Reduced gravitropic sensitivity in roots of a starch-deficient mutant of *Nicotiana sylvestris*

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Abstract. Gravitropism was studied in seedlings of *Nicotiana sylvestris* Speg. et Comes wild-type (WT) and mutant NS 458 which has a defective plastid phosphoglucomutase (EC 2.7.5.1). Starch was greatly reduced in NS 458 compared to the WT, but small amounts of starch were detected in root-cap columella cells in NS 458 by light and electron microscopy. The roots of WT are more sensitive to gravity than mutant NS 458 roots since: (1) in mutant roots, curvature was reduced and delayed in the time course of curvature; (2) curvature of mutant roots was 24–56% that of WT roots over the range of induction periods tested; (3) in intermittent-stimulation experiments, curvature of mutant roots was 37% or less than that of WT roots in all treatments tested. The perception time, determined by intermittent-stimulation experiments, was ≤5 s for WT roots and 30–60 s for mutant roots. The growth rates for WT and NS 458 roots were essentially equal. These results and our previous results with WT and starchless mutant *Arabidopsis* roots (Kiss et al. 1989, *Planta* **177**, 198–206) support the conclusions that a full complement of starch is necessary for full gravitropic sensitivity and that amyloplasts function in gravity perception. Since a presumed relatively small increase in plastid buoyant mass (*N. sylvestris* mutant versus *Arabidopsis* mutant) significantly improves the orientation of the *N. sylvestris* mutant roots, we suggest that plastids are the likeliest candidates to be triggering gravity perception in roots of both mutants.

Key words: Amyloplast – Gravitropism – Mutant (gravitropism) – *Nicotiana* (gravitropism) – Plastid

and graviperception – Starch and graviperception – Statolith

Introduction

For the past century, the starch-statolith hypothesis has been used to explain gravity perception in plants. This theory proposes that the displacement of and-or pressure from amyloplasts is the initial signal for gravity perception (for reviews see Volkmann and Sievers 1979; Björkman 1988; Sack and Kiss 1989b).

Starch-deficient mutants have been employed in tests of the starch-statolith hypothesis (Hertel et al. 1969; Roberts 1984; Moore and McClelen 1985; Moore 1987). However, since data about the molecular nature of the mutations are not available, it is difficult to make conclusions about the gravitropic sensitivity of these mutants relative to the wild-types (WT). Recently, Caspar and Pickard (1989) studied gravitropism in a mutant of *Arabidopsis* which is starchless as a result of a deficiency in plastid phosphoglucomutase (EC 2.7.5.1). They concluded that starch was not necessary for gravity perception in *Arabidopsis* and questioned the starch-statolith hypothesis (Caspar and Pickard 1989). However, using the same mutant, we demonstrated that the absence of starch markedly decreased gravitropic sensitivity in the roots (Kiss et al. 1989).

In this paper, we further clarify the relationship between starch and gravitropic sensitivity by studying gravitropism in the WT and in a starch-deficient mutant of *Nicotiana sylvestris*. This mutant, isolated by Hanson and McHale (1988), is also deficient in plastid phosphoglucomutase. We

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Abbreviations: IKI = iodine potassium iodide; WT = wild-type

report here that roots of the *N. sylvestris* mutant are less sensitive to gravity than are WT roots. These results are consistent with the conclusion that amyloplasts function in gravity perception.

Material and methods

Plant material and culture conditions. Seeds of the WT of *Nicotiana sylvestris* Speg. et Comes, and of the starch-deficient mutant NS 458 (F_3 generation from the first backcross) were generous gifts of Dr. Kenneth R. Hanson (Connecticut Agricultural Experimental Station, New Haven, USA). NS 458 has a recessive mutation in a single nuclear gene which makes it grossly deficient in the activity of plastid enzyme phosphoglucomutase (Hanson and McHale 1988).

Culture conditions were essentially as described in Kiss et al. (1989). Briefly, for curvature and growth studies, seeds were surface sterilized, and seedlings were grown under sterile conditions in Petri dishes on 1% (w/v) agar containing nutrients and 1% (w/v) sucrose (under continuous illumination of 90–100 μmol photons$\cdot$m$^{-2}\cdot$s^{-1} from 40-W "cool-white" fluorescent lamps).

