Planta (1989) 177:185–197

Planta

Gravitropism in a starchless mutant of *Arabidopsis*

Implications for the starch-statolith theory of gravity sensing

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Abstract. The starch-statolith theory of gravity reception has been tested with a mutant of *Arabidopsis thaliana* (L.) Heynh. which, lacking plastid phosphoglucomutase (EC 2.7.5.1) activity, does not synthesize starch. The hypocotyls and seedling roots of the mutant were examined by light and electron microscopy to confirm that they did not contain starch. In upright wild-type (WT) seedlings, starch-filled plastids in the starch sheath of the hypocotyl and in three of the five columellar layers of the root cap were piled on the cell floors, and sedimented to the ceilings when the plants were inverted. However, starchless plastids of the mutant were not significantly sedimented in these cells in either upright or inverted seedlings. Gravitropism of light-grown seedling roots was vigorous: e.g., 10° curvature developed in mutants rotated on a clinostat following a 5 min induction at 1·g, compared with 14° in the WT. Curvatures induced during intervals from 2.5 to 30 min were 70% as great in the mutant as the WT. Thus under these conditions the presence of starch and the sedimentation of plastids are unnecessary for reception of gravity by *Arabidopsis* roots. Gravitropism by hypocotyls of light-grown seedlings was less vigorous than that by roots, but the mutant hypocotyls exhibited an average of 70–80% as much curvature as the WT. Roots and hypocotyls of etiolated seedlings and flower stalks of mature plants were also gravitropic, although in these cases the mutant was generally less closely comparable to the WT. Thus, starch is also unnecessary for gravity reception in these tissues.

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Abbreviations: PAR = photosynthetically active radiation; PAS = periodic acid-Schiff's reagent; PGM = phosphoglucomutase; WT = wild-type

Key words: Amyloplast – *Arabidopsis* – Gravitropism – Mutant (gravitropism) – Phosphoglucomutase – Starch – Statolith

Introduction

Since the turn of the century it has been widely accepted that the first step in gravitropism by higher plants requires statoliths. As proposed by Haberlandt and Němec (reviewed in Audus 1962), these have been thought to be starch-filled plastids, relatively dense organelles that often settle to the lower sides of cells in tissues displaced from their upright position of equilibrium (e.g. Larsen 1971; Shen-Miller and Hinchman 1974; Audus 1975; Juniper 1976; Volkmann and Sievers 1979; Jackson and Barlow 1981; Wilkins 1984; Feldman 1985; Moore and Evans 1986). According to some authors (e.g. Perbal and Rivière 1976; Hillman and Wilkins 1982; Sack et al. 1984; Wendt and Sievers 1986), the statoliths once settled might, press against or interact chemically or eletrically with the lateral plasmalemma itself, the "cortical gel", or associated layers of the endoplasmic reticulum. Alternatively, it has been suggested (e.g. Iversen and Larsen 1973; Clifford 1979) that statoliths act by their movement through the cytoplasm. Other authors (e.g. Filner et al. 1970) have considered that starch-laden plastids need not settle, but might act by exerting force on hypothetical cytoskeletal structures that restrain their movement.

Three classes of evidence have been amassed in support of the starch-statolith theory. First, mobile, starch-filled amyloplasts are usually present in gravitropic organs. Second, accumulation of starch is often correlated with the development of gravitropic competence (e.g. Barlow 1974; Perbal and Rivière 1976; Hillman and Wilkins 1982;

Wright 1986). Third, low starch content or low amyloplast mobility is correlated with impaired gravitropism in a number of mutants (Roberts 1984; Olsen et al. 1984; Mirza et al. 1984; Hertel et al. 1969; Filner et al. 1970; Miles 1981).

Disputing the starch-statolith theory, some authors have reported substantial gravitropic responses by plant organs naturally free of starch, low in starch or depleted of starch (e.g. Pickard and Thimann 1966; Westing 1971; Grenville and Peterson 1981; Moore 1987). It has also been argued that the kinetics of plastid displacement are inconsistent with the kinetics of gravitropic induction (Pickard 1973; Johnsson and Pickard 1979; Clifford and Barclay 1980). However, because of the mass of evidence consistent with the starch-statolith theory and because of the lack of a satisfying alternative hypothesis, the inconsistencies have generally been viewed as problems which could be reconciled by further experimentation (e.g. Volkmann and Sievers 1979; Moore and Evans 1986).

In the present paper we report on a study of a previously described (Caspar et al. 1985a) starchless mutant of *Arabidopsis thaliana* (Brassicaceae = Cruciferae). Both light-grown and etiolated mutant seedlings as well as mutant flower stalks were found to be capable of gravitropic curvature, precluding an obligatory role for starch in their detection of gravity.

Preliminary results of this study have been previously reported (Caspar et al. 1985b).

Materials and methods

Reagents. Agar was obtained from Difco Laboratories, Detroit, Mich., USA; $NaH^{14}CO_3$ was from ICN Radiochemicals, Irvine, Cal., USA; biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo., USA.

Plants. The starchless mutant lines TC7, TC9, TC21, and TL25 were independently derived from the Columbia WT of *Arabidopsis thaliana* (L.) Heynh. (Caspar et al. 1985a; Lin et al. 1988). The mutations in TC9 and TC21 were characterized as being allelic to that in TC7 and the mutation in TL25 as nonallelic to that in TC7. The single, recessive, nuclear mutation in TC7 causes a deficiency of the activity of the plastid isoenzyme of phosphoglucomutase (PGM; EC 2.7.5.1) (Caspar et al. 1985a) and that in TL25 causes a deficiency in the activity of ADP-glucose pyrophosphorylase (EC 2.7.7.27) (Lin et al. 1988). A single seed lot of the wild-type (WT) and of each mutant line was used for all experiments.

Seedlings of these plants were grown under sterile conditions in square, gridded $100 \cdot 100 \cdot 15\ mm^3$ Petri plates with a medium consisting of the nutrient salts described by Haughn and Somerville (1986) plus 1% (w/v) sucrose and solidified with 1% (w/v) agar. Seeds were surface-sterilized as described in Haughn and Somerville (1986). They were distributed on the agar medium at intervals of 2 mm in parallel rows and the Petri plates were sealed with Parafilm (American Can Co., Greenwich, Conn., USA). Seed sterilization and sowing were carried out in room light for both light- and dark-grown seedlings. In a few of the later experiments, after seeds were sown they were stored for 2 or 3 d at 4° C as this promoted uniform germination.

The plates were placed on edge, with the rows of seeds horizontal, at 24° C in the light or dark as indicated. Photosynthetically active radiation (PAR) was provided by "cool white" fluorescent tubes (F40CW/RS/EW-II, Westinghouse, Somerset, N.J., USA or F40/CW/RS/SS, Sylvania, Danvers, Mass., USA). For the experiment of Fig. 7 a flux density of 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR was used because it produced fast-growing hypocotyls; otherwise 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR was used because it produced vigorous root growth and compact shoots. Light was measured with a LI-188B or LI-185B meter with a LI-190SB quantum sensor (Li-Cor, Lincoln, Neb., USA). Roots of light-grown seedlings were used when their length was 6–10 mm, and hypocotyls when about 3–4 mm. For the WT, these lengths were achieved in about 85–90 and 90–95 h, respectively. Mutant seed were sown 14–16 h earlier because germination was delayed with respect to the WT.

