B inflorescence has a flower or a pistil-like structure at the top. Flowers of this type usually have no stamens and have petals with abnormal shapes (Figure 1D). The number of petals is variable (from two to six). In some cases, a pistil-like structure without sepals, petals, and stamens is formed at the top (Figure 1C). The pistil-like structures are sterile because the ovules are not developed (Figure 1G). The ratio of the type A and type B abnormal inflorescences is about 2 to 1. In some cases the inflorescence axes are fasciated. The degree of apical dominance seems to be normal because the axillary shoots begin to grow after the main shoots grow to about 25 cm high.

The *pin*1 mutant also shows structural abnormalities in leaves. Some leaves become much wider because the major vein is branched at the base (Figure 1F). Cotyledons are often fused or deformed (data not shown). Phyllotaxis on the inflorescence axes is also abnormal. In wild-type plants, the leaves on the axis are formed in "alternate" positions where the axillary shoots appear. In the mutant, leaves and axillary shoots are often formed in "opposite" positions along the inflorescence axis (Figure 1H).

Timing of bolting delays slightly in the *pin*1 mutant. It takes 4 to 5 weeks after germination until the inflorescence axes of the mutant start to elongate, whereas it takes 3 to 4 weeks for wild-type plants.

We have recently isolated a new *pin-formed* mutant (*pin*1-2), which carries a mutation allelic to the former mutant (*pin*1-1). The variety of phenotype of this mutant is almost the same as that of the *pin*1-1 mutant described above (data not shown).

Effects of the Auxin Polar Transport Inhibitors on Wild-Type Plants

To discover the roles of the auxin polar transport systems in flower development, we cultured wild-type *Arabidopsis* on agar plates containing auxin polar transport inhibitors: 9-hydroxyfluorene-9-carboxylic acid (HFCA) (morphactin) (Krelle and Libbert, 1968; Gaither, 1975), *N*-(1-naphthyl)phthalamic acid (NPA), or 2,3,5-triiodobenzoic acid (TIBA) (Thomson et al., 1973; Cande and Ray, 1976). The seeds germinated normally, but plant growth was retarded. Inflorescence axes with aberrant structure elongated after about 4 weeks of incubation. As shown in Figure 2C, no floral buds were formed at the top of the axes in some cases. In other cases, flowers with no stamens and structurally aberrant petals (Figure 2B) or a pistil-like structure (Figure 2D) were observed at the top of the axes. The former case resembled the type A inflorescence and the latter case resembled the type B inflorescence of the *pin*1 mutant. Phyllotaxis was also affected. In some cases, two leaves generated at the "opposite" position on the inflorescence axis (Figure 2E). The structural resemblance between the phenotypes of the *pin*1 mutant and the effect of polar transport inhibitors indicated strongly that the major genetic defect of the mutation is related to the auxin polar transport system(s). The inflorescence structure was not aberrant when wild-type plants were treated with 2-(*p*-chlorophenoxy)-isobutyric acid (CPIB), an auxin antagonist that represses several effects caused by auxin (McRae and Bonner, 1953; Foster et al., 1955; Evans and Hokanson, 1969) (see Table 1).

Reduced Auxin Polar Transport Activity in the *pin-formed* Mutant

Auxin polar transport activity in the inflorescence axes was tested using radioactive indole-3-acetic acid (IAA), a major component of endogenous auxin. The inflorescence axes of the *pin* mutants and wild-type plants were cut to pieces 2.5 cm long, put in plastic tubes in the normal or inverted orientation, and supplied labeled IAA at one end, as shown schematically in Figure 3A. For wild type, labeled IAA was transported from one end to the other through the inflorescence tissues in the inverted orientation only (Figures 3B, panel b and 3C, lanes b and f). This means that IAA was transported in a polar direction, i.e., from the apical side to the basal side of the pieces, against gravity. When

Figure 1. (continued).