Seeds were used for curvature and growth studies two to ten months after harvesting. Wild-type and NS 458 roots were used when they were 3–6 mm long, approx. 6–7 d after sowing (except the experiments summarized in Table 3).

Curvature and growth studies. These studies were performed in continuous illumination since we determined that the roots were not phototropic. In all experiments, for each root, curvature was measured as an increment over the starting value. Procedures used were as described in Kiss et al. (1989) except that: (1) in the induction experiments (Fig. 7; Table 2, 3), the horizontal exposures ranged from 5 to 60 min, and curvature was measured after a 2-h rotation on the clinostat; (2) growth rates were calculated from increases in root length over an 8-h period; (3) seedlings were excluded from all samples if their roots were not 3–6 mm long (except the experiments summarized in Table 3).

Microscopy. Light and electron microscopy was performed as described by Sack and Kiss (1989a). Briefly, the seedlings were fixed with glutaraldehyde and *para*formaldehyde, and postfixed with osmium ferricyanide. For light microscopy, 1.5-μm sections were stained with toluidine blue, and for electron microscopy, silver to gold sections were stained with lead citrate and uranyl acetate. In order to test for the presence of starch, fresh tissue was stained with iodine potassium iodide (IKI) and examined using light microscopy (O'Brien and McCully 1981).

Reagents. Agar and chemicals used for electron microscopy were purchased from Polysciences (Warrington, Penn., USA). All other biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo., USA).

Results

Starch content of the WT and mutant NS 458. Although Hanson and McHale (1988) biochemically characterized the amount of starch in the WT and mutant, they did not use microscopy to test for starch. Since the columella cells of the root cap are the probable sites of gravity perception in the

root (Behrens et al. 1985; Sack and Kiss 1989b), we determined the starch content of these cells by microscopy. Starch was detected in electron micrographs of plastids of the columella cells in both the WT (Fig. 1) and the mutant (Fig. 2). The mutant contains much less starch than the WT, but starch grains were present in most sections and positive staining with IKI confirmed the presence of reduced starch in these plastids. Based on microscopic observations of roots, we estimated that mutant plastids contain 10% or less of the starch of WT plastids. Small amounts of starch also were detected in the hypocotyl and cotyledons of the mutant seedlings by IKI staining (data not shown).

In addition to the plastoglobuli (presumably composed of lipid), the plastids in the root-cap columella cells also contained larger, membrane-bound, osmiophilic structures (Figs. 1, 2). Although initially more obvious in mutant plastids because of reduced starch (Fig. 2), these larger structures were also present in WT amyloplasts (Fig. 1). Vigil and Ruddat (1985) have demonstrated that these structures are protein bodies in *N. tabacum*. We found that the protein bodies were similar in size in WT and mutant plastids.

Columella cell polarity. Columella cells in WT *N. sylvestris* have a polar organization similar to that found in columella cells of other plants (Volkmann and Sievers 1979; Sack and Kiss 1989a). The nucleus was found in the proximal (top in vertical roots) part of the cell, and the plastids were sedimented in the distal (bottom) part of the cell in the WT (Fig. 3). NS 458 plastids can be identified in the light microscope in toluidine-blue-stained sections (Fig. 4). Based on qualitative observations of light- and electron-microscopic sections, the mutant plastids were not found to be sedimented to the bottom of columella cells in vertically oriented roots.

Vertical growth of seedlings. Both WT and mutant seedlings had roots that were closely oriented around the gravity vector during vertical growth. The distribution around and the extent of deviation from the gravity vector of vertically grown roots are shown in Fig. 5. Both WT and mutant roots had essentially the same negligible mean divergence from the vertical (1.4° and 1.3° for WT and mutant, respectively). The standard deviation for mutant roots ($\pm$12.8°) was slightly higher than for the WT ($\pm$10.8°).