For production of leaves and flower stalks, sets of 15–30 plants were grown in 130-mm-diameter pots (as in Haughn and Somerville 1986) at 22° C with continuous illumination from "cool white" fluorescent tubes (FR72T12/CW/VHO/135, Sylvania) (125 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR). For biochemical assays, plants were used at the rosette stage (about three weeks old). For studying gravitropism of flower stalks, plants were used when six weeks old, with flower stalks 50–150 mm in length.

Microscopy. Fresh seedling and flower-stalk tissue was stained with iodine as described in Caspar et al. (1985a). For fixation, uniform seedlings were placed upright on blocks of agar, approx. $10 \cdot 20 \cdot 30\ mm^3$, and gently secured to the blocks with moistened gauze. Care was taken to maintain the vertical orientation of the seedlings throughout these manipulations. Half the assemblies were placed upright and half were placed inverted in darkened, sealed beakers. For study of roots, the assemblies were left for 2 h; for hypocotyls, 3 h. The seedlings were then fixed without altering their positions by filling the beakers with ice-cold 4% (v/v) glutaraldehyde in 0.1 M sodium phosphate (pH 7.2). After 90 min, the root tips or hypocotyls were excised from the seedlings and fixed for an additional 60 min. Following a rinse in the phosphate buffer, the sections were post-fixed for 90 min in 1% (w/v) OsO_4 in the same buffer and the tissues were then dehydrated, and embedded in a 1:1 (w/w) mixture of Mollenhauer's and Spurr's resins (Klomparens et al. 1986, chptrs. 4, 5, Append. II). Longitudinal 2-μm sections were cut serially until the midplane of each organ was reached; then, ultrathin (approx. 80 nm) sections were cut. The 2-μm sections were stained with periodic acid-Schiff's reagent (PAS) and counterstained with toluidine blue (Feder and O'Brien 1968). The ultrathin sections were stained 30 min with uranyl acetate and lead citrate (Reynolds 1963) and viewed with either a Philips (Eindhoven, The Netherlands) EM 201 electron microscope at 60 kV or a JEOL (Tokyo, Japan) 100CX II electron microscope at 100 kV.

Morphometric assessment of plastid distribution. Root caps of the WT were analyzed by light microscopy; analysis of a small sample of electron micrographs gave similar results. The plastids in the columella of TC7, however, were not readily visible by light microscopy, so electron micrographs were used. The *total area* occupied by the plastids in a single section of a columella cell in the root cap was measured by a point-counting method, using a square lattice (Weibel and Bolender 1972).

The *average position* of the plastids in each micrograph of a columella cell was determined by a modified point-counting method in which the lattice was used to calculate the distance from the ends of the cell to each unit square of the lattice occupied by plastid material. The *relative plastid position* was calculated by counting the number of unit squares occupied by plastid material within each transverse row of the lattice and multiplying by the rank of the row; the sum of the products for all rows was divided by the sum of the unit squares occupied in all rows; finally, the resultant value was divided by the number of rows in the cell (i.e. its height) and expressed as a percentage. (This method, based on the size rather than the number of plastid sections, was chosen because, without serial reconstruction of the plastids in each cell, it was not possible to determine for the irregularly-shaped plastids whether adjacent sections of plastid in a micrograph were part of the same or different plastids). For each experimental treatment the total area occupied by plastids and the relative mean plastid position determined for all cells within a given columella layer from three to six seedlings were further averaged to produce the overall average values for that layer.

Assays for chloroplast enzymes. Plants were placed in the dark for 16 h, assuring depletion of starch in the WT. Extracts were prepared at 4° C. For crude extracts, 500 mg of leaves were ground with a mortar and pestle in 5 ml of buffer (28 mM imidazole-HCl, pH 7.4; 3.3 mM $MgCl_2$; 40 mM 2-mercaptoethanol; 0.1%, w/v, defatted bovine serum albumin; 2 mM glucose 6-phosphate) and filtered through Miracloth (Behring Diagnostics, La Jolla, Cal., USA).

For preparation of chloroplast extracts, washed leaves were homogenized in 20 volumes of buffer (20 mM N-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine (Tricine)-KOH, pH 8.4; 10 mM ethylenediaminetetraacetic acid (EDTA); 10 mM $NaHCO_3$; 0.1%, w/v, defatted bovine serum albumin; 450 mM sorbitol) for 6 s at the maximum speed with a Tekmar (Cincinnati, Oh., USA) homogenizer. After filtration through Miracloth, the extracts were centrifuged at 475·g for 4 min. The pellets were gently suspended in suspension buffer (300 mM sorbitol; 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)-KOH, pH 7.4; 5 mM $MgCl_2$; 2.5 mM EDTA; 0.1%, w/v, defatted bovine serum albumin; 10 mM $NaHCO_3$; 2 mM glucose 6-phosphate), using 0.5 ml per 1 g of leaf starting material, and centrifuged at 275·g for 90 s. The pellets were resuspended (0.04 ml suspension buffer per 1 g leaf) and used for assays.

Phosphoenolpyruvate carboxylase (EC 4.1.1.31) and PGM were assayed at 22° C according to Stitt et al. (1978) and ribulose-1,5-bisphosphate carboxylase (EC 4.1.1.39) was assayed at 22° C according to Pierce et al. (1982) except that the reaction was initiated by the addition of ribulose 1,5-bisphosphate. Octylphenoxypolyethoxy ethanol (Triton X-100; 0.1%, v/v) was included in all enzyme assays to ensure complete lysis of organelles. Chloroplast intactness was measured by ferricyanide-dependent oxygen evolution (Somerville et al. 1981). Starch gels were run as described in Caspar et al. 1985a). Chlorophyll was assayed in 80% (v/v) acetone according to Mackinney (1941).

Kinetics of tropism. Gravitropism of light-grown roots was assessed at 24° C in white light of 50–60 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR (tubes were of the same types as used for growth). For the experiment of Fig. 6, induced plants were mounted parallel to the axis of a 3-rpm clinostat and provided with an axial light source of about 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR and a lateral source of about 15 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. It was carefully checked that the roots were not phototropic even in continuous bright unilateral white light. Gravitropism of light-grown hypocotyls and flower stalks was assessed in the dark (except for light required for photography).

Gravitropic stimulation of hypocotyls and roots was achieved by rotating the square Petri plates on edge so that the seedlings were horizontal, or, when specified, 30° from the vertical. Stimulation of flower stalks was achieved by placing the pot with the plants on its side in a room of high relative humidity (>95%).

For phototropic stimulation of hypocotyls, a fan-cooled 80·360-mm^2 panel of blue filter glass (No. 5543; Corning Glass Works, Corning, N.Y., USA) was sealed into a window of a light-tight shield placed immediately in front of two adjacent fan-cooled "cool-white" fluorescent tubes. Petri plates were set with seedlings in their normal orientation but with the surface of the agar at an angle of 24° from the axis of incident light; this orientation prevented the shoots from shading each other. Photon flux density at the surface of the agar in the center of each Petri dish was 1 $\mu mol \cdot m^{-2} \cdot s^{-1}$. A thermistor placed in a dummy assembly indicated that the air temperature inside the Petri dishes was maintained constant at 24° C.