(A) Tops of the inflorescence axis of *Arabidopsis* wild type (right) and the *pin*1-1 mutant (left).
(B) A mutant inflorescence with no floral buds.
(C) A mutant inflorescence with a pistil-like structure at the top.
(D) A mutant inflorescence with aberrant petals and no stamens.
(E) Transverse section of the top region of the inflorescence axis with no floral buds shown in **(B)**.
(F) A deformed leaf.
(G) Transverse section of the pistil-like structure shown in **(C)**. Arrowheads show immature primordia of ovules.
(H) A mutant plant with two leaves and two axillary shoots in opposite positions on the inflorescence axis (arrowhead).

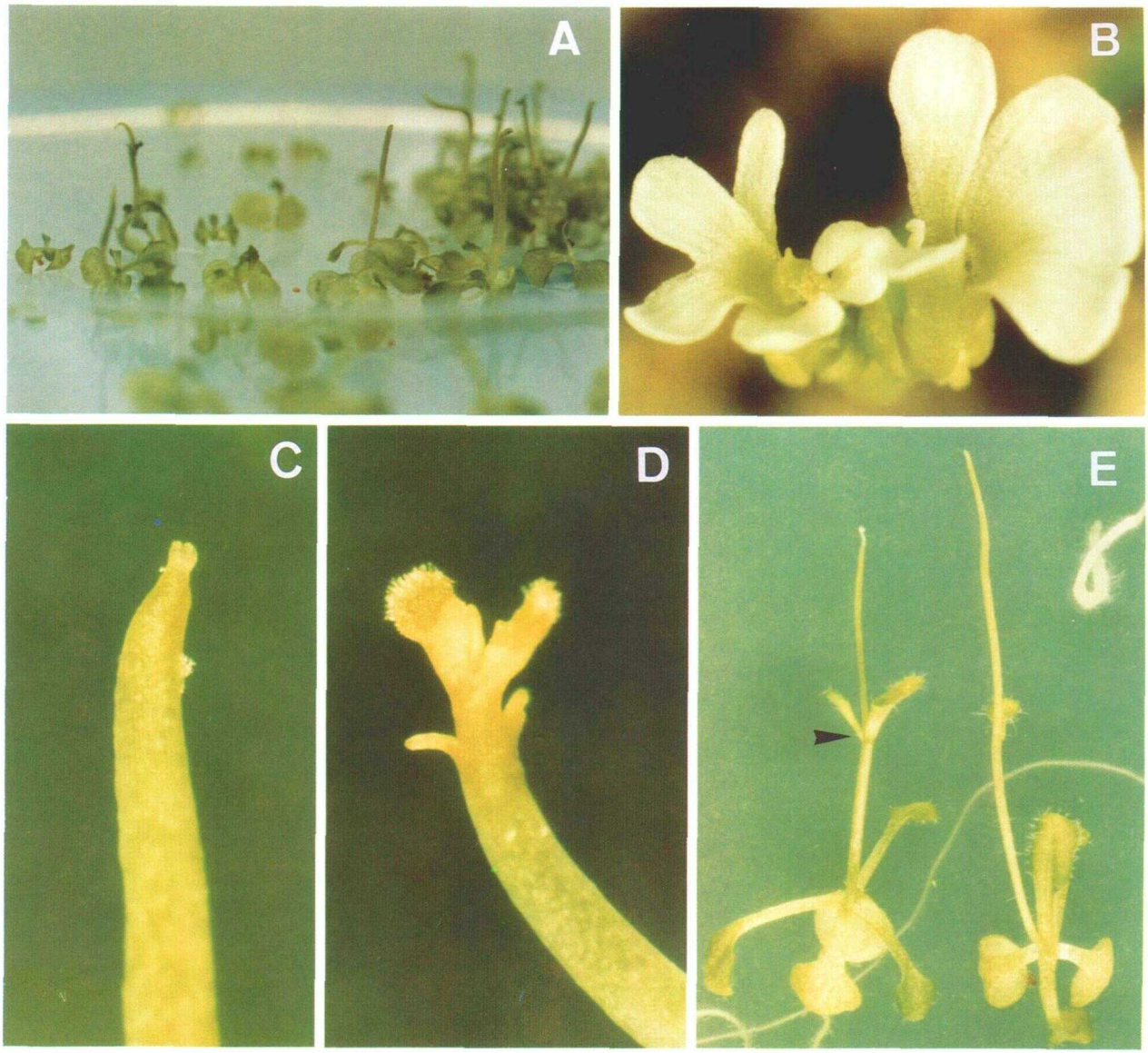


Figure 2. Structural Abnormalities in Wild-Type Plants Cultured in the Presence of Auxin Polar Transport Inhibitors.