Growth rate. The growth rates of roots of both genotypes were essentially equal for vertically
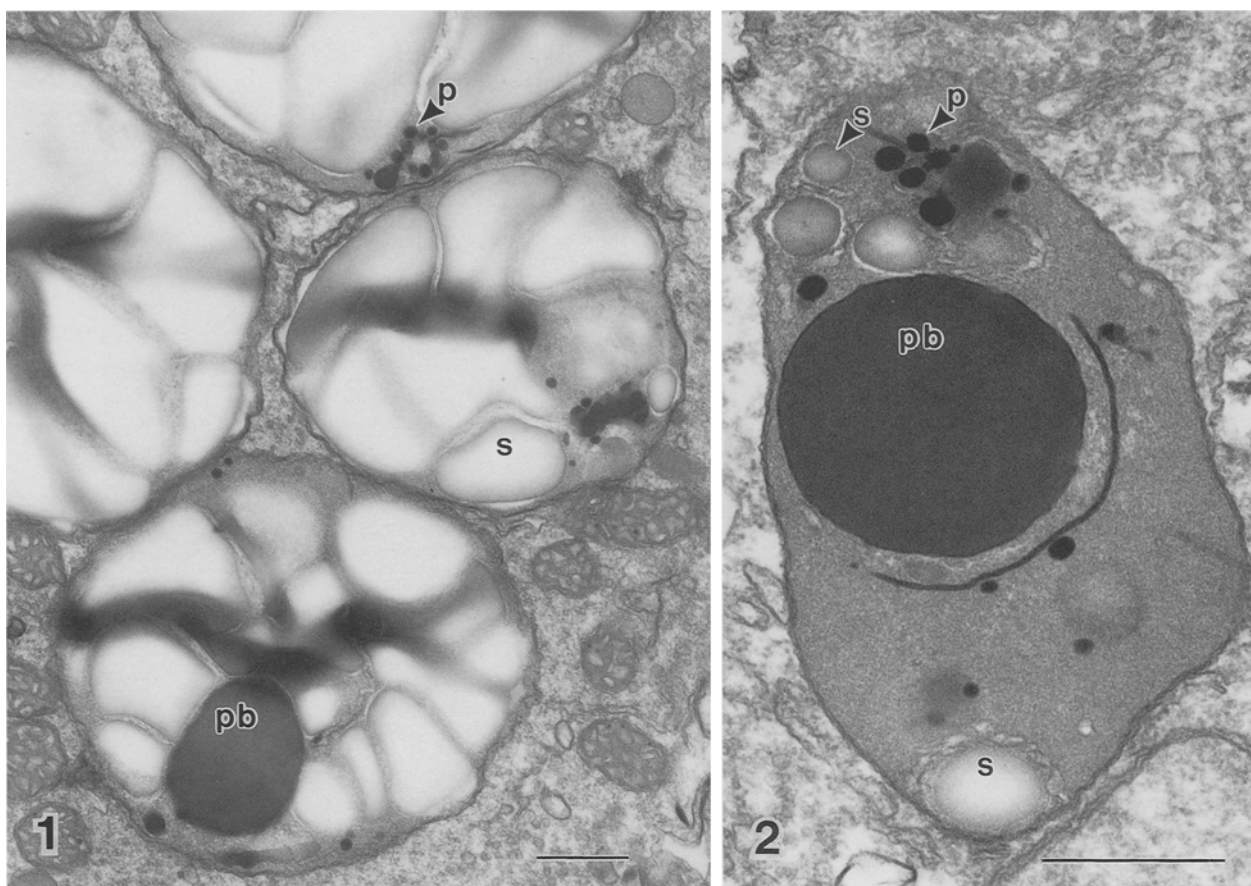
Figs. 1, 2. Electron micrographs of plastids from root-cap columella cells in *Nicotiana sylvestris*. Plastids from both genotypes contain three types of inclusions: starch (*s*), plastoglobuli (*p*), and protein bodies (*pb*). Scale bars = 0.5 μm

Fig. 1. Wild-type. × 23000

Fig. 2. Mutant NS 458. The amount of starch is greatly reduced compared to the WT. × 46000

grown seedlings and for seedlings grown on a rotating clinostat (Table 1).

Time course of curvature. The time course of curvature of roots following gravistimulation is shown in Fig. 6. Wild-type roots curved more rapidly than mutant roots throughout the course of curvature, but only slightly so. Measurable curvature was first detected after 10 min in WT roots and only after 20 min in mutant roots.