Seedlings were photographed in the vertical position at the beginning of each experiment and at all designated times for plants gravitropically reacting at 30° from the horizontal or on the clinostat, or undergoing phototropism. They were generally photographed in the horizontal position if reacting from the horizontal position. The photographic light source was white fluorescent tubes except for experiments carried out in the dark, for which a 4.8-mm-thick sheet of red, acrylic plastic (No. 2444; Rohm and Haas, Philadelphia, Penn., USA) was placed in front of the tubes. The red light was confirmed to be phototropically inactive. Film was 35 mm Kodak Plus-X Pan (Eastman Kodak, Rochester, N.Y., USA); prints were made on Kodak Polycontrast Rapid II RC FM paper. Plants were reduced by a factor of 0.5 in the negative image, and enlarged by a factor of 3.5 in the prints. Images of plants which had germinated too late to produce roots at least 4 mm long, and plants which had grown into contact with their neighbors, were marked for exclusion. Flower stalks were photographed using infrared-sensitive film (Kodak 2481) and an electronic flash covered with a far-red filter (Kodak Wratten 87C). It was confirmed that the flash did not induce a phototropic response. Curvatures were measured by extending the axis of the apical 1 mm of the enlarged image of the root or hypocotyl with a straight-edge and a sharp pen, and measuring the angle formed with the originally vertical grid lines of the Petri plate. All angles are given as increments over the starting values. All error bars in figures are standard errors of the means (SE) unless otherwise specified. All figures represent pooled data from at least two consecutively performed experiments.

Seedling orientation in the experiment of Fig. 8 was quantified by determining the angle from vertical of the line connecting the root-hypocotyl junction with either the root tip or a point just proximal to the hypocotyl hook.

Results

Phosphoglucomutase activity in isolated chloroplasts. The previous characterization of mutant line TC7 as lacking starch because of a deficiency of the chloroplast isoenzyme of PGM (Caspar et al. 1985a) was based on a starch gel assay of leaf extracts. In order to determine more rigorously whether any chloroplast PGM activity remained in the mutant, PGM activity in extracts from iso-

Table 1. Enzyme activities in extracts of WT and mutant TC7 *Arabidopsis*. Values are the means of two to eight assays. Ferricyanide-dependent oxygen evolution assays indicated that 22% of the WT and 27% of the mutant chloroplasts were intact. Activities in $\mu mol \cdot min^{-1} \cdot (mg\ chlorophyll)^{-1}$

Enzyme	WT		TC7	
	Crude	Chloroplast	Crude	Chloroplast
Phosphoglucomutase	2.7	0.28	2.1	0.010
Ribulose-1,5-bisphosphate carboxylase	2.2	0.67	2.4	0.95
Phosphoenolpyruvate carboxylase	0.14	0.001	0.14	0.001

lated chloroplasts from mutant and WT plants was measured. As shown in Table 1, the chloroplast preparation from the mutant had less than 4% of the PGM activity of the WT chloroplast preparation. Based on the activities of ribulose-1,5-bisphosphate carboxylase (a stromal enzyme) and phosphoenolpyruvate carboxylase (a cytosolic enzyme) in the chloroplast preparations, the residual PGM activity in the chloroplast extract from the mutant was entirely attributable to contamination of the chloroplasts by the cytosolic isoenzymes of PGM. This conclusion was confirmed by electrophoresis of the chloroplast extracts on starch gels followed by staining for PGM activity. On these gels only the cytosolic PGM isoenzymes were observed in chloroplast preparations from the mutant, whereas the plastid isoenzyme was the major species in preparations from the WT (data not shown).

Determination of starch by histochemistry and electron microscopy. Because the previous determinations of starch (Caspar et al. 1985a) and the determinations of PGM activity shown in Table 1 were made with leaves, it remained possible that starch could be present in specialized tissues of gravitropically receptive organs – tissues such as the columella of the root cap and the starch sheaths of the hypocotyl and flower stalk – and even here, only at special stages of development. Therefore, these tissues were examined for starch in plants of the same age and grown under the same conditions as those used for measurements of gravitropism.

Initially, fresh root caps, hypocotyls, and flower stalks were stained with iodine. Starch was clearly evident in the WT organs, but none was evident in those of the mutant ($n > 20$; data not shown).

To improve the sensitivity of the assay, fixed, sectioned roots were examined. The Columbia WT root cap (Fig. 1A) generally resembles that of the Landsberg race of *Arabidopsis* described by Olsen et al. (1984). Five (or ocasionally six) distinct columellar layers were present. The outermost layer was complete rather than represented by only a single apical cell as in Landsberg. The columella layers were numbered from 1 to 5, with 5 being outermost (Olsen et al. 1984). In roots with six intact columella layers, the innermost layer was designated layer zero. A layer of detaching cells (which lacked starch) was often seen incompletely separated from the root cap; these were not included in the numbering system.

The WT contained large plastids in columella layers 2, 3, 4, and 5 (and occasionally in layer 1). They were filled with starch which reacted intensely in the PAS test as judged by light microscopy (Fig. 1A, B), and stained densely in electron micrographs (Fig. 2A). In contrast, the mutant showed no evidence of starch in the cap or in any other tissue of the nine roots for which serial sections were viewed by light microscopy (Fig. 1C) and median longitudinal sections were viewed by electron microscopy (Fig. 2B, C, D). The mutant plastids were small and irregularly shaped, and frequently contained internal membranes similar to those found in proplastids. Plastoglobuli, present in both the WT and mutant, were readily distinguished from small starch grains by their characteristic size and uniform round shape. Internal membranes and plastoglobuli were less apparent in WT than in mutant plastids (Fig. 2A, B). Possibly they were less obvious in the WT because of the proportionately smaller volume they occupied in the starch-filled plastids, or perhaps they were more abundant in the mutant because more carbon is channelled into lipid synthesis when starch synthesis is blocked.

Longitudinal sections of fixed hypocotyls were also examined. Large, starch-filled plastids were present in the WT (Figs. 1D, E, and 3A). However, in each of the three mutant seedlings examined, the plastids were small and showed no starch by either light (Fig. 1F) or electron (Fig. 3B) microscopy. Otherwise, they appeared similar to those in the WT.

The mutant plastids do not settle. Preparatory to examining whether starch-free plastids would sediment in response to gravity, the distribution of plastids was checked in undisturbed and reoriented WT seedlings. As expected, sedimented plastids were observed in WT seedlings only in the root-cap columella and the hypocotyl starch sheath. Thus, these tissues were subjected to more detailed study.