(A) to **(D)** 28-day-old wild-type plants (Landsberg ecotype) grown on agar containing 20 μM HFCA.
(A) Plants on agar medium.
(B) Two flowers on the top of the inflorescence axis. Petals are wide and stamens are missing.
(C) An inflorescence axis with no floral organs at the top.
(D) An inflorescence axis with two pistil-like structures carrying stigmatic hairs at the top.
(E) 30-day-old wild-type plants (Columbia ecotype) grown in the presence of 50 μM NPA. Two leaves appear at the opposite position in the plant at the left (arrowhead). No flowers are formed at the top of the inflorescence axes.

the pieces were incubated in the normal orientation, labeled IAA was accumulated at the basal end but was not transported (Figure 3B, panel a).

Polar transport activity in the inflorescence axes of both the *pin*1-1 mutant and the *pin*1-2 mutant was found to be reduced to about 14% and about 7% of wild type, respectively (Figures 3B, panel d and 3C, lanes d and h). This finding supports the hypothesis that the aberrant mutant phenotypes would result from the decreased activity of the auxin polar transport in the inflorescence axis.

Table 1. Efficiency of Chemical Compounds on Formation of the *pin*1 Mutantlike Inflorescence

		HFCA		NPA	TIBA		CPIB	
	None[a]	5 μM	20 μM	50 μM	10 μM	30 μM	10 μM	30 μM
Total no. of plants tested	100	36	75	16	25	25	25	25
Plants not bolted[b]	0	17	36	0	14	19	16	11
Plants with normally shaped inflorescence	100	0	0	0	7	3	9	14
Plants with the mutant like inflorescence[c]	0	19	39	16	4	3	0	0

The chemicals were dissolved in ethanol and added to the agar medium before solidification. The final concentration of ethanol was 0.1%. The structure of inflorescence was checked 5 to 6 weeks after germination.

[a] Ethanol was added to 0.1%.

[b] Neither vegetative nor reproductive growth of the plants was observed.

[c] Inflorescences resembling either type A or type B of the *pin*1 mutant (see text) are counted together.

Inhibition of IAA Polar Transport by Chemical Compounds

We tested the effects of chemical compounds on the IAA polar transport activity in the inflorescence axis of wild-type plants (Figure 3C, lanes i, j, k, and l). Polar transport of radioactive IAA was inhibited completely by HFCA and NPA. Inhibition by TIBA was less effective. CPIB showed no inhibitory effect.

Reduced Amount of Free IAA in the *pin-formed* Mutant

We measured the amount of free IAA in the *pin*1 mutant and in wild type. The result is summarized in Table 2. The amount of free IAA in the wild-type plant (327 ng/g of fresh weight of starting tissue including the rosette leaves and inflorescences) was roughly the same as the amount of free IAA in fresh tissues of *Brassica juncea* (116 ng/g in apical inflorescence, 292 ng/g in mature rosette leaves) (Ueda et al., 1991). The two plants belong to the Brassicaceae family. Free IAA in the *pin*1 mutant was decreased to about 8% of wild-type *Arabidopsis* (Table 2). This result showed that the *pin*1 mutation affects both IAA polar transport and IAA synthesis.