Induction experiments. In an attempt to separate graviperception from later phases of gravitropism, seedlings were given a brief horizontal stimulation (induction time) and then permitted to develop

curvature on a clinostat. Curvature was measured after a 2-h rotation on the clinostat since preliminary experiments established that this was the optimum time to measure curvature (data not shown). Curvature was plotted against the logarithm of the induction time (Fig. 7). Curvature of mutant roots was 24–56% that of WT roots over the range of times tested (Fig. 7).

The *presentation time*, which has been used as a measure of the single threshold period of gravistimulation beyond which curvature can be detected, was calculated from the regression equation for $y = 0°$ (Johnson and Pickard 1979; Kiss et al. 1989); these values were approx. 4.8 min for WT and 5.1 min for the mutant. Calculations of presentation time are affected by which data points are included in the regression equation (Table 2). The presentation time of WT roots was slightly shorter than that of mutant roots for most calculated values. The effect of seedling age on the development of curvature was tested for a selected induction period (Table 3). Curvature of the emerging roots from 5-d-old seedlings was difficult to measure. The difference in curvature between mutant and WT roots was greater in 9-d-old than in 7-d-
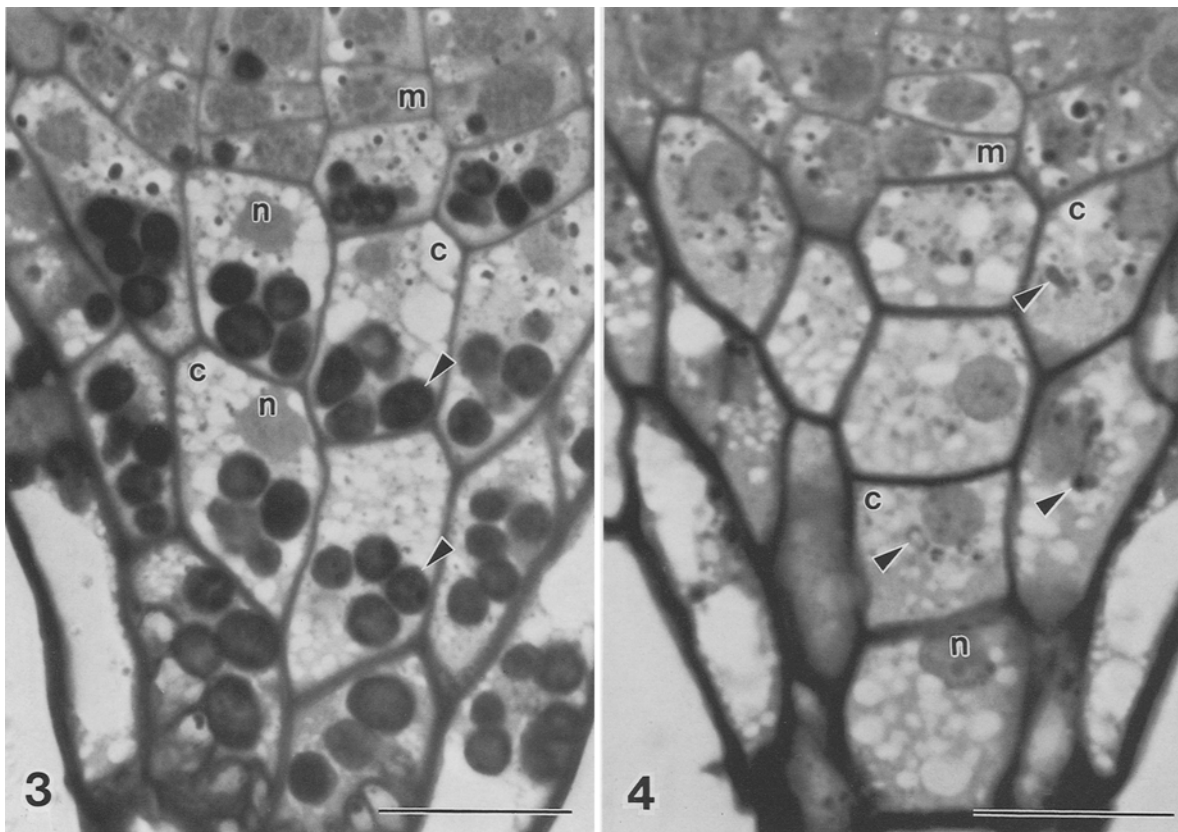
Figs. 3, 4. Light micrographs of toluidine-blue-stained sections of root caps from vertically-oriented *N. sylvestris* seedlings. Most nuclei (*n*) are found in the top (proximal) part of the columella cells (*c*). The gravity vector is towards the bottom of each micrograph (*m*=meristematic cell). ×1300; scale bars=20 μm