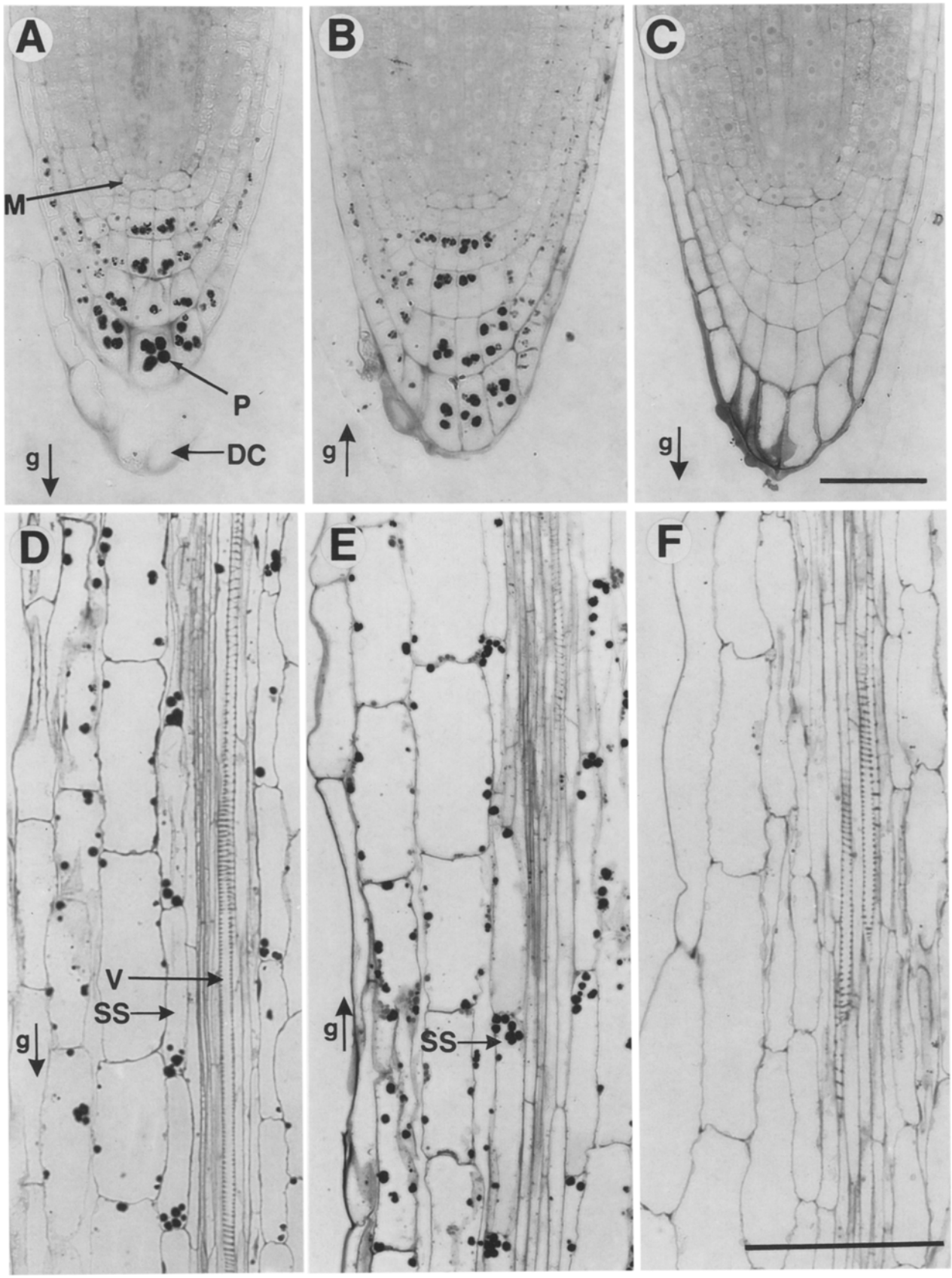

Fig. 1A–F. Light micrographs of median longitudinal sections of WT and TC7 *Arabidopsis* root tips and hypocotyls stained with PAS and toluidine blue. Sections are positioned according to their orientation during growth; arrows marked *g* indicate the direction of the gravity vector for the 2 h (roots) or 3 h (hypocotyls) immediately before fixation. **A** Upright WT root tip. **B** WT root tip inverted for 2 h before fixation. **C** Upright TC7 root tip. **D** Upright WT hypocotyl. **E** WT hypocotyl inverted for 3 h before fixation. **F** Upright TC7 hypocotyl. *SS*, starch sheath; *V*, vascular tissue; *M*, meristematic zone; *DC*, detaching cells; *P*, plastid. **A, B, C** ×460; scale bar = 50 μm. **D, E, F** ×355; scale bar = 100 μm.

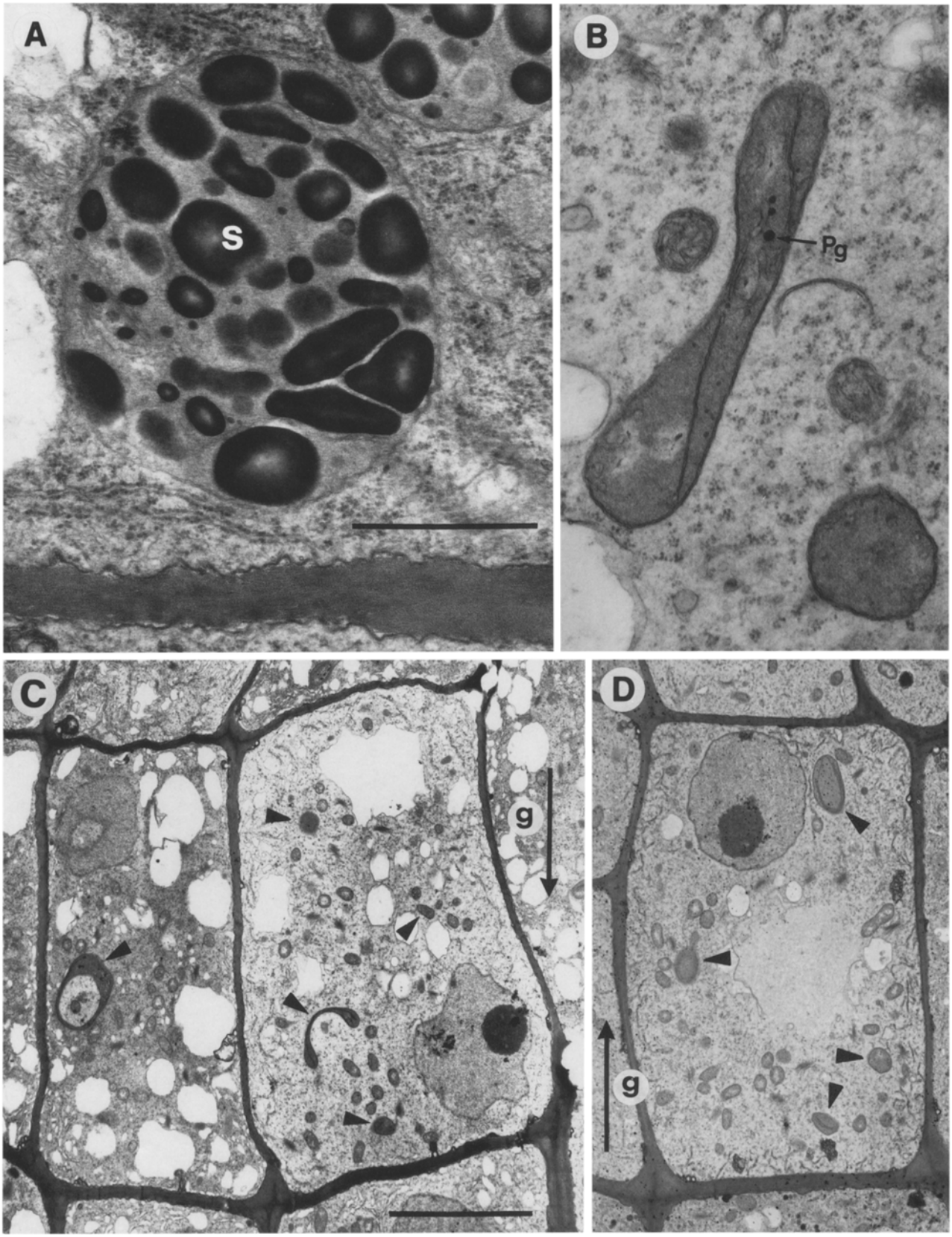

Fig. 2A–D. Electron micrographs of WT and TC7 *Arabidopsis* root cap columella cells. Panels C and D are positioned according to their orientation during growth and the *arrow* marked *g* indicates the direction of gravity during the 2 h before fixation. **A** Plastid from layer 3 of the root cap columella of a WT seedling. **B** Plastid from layer 2 of the columella of a TC7 seedling. **C** Cells from layer 3 of the columella from an upright TC7 seedling. **D** Cell from layer 3 of the columella from an inverted TC7 seedling. *N*, nucleus; *Pg*, plastoglobulus; *S*, starch grain; *arrow heads* in **C** and **D** point to plastids. **A, B** ×24200; scale bar = 1 μm. **C, D** ×5100; scale bar = 5 μm.