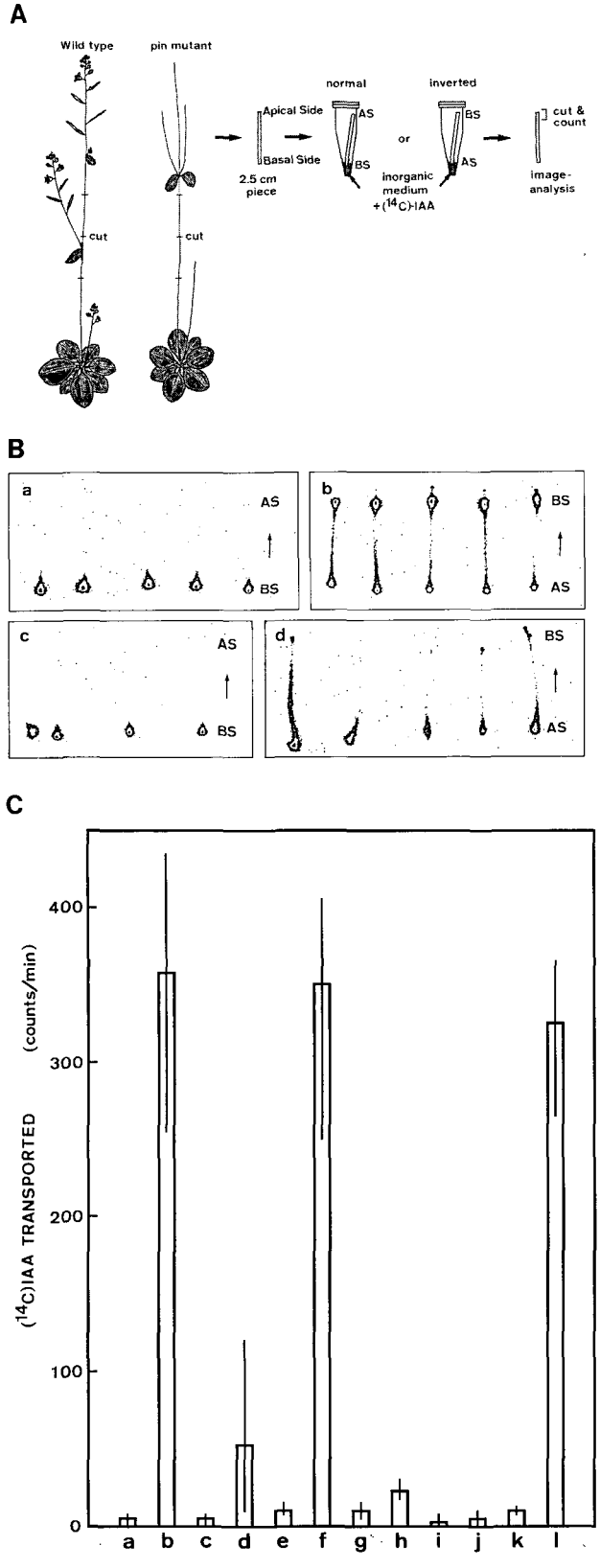
DISCUSSION

Phenotypic Variation of the *pin-formed* Mutants

Two types of inflorescence were observed with homozygous *pin*1 mutant plants: type A had no floral buds and type B had a pistil-like structure or a flower with deformed floral organs. Our interpretation is that the type A inflorescence is generated by halting the flower development at the stage where floral meristems are formed and that the type B inflorescence is generated at the stages where floral organ primordia are formed or where the primordia develop to mature functional organs. Because the two types of inflorescence are often seen in the same plant, we postulate that the major genetic defect of the *pin*1 mutation resides in the floral meristem formation and that the minor or secondary genetic defect is disturbance of the primordia formation and development. We presume that the type B inflorescences are formed when the inflorescences somehow pass through the developmental stage of floral meristem formation. Some environmental or metabolic perturbation could be responsible for the escape from a major defect.