Fig. 3. Wild-type. The amyloplasts (*arrowheads*) appear to be sedimented in the columella cells (*c*)

Fig. 4. Mutant. The plastids (*arrowheads*) do not appear to be sedimented in the columella cells (*c*)

old seedlings. Six- to seven-day-old seedlings were used in all other experiments since a greater number could be analyzed per Petri dish. At all ages studied, curvature was greater in the WT than in the mutant.

Intermittent stimulation. In intermittent-stimulation experiments, seedlings were repeatedly gravistimulated for short periods that alternated with periods of rotation on the clinostat (Table 4).
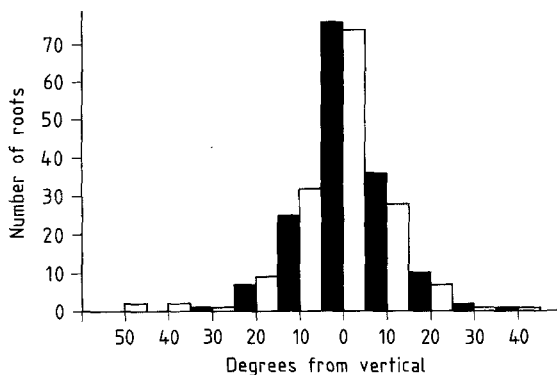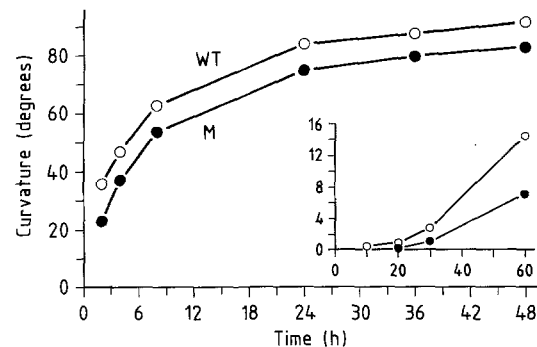

Fig. 5. Histogram of the deviation of populations of roots of vertically grown WT (*n*=158) and mutant NS 458 (*M*; *n*=157) seedlings of *N. sylvestris* from the gravity vector. Each bar represents a 10°-interval, and 0° represents the gravity vector. The SD of mutant roots (±12.8°) is slightly higher than that of the WT (±10.8°). ■ WT; □ M

Fig. 6. Time course of curvature of roots of *N. sylvestris* WT and mutant NS 458 (*M*) seedlings. The downward curvature of roots was measured following placement in a horizontal orientation. *Inset* shows early stages of curvature, and the abscissa scale is in minutes. Curvature was first detected after 10 min in WT roots and after 20 min in mutant roots. All SE bars are shorter than the diameter of the symbols (37<*n*<81)

Table 1. Growth rates (in $\mu m \cdot h^{-1} \pm SE$) of *Nicotiana sylvestris* WT and mutant NS 458 roots. The growth rates were measured over an 8-h period and were essentially equal for both genotypes. These rates were measured (from separate samples) at the same time kinetics of gravitropism experiments were performed, using seedlings of comparable age and size. Sample size is in ( )

	WT	NS 458
Vertical	94 ± 3.4 (53)	96 ± 3.9 (54)
Clinostat	79 ± 3.4 (77)	82 ± 3.0 (81)

Table 2. Calculations of presentation times (in min) of *N. sylvestris* roots. This table illustrates how the values calculated for presentation time depend on which data points (induction times) are included or excluded. The induction plot for all points (i.e. no data points are excluded) is shown in Fig. 7. The correlation coefficients are $0.92 \leq X \leq 0.99$