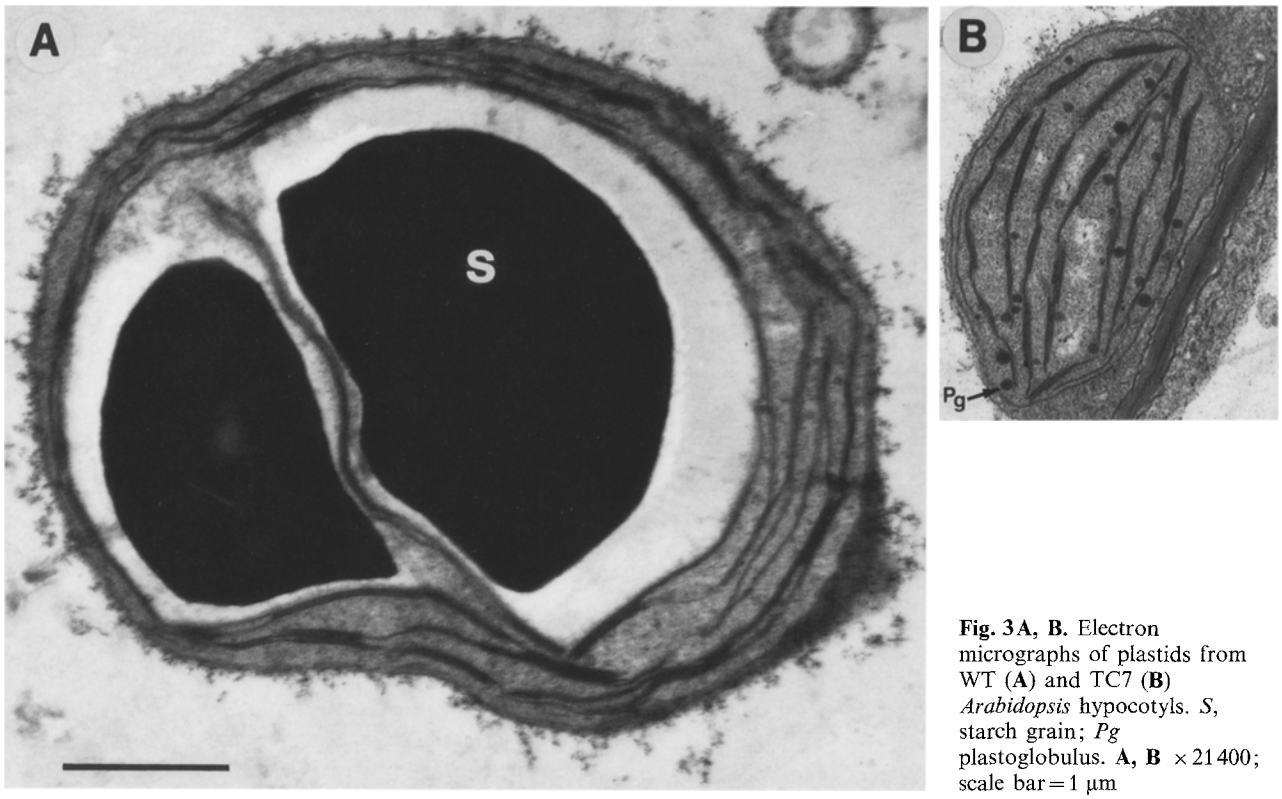

Fig. 3A, B. Electron micrographs of plastids from WT (**A**) and TC7 (**B**) *Arabidopsis* hypocotyls. *S*, starch grain; *Pg* plastoglobulus. **A, B** ×21400; scale bar = 1 μm

In the upright WT root, the plastids in columella layers 2 through 4 (and layer 1 when plastids were evident) appeared by simple inspection to be sedimented on the floors of the cells (Fig. 1A). Following a 2-h inversion, the plastids in layers 1 through 3, but not in layers 4 and 5, had settled to the ceilings of the cells (Fig. 1B). These observations were confirmed by morphometric analysis (Fig. 4A). It should be noted that, because of the large fraction of the WT columella cells occupied by plastids (13–17% in layers 2 through 5) and the loose packing of the plastids which resulted from their large size, the average position of the sedimented plastids was about a third of the distance from the lower to the upper cell wall. Combining the data on average plastid position with the average height of the cells in each layer, the average distance moved by the plastids following inversion was calculated to be 2.4, 3.2 and 4.3 μm for, in this order, layers 1, 2 and 3. These estimates agree reasonably well with the value of 4.2 μm previously reported (Olsen et al. 1984) for a 40-min inversion of seedlings of the Landsberg ecotype of *Arabidopsis* (the columella layers used for these measurements were not noted). Thus it is likely that sedimentation in our experiments was essentially complete within 40 min.

Both in upright and inverted mutant seedlings, plastids in all columella layers appeared by simple inspection to be randomly distributed (Fig. 2C, D). Morphometric analyis (Fig. 4B) showed that in layers 1 through 3, which contained mobile plastids in the WT, mean plastid positions were similar for upright and inverted plants and were fairly close to the midpoint of the cell. If sedimentation had been occurring in the mutant, it would likely have resulted in a much greater displacement of the mean position than for the WT, because of the considerably smaller total volume of the mutant plastids (1–3% of the total cell volume). In layer 5, the mean positions were likewise indistinguishable with plant orientation, but both deviated toward the proximal wall to about the same extent as the mean for the WT. The larger variability of the average positions for TC7 than for the WT attests to the more random position of the starch-free plastids in the mutant. Only cell layer 4 showed any significant difference in average position of the plastids between the upright and inverted treatments, and this difference, though ostensibly reproducible ($p > 99\%$), may well be artifactual: much of it could be attributed to a single one of the six inverted seedlings measured. It is possible that in this particular seedling layer 4 was in developmental transition to layer 5 in which the mean position in both WT and mutant for both