The Auxin Polar Transport System Is Required in Floral Meristem Formation as well as in Primordia Formation and Development

Two different pieces of evidence led us to conclude that the auxin polar transport system plays an important role in an early stage of *Arabidopsis* floral bud formation. The first is that the activity of IAA polar transport was reduced to about 10% in the inflorescence axis of the two independently isolated *pin*1 mutants. The second is that auxin polar transport inhibitors distorted the formation and development of the floral meristem and that the resulting phenotype of wild-type plants treated with such inhibitors resembled the phenotype of the *pin*1 mutants. The auxin antagonist CPIB, which has no effect on polar transport, did not generate the mutantlike phenotype. These results indicated strongly that the normal level of the auxin polar transport system is required in the development of inflorescence and floral buds. The molecular mechanism of the polar transport system is not yet sufficiently elucidated (Goldsmith, 1977; Rubery, 1981). Neither the energy source nor the carrier molecule(s) is known. Therefore, it



remains to be resolved whether transported auxin controls directly the flower development or whether some cell-to-cell communication networks that share the auxin polar transport are required.

It has been reported that gravitropism and phototropism of shoots and roots are related to transport and distribution of auxin (Scott, 1972; Juniper, 1976). Addition of the auxin transport inhibitors to wild-type plants causes reduction of gravitropic responses. However, the tropic responses of shoots and roots of the *pin*1 mutant seem to be normal (data not shown). This observation indicates either that the reduced activity of auxin transport in the mutant is sufficient for the tropic responses or that the genetic regulatory mechanisms of the tropic responses are different from that defective in the *pin*1 mutant.

Reduced Amount of Endogenous IAA in the *pin-formed* Mutant

The endogenous amount of free IAA in the *pin*1-1 mutant is reduced to about 8% of that of wild type. Although the amount of IAA bound tightly to macromolecules was not assayed, these results indicated that the biosynthesis of IAA is also affected by the mutation. It is not known whether the *pin*1 gene product is involved in both the polar transport system and IAA biosynthesis, or whether the reduction of the IAA level is a secondary effect of the reduced polar transport activity. Because the major sources of IAA in the plant body are the apical meristem, young floral buds, and leaves (Matthysse and Scott, 1984;

Figure 3. Assay for Transport Activity in the Inflorescence Axis.

(A) Experimental procedures. See Methods for details.

(B) Distribution of the transported radioactive IAA in the pieces of the inflorescence axis. After incubation for 18 hr, distribution of ^{14}C-labeled IAA was monitored using the Fujix image analyzer. AS, apical side; BS, basal side. Arrows indicate the direction of transport. Panel a, wild type (Enkheim ecotype) incubated in the normal orientation; panel b, wild type (Enkheim ecotype) in the inverted orientation; panel c, *pin*1-1 mutant in the normal orientation; panel d, *pin*1-1 mutant in the inverted orientation.

(C) Amounts of radioactive IAA transported to the upper end of the pieces of the inflorescence axis after incubation for 18 hr. Lane a, wild type (Enkheim ecotype) incubated in the normal orientation; lane b, wild type (Enkheim ecotype) in the inverted orientation; lane c, *pin*1-1 mutant in the normal orientation; lane d, *pin*1-1 mutant in the inverted orientation; lane e, wild type (Landsberg ecotype) in the normal orientation; lane f, wild type (Landsberg ecotype) in the inverted orientation; lane g, *pin*1-2 mutant in the normal orientation; lane h, *pin*1-2 mutant in the inverted orientation; lanes i to l, wild type samples (Enkheim ecotype) in the inverted orientation incubated with chemical compounds. The added chemicals and their final concentrations are: lane i, 15 μM HFCA; lane j, 15 μM NPA; lane k, 15 μM TIBA; and lane l, 15 μM CPIB.

Table 2. Endogenous IAA Levels in Wild Type and the *pin*1-1 Mutant

	IAA in ng/g Fresh Wt
Wild type[a]	326.7 ± 2.6
*pin*1-1[b]	25.8 ± 1.7

Plant materials including rosette leaves and inflorescence were used for the measurement.
[a] Plants including *pin*1-1/+ heterozygotes and +/+ homozygotes, which are segregated in the self-fertilized progeny of the heterozygous parent.
[b] *pin*1-1/*pin*1-1 homozygous plants.