Data points (min) excluded	Calculated presentation time (min)	
	WT	NS 458
None	4.8	5.1
5	5.8	6.6
5, 7.5	7.1	7.5
60	4.0	4.1
40, 60	3.1	3.0
5, 60	4.6	5.4
Range	3.1–7.1	3.0–7.5

Table 3. Effect of seedling age on curvature (degrees ± SE) in *N. sylvestris* roots after a 20-min induction period and 2 h rotation on the clinostat. Mean root length (which was equal for WT and NS 458) is given in [ ], and sample size in ( )

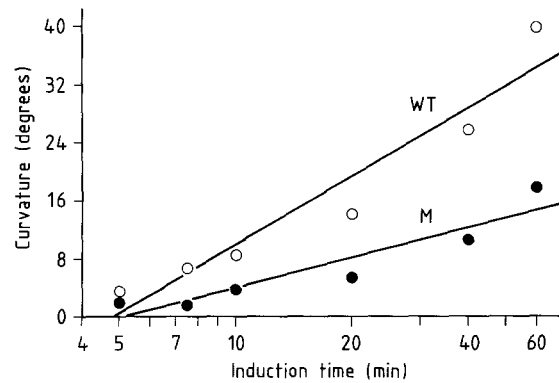|	5 d [0.9 mm]	7 d [4.9 mm]	9 d (10.0 mm)
WT	3.2 ± 0.6 (33)	17.3 ± 1.3 (65)	24.3 ± 1.8 (43)
NS 458	0.8 ± 0.3 (28)	7.2 ± 1.3 (52)	5.3 ± 1.1 (41)

Fig. 7. Induction curve for roots of *N. sylvestris* WT and mutant NS 458 (*M*) seedlings. Plants were placed horizontally for the single period indicated on the abscissa and then rotated axially on a 1-rpm clinostat. Curvature of the roots was measured 2 h after the plants were placed on the clinostat. Correlation coefficients for the regression lines were 0.96 for WT and 0.94 for mutant. Regression lines are extrapolated to 0°, and the presentation times were calculated (from the regression equation) to be 4.8 min for WT and 5.1 min for mutant. All SE bars are shorter than the diameter of the symbols ($67 < n < 86$)

Curvature of mutant roots was 37% or less than that of WT roots in all treatments tested. For example, in a 2:8 treatment (a 2-min horizontal exposure followed by 8 min of rotation on the clinostat, repeated 12 times), WT roots curved 35.6° while mutant roots curved only 9.6°.

The *perception time*, in addition to the presentation time, can be used to estimate gravitropic sensitivity and is defined operationally as the smallest period of gravistimulation that can be summed to produce curvature following intermittent stimulation (Pickard 1973; Volkmann and Sievers 1979; Sack and Kiss 1989b). The shortest repeated period of horizontal exposure that resulted in curvature compared to the controls (0:10 cycle, which is continuous rotation on the clinostat) was 5 s for WT (5 s:9 cycle) and 30 s or 1 min (30 s:9 or 1:9 cycle) for mutant roots. Thus,

Table 4. Effects of intermittent stimulation on curvature (degrees ± SE) in *N. sylvestris* roots. Seedlings were stimulated (placed horizontally) for the times (in min, except for 5 s, 10 s, and 30 s) indicated (in top row) to the left of the colon and then rotated on a clinostat at 1 rpm for the times (in min) indicated to the right of the colon. Each cycle totalled 10 min (except for 5 s:9, 10 s:9, and 30 s:9) and was given a total of 12 times. 0:10 was rotated on a clinostat continuously for 2 h, and 10:0 was placed horizontally for 2 h and not rotated on a clinostat. Curvature was measured at the end of the last cycle, i.e. at 2 h. Sample size in ( )

Geno-type	min horizontal:min on clinostat							
	0:10	5 s:9	10 s:9	30 s:9	1:9	2:8	4:6	10:0
WT	1.0 ± 0.6 (109)	4.2 ± 1.0 (82)	3.8 ± 0.7 (90)	13.9 ± 1.2 (107)	22.7 ± 1.5 (86)	35.6 ± 1.0 (106)	34.3 ± 1.0 (102)	38.8 ± 1.0 (115)
M	0.0 ± 0.6 (74)	0.3 ± 0.7 (61)	0.7 ± 0.6 (82)	2.4 ± 0.7 (91)	4.3 ± 0.7 (73)	9.6 ± 1.2 (79)	12.8 ± 1.0 (93)	20.8 ± 1.0 (106)

the perception time of WT roots is 5 s or less and that of mutant roots is between 30 s and 1 min.