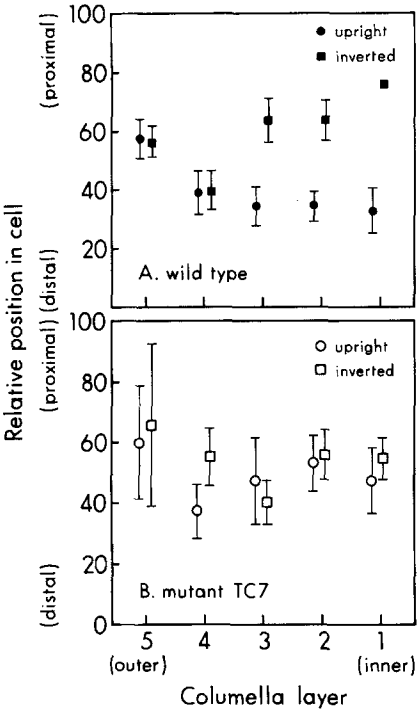

Fig. 4A, B. The effect of gravity on the position of plastids in root-cap columella cells of WT (**A**) and TC7 (**B**) *Arabidopsis*. Seedlings were either fixed in their normal upright orientation or were inverted for 2 h and then fixed. The average positions of plastids relative to the proximal (basal) and distal (apical) ends of the cell were determined in the central columella cells from three to six roots for each treatment. Error bars represent the 95% confidence limits. The WT layer 1 inverted-treatment point has no error bars since it represents only one cell (only one out of 16 cells examined from this layer contained starch)

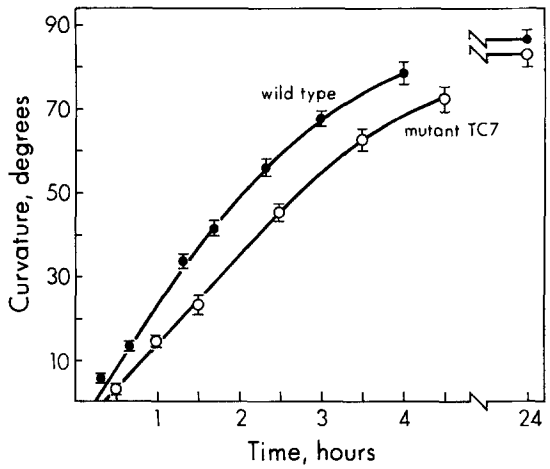

Fig. 5. Gravitropism by roots of WT and TC7 *Arabidopsis* seedlings maintained in 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR. Plants were placed horizontally at time zero. Interpretive curves were drawn based on visual impressions to intercepts of 20 min for the WT and 30 min for TC7. $n = 35$

treatments is more proximal. Moreover, the plastids in this layer of the WT did not sediment; since they are as large as or larger than those in layers 1 through 3, this is presumably a consequence of cytoskeletal constraint. There is no reason to suppose that this constraint present in the WT is absent in the starchless mutant.

Examination of PAS-stained longitudinal sections of WT hypocotyls by light microscopy showed plastids of the starch sheath sedimented in both upright (Fig. 1D) and 3-h-inverted plants (Fig. 1E). In the mutant no plastid sedimentation was observed in electron micrographs of longitudinal sections through the region of the starch sheath in either upright or inverted hypocotyls. Morphometric analysis of plastid sedimentation in hypocotyls was not performed because of the difficulty in unambiguously identifying the starch sheath in the starchless mutant.

Gravitropism of light-grown seedling roots. Figure 5 shows the time-course of downward bending by horizontally displaced roots of light-grown seedlings stimulated in the light: both the WT and mutant TC7 display vigorous gravitropism. During the period of most rapid response, the mutant lagged slightly: at 2 h, for example, it had attained only 70% as much curvature as the WT. By 4 h the mutant had almost caught up, and ultimately mutant and WT had achieved the same curvature.

In order to exclude the possibility that the ability of TC7 to respond gravitropically is atypical of starchless mutants, the independently derived allelic mutants TC9 and TC21, which are also defective in plastid PGM activity, were gravitropically stimulated in assays comparable to that shown in Fig. 5. Roots of TC9 and TC21 produced curvatures closely comparable to those of TC7 (e.g. at the representative response time of 3 h, mean responses were $39.7 \pm 1.1°$, $40.4 \pm 1.0°$, and $38.2 \pm 1.0°$, for, in this order, TC9, TC21, and TC7). Another starchless mutant (TL25) of *Arabidopsis* which is completely deficient in ADP-glucose pyrophosphorylase activity (Lin et al. 1988) also had vigorously gravitropic roots (data not shown).

Induction in the root. Gravitropic reception was separated from the late phases of response by stimulating plants for short intervals and then permitting curvature to develop on a clinostat.

Previous studies on *Artemisia* roots (Larsen 1957) and *Avena* coleoptiles (Dolk 1936; Pickard 1973) showed that the time on the clinostat required to achieve maximum response under a given set of conditions is independent of the duration of the stimulus. Thus, for a population with a given

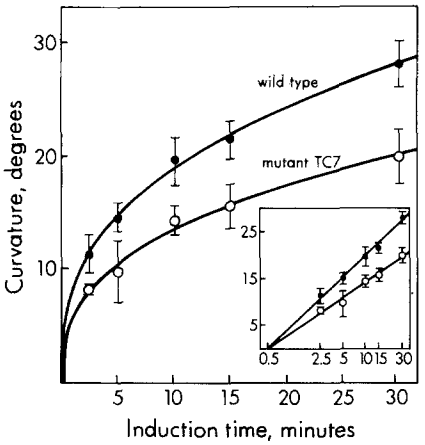

Fig. 6. Gravitropic curvature developed by *Arabidopsis* roots on a clinostat following brief stimulation in the horizontal position. $47 < n < 121$. Zero stimulus values were not assessed in the experiments of the graph; however, supplementary experiments with the WT indicated that comparably large sets of unstimulated plants could show both positive and negative mean curvatures as large as 2° during a 3-h period on the clinostat. Interpretive curves were generated by linear regression of logarithmically-transformed data; a replot of the data with semilogarithmic coordinates and with interpretive curves generated by linear regression of semilogarithmically-transformed data is shown in the inset.

Analytical details: Dimensional analysis was used to fit the data with equations. Application of the Buckingham Pi Theorem (Langhaar 1951, e.g. pp. 18–19, 55–59) yields $\mathscr{C} = G f[(t-\tau_0)/\tau_0]$, where $\mathscr{C}$ is curvature, G is a constant, t is stimulus time, τ is threshold time, and τ_0 is reference threshold time. A fairly general monotonically increasing function which meets the constraints is the power law $\mathscr{C} = D[(t-\tau_0)/\tau_0]^{\alpha}$. To facilitate computation this was considered in the form $\mathscr{C} = A(t-\tau_0)^{\alpha}$, where $A = D/\tau_0^{\alpha}$. Linear regression of logarithmically transformed data yields $A = 8.15°/\text{min}^{\alpha}$, $\alpha = 0.364$, $r = 0.998$ for the WT, and $A = 5.76°/\text{min}^{\alpha}$, $\alpha = 0.368$, and $r = 0.992$ for the mutant. The significance, if any, of the roughly three-eights power-law dependence is unknown. Another function which meets the constraints is the semilogarithmic equation $\mathscr{C} = B \log_{10}(t/\tau_0)$. Linear regression of semilogarithmically-transformed data yields $B = 15.22°$, $\tau = 0.51$ min, and $r = 0.991$ for the WT, and $B = 11.00°$, $\tau = 0.52$ min, and $r = 0.989$ for the mutant. Both fits are quite good; however, although the power law seems to fit slightly better, the semilogarithmic equations are less noisy near the lower limits. Moreover, they are particularly useful because data in the gravitropic literature have often been presented in semilogarithmic plots. Thus, the semilogarithmic forms have been used for extrapolation of induction thresholds.

growth rate, induction (which is a measure of reception) can be measured as a function of stimulus time. In order to determine when to measure response on the clinostat, an experiment was performed to check the development of curvature as a function of time on the clinostat for both mutant and WT. Contrary to previous observations with the Landsberg ecotype of *Arabidopsis* (Mirza et al. 1984), curvature increased rapidly during the first 2 h on the clinostat, slowly reached a peak during the next 2 h, and then declined gradually (data not shown). Considerable nutation was superimposed on these curvatures; also, the mutant appeared to reach its peak somewhat more slowly than the WT, and to decline a little later. In order to minimize the effects of nutation and of subtle differences in curvature development, curvatures for the induction experiment were assessed at both 2.25 and 3.5 h, and averaged.