Ueda et al., 1991), the reduction of endogenous IAA in the mutant could be due to the lack of actively synthesizing cells in the meristem and floral buds or by some feedback regulatory system that links IAA biosynthesis and transport. The structural abnormalities of the *pin*1 mutant were not restored by supplying IAA (J. Ueda, M.K. Komaki, K. Okada, and Y. Shimura, unpublished results).

METHODS

Plant Materials and Growth Conditions

Two wild-type *Arabidopsis thaliana* strains, Enkheim ecotype and Landsberg ecotype, and the *pin-formed*1-1 mutant *pin*1-1 were obtained from the Arabidopsis Information Service (Dr. K.R. Kranz, Botanisches Institut, J.W. Göethe Universität, Frankfurt am Main, FRG). The *pin*1-1 mutant was derived from the Enkheim ecotype. The *pin*1-2 mutant was isolated from ethyl methanesulfonate-mutagenized seeds of the Landsberg ecotype. Mutagenesisand mutant isolation procedures were described in Okada and Shimura (1990).

Seeds were sown in pots or in plastic containers with agar medium. Plants were incubated in the laboratory under continuous illumination of about 1000 lux at 22°C. Chemical compounds dissolved in ethanol were added to the hot agar medium (0.7% agar w/v) containing 0.5 × *Arabidopsis* inorganic medium before solidification in plastic containers. The final concentration of ethanol in agar medium was 0.1%. *Arabidopsis* inorganic medium (1 x) contains 5 mM KNO_3, 2 mM $MgSO_4$, 2 mM $Ca(NO_3)_2$, 2.5 mM $K\text{-}PO_4$ adjusted to pH 5.5, 50 μM Fe-EDTA, 70 μM H_3BO_3, 14 μM $MnCl_2$, 0.5 μM $CuSO_4$, 1 μM $ZnSO_4$, 0.2 μM $NaMoO_4$, 10 μM NaCl, 0.01 μM $CoCl_2$. Before sowing onto the agar medium, seeds were sterilized by immersing in a solution containing 10% liquid bleach (Haitar; Kao Co., Ltd., Tokyo, Japan) and 0.02% Triton X-100 for 3 min, followed by extensive washing with sterilized water.

Chemicals

HFCA, TIBA, and CPIB were purchased from Sigma, and NPA was from Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan.

Radioactive IAA (1-^{14}C-IAA) was purchased from American Radiolabeled Chemicals, Inc., St. Louis, MO.

Assay of IAA Transport Activity in Inflorescence Axes

The experimental procedure is schematically illustrated in Figure 3A. The inflorescence axes of the *pin-formed* mutant and wild-type plants were cut to pieces of 2.5 cm in length and put into 1.5-mL Eppendorf plastic tubes in normal or inverted orientation. Thirty microliters of 0.5 x *Arabidopsis* inorganic medium containing ^{14}C-labeled IAA with or without polar transport inhibitors was supplied at the bottom of the tubes. The final concentration of IAA was adjusted to 1.45 μM (0.08 μCi/mL). After incubation at room temperature for 18 hr, the pieces were washed with the medium several times. Distribution of the labeled IAA in the pieces was visualized by the Fujix image analyzer (BAS2000, Fuji Photo Film Co., Ltd., Tokyo, Japan) by arraying the pieces on the plastic wrapping sheet and exposing to the imaging plate for 12 hr. After exposure, small slices of about 5 mm thickness were cut at the upper end of the pieces. Radioactivity of the small slices was counted by a liquid scintillation counter.

Measurement of Free IAA

Wild-type (Enkheim ecotype) and the *pin*1-1 mutant plants were grown in pots for 4 to 6 weeks. The bolted plants including rosette leaves and inflorescences (about 100 g) were used for the measurement. Endogenous amount of free IAA was measured using gas-liquid chromatography-selected ion monitoring with d_5-IAA as an internal standard. The procedures for extraction, purification, and measurement were described by Ueda et al. (1991).

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