Induction plots for TC7 and WT roots are presented in Fig. 6. For each pair of points plotted, the performance of the mutant was about 70% of the WT (73, 68, 73, 73, and 71% for successive values of induction time). More generally, after determining that the two sets of points accord well with full-logarithmic and semilogarithmic equations (legend, Fig. 6), the respective rate-determining coefficients for the mutant were found to be 72% of those for the WT. This emphasizes that the gravitropic impairment of the mutant is slight.

Responses of roots to brief and weak stimuli. In Fig. 6, it is noteworthy that mutant and WT responded by curvatures of 8.7° and 11.3°, respectively, following a 2.5-min stimulation at $1 \cdot g$. Moreover, induction thresholds determined by extrapolation are brief. The inset shows that regression plots for semilogarithmically-expressed induction data for the WT and mutant extrapolate to thresholds of 0.5 min. Because of the possibility that brief inductions might reflect reception more directly than extended inductions, the approx. 70% ratio of mutant to WT performance following the 2.5-min stimulus and the brief, closely similar threshold estimates are of particular importance in establishing the mutant's relative gravitropic effectiveness.

The mutant also responded slightly more than 70% as well as the WT after weak rather than brief stimuli: seedlings stimulated with a perpendicular vector of $0.5 \cdot g$ by displacing them 30° from the vertical (compare Pickard 1971, 1973) curved $15.9 \pm 1.6°$ versus $21.4 \pm 1.0°$ within 3 h ($n = 100$ and 172, respectively; curvatures should not be compared with those of other experiments because these seedlings were shorter than normal). As $1 \cdot g$ is the maximum gravitropic stimulus plants receive in nature, it is reassuring that stimulation with a more moderate effective force yielded comparable results.

Gravitropism and phototropism of hypocotyls. Figure 7 A shows the gravitropic response in the dark by hypocotyls of light-grown mutant and WT seedlings. Curvature of the mutant at 1.5 and 3 h was

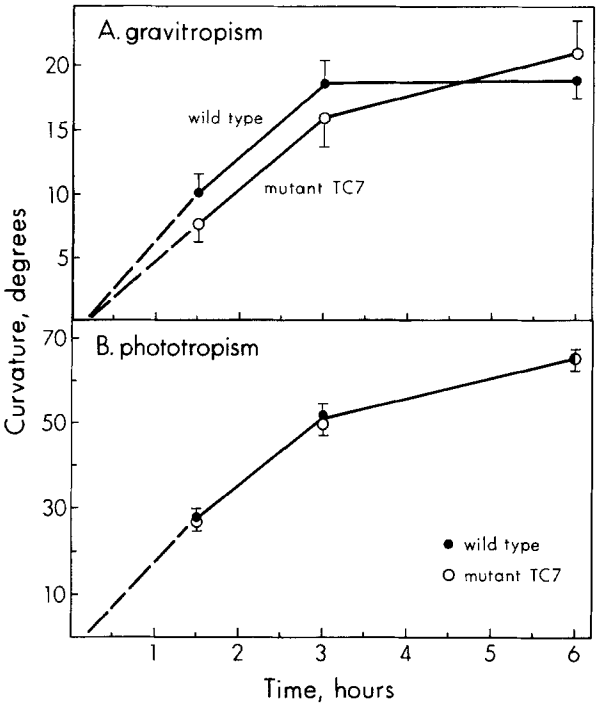

Fig. 7A, B. Gravitropism (**A**) and phototropism (**B**) by hypocotyls of WT and TC7 *Arabidopsis* seedlings. Seedlings were grown in 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR and pre-equilibrated in the dark for 1.5 h before being placed horizontal (**A**) or illuminated with blue light (1 $\mu mol \cdot m^{-2} \cdot s^{-1}$) (**B**) at time zero. For phototropism the plates, bearing the upright seedlings, were oriented with the agar slab at an angle of 24° from the light beam, but photographs were taken perpendicular to the slabs; thus the component of curvature measured is only 90% the value in the axis of the beam. Photographs were taken with a red worklight. $n > 50$.

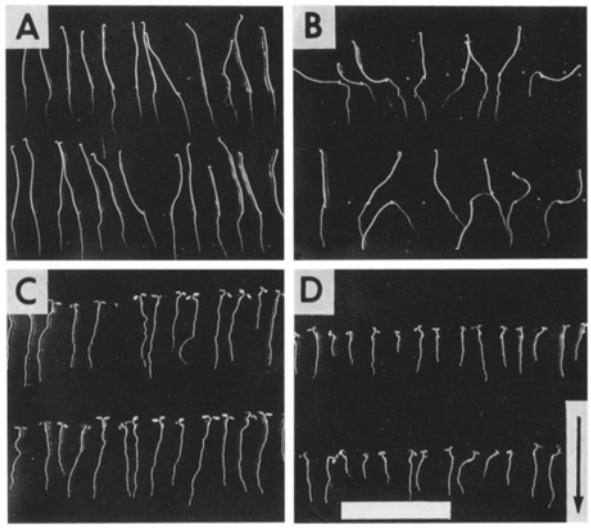

Fig. 8A–D. Orientation of dark- and light-grown WT (**A, C**) and TC7 (**B, D**) *Arabidopsis* seedlings of identical ages. (Normally, the mutants were grown longer than the WT in order to permit comparison of seedlings of identical sizes.) Seeds were sterilized and sown in Petri plates in room light (less than 10 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR). Within 1 h of initiating imbibition, they were placed in complete darkness at 4° C for 60 h. Plates were then placed vertically in darkness (**A, B**) or in white light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) (**C, D**) for 90 h at 22° C. Arrow indicates the direction of the gravity vector during growth. Scale bar = 30 mm, magnification = $\times 0.48$. Measurement of the orientation relative to vertical of a larger sample ($n > 87$) of roots and hypocotyls of the dark-grown WT and TC7 seedlings produced the following results (values are the mean orientation from vertical $\pm$ SE):

	WT	TC7
Root	7 $\pm$ 0.5°	21 $\pm$ 2.3°
Hypocotyl	8 $\pm$ 0.6°	37 $\pm$ 4.2°

70–80% that of the WT. By 6 h the response by both had slowed and the mutant's curvature slightly, though not significantly, exceeded that of the WT. In one large replicate experiment, performance by mutant and WT hypocotyls was essentially identical although absolute curvatures were lower than those in Fig. 7A, while in another large replicate absolute curvatures were considerably higher but initial curvature by the mutant averaged only 70% of the WT. Figure 7B shows that mutant and WT hypocotyls had vigorous, indistinguishable phototropic responses to strong, continuous blue light. This indicates that the potential for tropic response of the mutant hypocotyls is not greater than that of the WT.

Gravitropism by flower stalks. The flower stalks that are formed by the plants after a period of rosette growth showed a gravitropic response in both mutant and WT plants. In darkness, stalks of the mutant responded more slowly than those of the WT. For example, in one experiment with more than 75 plants in each set, WT stalks achieved 60° curvature in 80 min whereas the mutant required 240 min to reach the same curvature. Variability was so great, however, that quantitative comparisons would be of dubious value.

Gravitropism by etiolated seedlings. Both roots and hypocotyls of mutant and WT seedlings grown in total darkness were oriented with respect to gravity (Fig. 8), although alignment by the dark-grown mutant was less accurate than by either the dark-grown WT or the light-grown mutant. Growth in white light (50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR) in an atmosphere containing less than 15 $\mu l \cdot l^{-1}$ CO_2 improved the gravitropic alignment in the mutant, indicating that the light was not required simply to support photosynthetic CO_2 fixation.

Discussion

Starch is not required for gravitropism by Arabidopsis. Leaves of the *Arabidopsis* mutant TC7 completely lack starch and any detectable activity of the chloroplast isoenzyme of PGM (Table 1 and Caspar et al. 1985a). This enzyme is required for starch biosynthesis, and a cosegregation analysis indicated that a single mutation is responsible for the lack of both starch and chloroplast PGM activity (Caspar et al. 1985a). Seedlings of the mutant also contain no starch in the root cap, hypocotyl starch sheath or elsewhere as judged by both light and electron microscopy (Figs. 1–3), indicating that both amyloplasts and chloroplasts utilize the *pgmP* gene to produce PGM activity in the plastid.

Despite the complete absence of starch in hypocotyls, flower stalks and seedling roots of the mutant, these organs are all gravitropic. Thus, starch is not required for gravitropism in these organs in the mutant or, by extension, in the WT.

It might be suggested that a second mutation is present in the background of TC7 which in some way permits gravitropism by compensating for the lack of starch. This possibility is effectively ruled out by the independent isolation of mutant lines TC9 and TC21 which are also starchless because of allelic mutations at the *pgmP* locus, and are also gravitropically competent. A related question is whether mutations in the *pgmP* locus are unusual in that they themselves promote a compensatory gravitropic capacity. This possibility is excluded by the observation that the mutant line TL25, which is starchless because of a lack of ADP-glucose pyrophosphorylase, is also gravitropically responsive.

Gravitropism by etiolated seedlings. Gravitropism of seedlings is disproportionately weaker for the mutant than the WT in the absence of light. The reason is unclear. Perhaps in gravity reception by dark-grown seedlings there is a reliance on plastids which is suppressed in light-grown seedlings. Alternatively, a potential reduction in the intensity of the gravitropic response of the mutant based on its altered carbohydrate reserves might be most strongly realized under the suboptimal conditions of growth in darkness. At present it is not possible to distinguish between these and other possibilities.

Variability with age, conditions and seed lot. All the experiments reported in this paper were conducted during a two-year period with single lots of each type of seed. However, a few qualitative observations on gravitropism of light-grown seedling roots made at a later time with a variety of seed lots, as well as recent results of Kiss et al. (1989) indicate the need for caution in interpreting the reported results. In contrast to ours, the results of Kiss et al. show relatively lower response rates for both TC7 and the WT, with the TC7 usually being disproportionately lower. The sources of this variability have not been identified; there are several possibilities. First, as a seed lot ages germination can become less well synchronized and the uniformity of seedling growth and gravitropism as well as gravitropic sensitivity can decrease. The onset of these changes occurs well before viability is lost. Second, even fresh seed lots can differ in viability, synchrony of germination, and regularity and rate of growth and gravitropism. Environmental and maternal conditions during seed maturation may well play critical roles. Third, it appears that the rate of gravitropism is critically dependent on the precise conditions of growth and experimentation; our experiments were carried out under favorable conditions established by preliminary testing, but obviously the full range of relevant conditions has not been explored. Nevertheless, the high gravitropic sensitivity of both WT and TC7 roots, the reproducibility of gravitropic parameters with the given lots of WT and TC7 over the two-year experimental period, and the close similarity of gravitropism by TC7, TC9 and TC21 under identical conditions underscore the meaningfulness of our data, although caution should be exercised in precise quantitative comparisons between plants of different seed lots. Particularly because the mutant with its impaired carbohydrate metabolism is potentially more sensitive to environmental conditions than the WT, careful optimization of the environment of the maternal plant and the conditions of seed harvesting and afterripening as well as the conditions for seedling growth may be required.

Contribution of starch in gravitropism by light-grown Arabidopsis roots and hypocotyls. Although starch is not required for gravitropism, the reduced response of the starchless mutants indicates that starch does make some gravitropic contribution. This contribution might occur at the level of the first step in signal reception or of later processes.

Impairment occurring at the first step of gravity reception would support the starch-statolith theory, according to which decreases in the size and presumed density of the plastids should reduce the effective signal force. It is worth noting, however, that the roughly 30% reduction in response caused by the *pgmP* mutations is much less than the reduction in total buoyant mass (i.e. force ex-

erted on the cytoplasm) of the root-cap plastids. The buoyant mass of the starch-free plastids may be estimated by assuming densities of 1.0 mg·mm^{-3} for cytoplasm and 1.5 mg·mm^{-3} for starch-laden plastids (Audus 1962) and assuming that the density of starch-free plastids is 1.23 mg·mm^{-3} as found for proplastids (Quail 1979), which they resemble ultrastructurally. Given that the total fraction of cell volume occupied by plastids, averaged for the entire columella region, is about 14% for the WT and 2% for the mutant, the total force exerted by the plastids in a mutant columella cell is only about 6% that for the WT. Even taking into account an approximately logarithmic dependence for response on quantity of stimulus, it is not obvious why a 94% loss of putative signal force would result in only a 30% loss of response, particularly for inductions of only a few minutes duration. Similarly, the 30% reduction in response caused by the mutation is small in relation to the essentially full loss of plastid sedimentation.

Alternatively, impairment in the mutant of only secondary processes would invalidate the starch-statolith theory. However, so far receptive and later mediational events have been only partially separated, and no satisfactory way to discriminate signal transduction and secondary steps of reception has been proposed. Furthermore, problems posed by seed lot variability and possible differential sensitivity of the mutant and WT to environmental conditions have yet to be overcome. Nevertheless, the starchless mutants can be much further exploited in dissecting the stages of gravitropic response and factors controlling them, and quite possibly they can be utilized for a decisive evaluation of the role of amyloplasts in gravitropic signal transduction.

This work was supported in part by grants from the U.S. Department of Energy (DE-AC02-76ER01338) and U.S. Department of Agriculture (86-CRCR-1-2046) to C.R. Somerville (Michigan State University) and the National Science Foundation (PCM-8206147) to B.G.P. T.C. was supported by a National Science Foundation Predoctoral Fellowship. We thank Jane Schuette, Karen Klomparens (Center for Electron Optics, MSU), and Ljerka Kunst (MSU) for assistance with microscopy, and Chris Somerville, Rainer Hertel (Biology III, University of Freiburg, GDR), Fred D. Sack and John Z. Kiss (Department of Botany, Ohio State University), and Ken Poff (PRL, MSU) for valuable discussions, and William Pickard (Department of Electrical Engineering, Washington University) for help with kinetic analyses.

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Received 23 December 1987; accepted 3 October 1988