In most experiments, curvature was measured at the end of the twelfth cycle. Further rotation on the clinostat did not change the large differences in curvature between WT and mutant roots. For example, when the seedlings were rotated on a clinostat for another 2 h after the 1:9 treatment, the resulting curvatures were 23.9±2.9° (*n*=35) for WT and 3.5±1.8° (*n*=34) for the mutant.

Discussion

Nicotiana sylvestris mutant NS 458 contains small amounts of starch. The mutant was characterized biochemically as starchless by Hanson and McHale (1988). However, we detected (by light and electron microscopy) small starch grains in seedlings grown under continuous illumination on a nutrient agar. This is in contrast to the *Arabidopsis* mutant (TC7) that is completely starchless (Caspar and Pickard 1989; Kiss et al. 1989; Sack and Kiss 1989a).

A full complement of starch is required for full gravitropic sensitivity in N. sylvestris roots. Roots of the WT are more responsive to gravity than roots of the mutant NS 458 by these parameters:

(1) In mutant roots, curvature was reduced and delayed compared to WT roots in the *time course of curvature*.

(2) Curvature of mutant roots was 24–56% that of WT roots over the range of *induction periods* tested.

(3) In *intermittent-stimulation* experiments, curvature of mutant roots was 37% or less than that of WT roots in all treatments tested. Additionally, WT roots curved after repeated 5-s exposures, the lowest period tested, but mutant roots only showed curvature after repeated 30–60-s exposures.

As in the case of the starchless *Arabidopsis* mutant (Kiss et al. 1989), it is uncertain whether the reduced curvature in mutant roots results from lowered sensitivity (perception), or whether impairment of the responding and-or transducing systems contributes as well. However, we suggest that a major effect of the NS 458 mutation (as well as the TC7 mutation in *Arabidopsis*) is an impairment of gravity perception since (1) the growth rates of WT and NS 458 are essentially equal, and (2) longer periods on the clinostat after intermittent stimulation did not result in increased curvature. We therefore conclude that NS 458 roots are less sensitive to gravity than WT roots, that a full complement of starch is necessary for full gravi-

tropic sensitivity, and that amyloplasts function as statoliths in WT roots. Furthermore, initial observations indicate that starch-deficiency seems to reduce gravitropic sensitivity even more in *N. sylvestris* hypocotyls than in roots (Sack and Kiss 1989b).

Estimates of gravitropic sensitivity. The *presentation time* has been considered as the threshold time beyond which a gravitropic response occurs (Larsen 1962; Johnsson and Pickard 1979). The presentation time can be determined by extrapolation to, or calculated from a regression equation for zero curvature in an induction curve with axes (curvature versus duration of stimulation) on a linear, semilogarithmic, or logarithmic scale (Johnsson 1971; Johnsson and Pickard 1979; Kiss et al. 1989; Sack and Kiss 1989b). If linear scales are used for both axes in the induction curve for *N. sylvestris*, almost all derived values for presentation time are meaningless (negative). The choice of which time points to include in calculations of presentation time has not been rigorously evaluated in the literature and usually only one value is given. Presentation times for *N. sylvestris* roots range from 3.0 to 7.5 min, depending on the induction times included (Table 2). Thus, the presentation time should be expressed as a range rather than one value. Furthermore, the presentation time itself is not a useful index of gravitropic sensitivity in *N. sylvestris*, although there was a difference in response between the two genotypes at each induction time tested.

Intermittent-stimulation experiments demonstrated the greatest difference in sensitivity between mutant and WT *N. sylvestris* roots, i.e. the *perception time* is 30–60 s for mutant roots and is 5 s or less for WT roots (Table 5). Furthermore, WT roots can sum repeated 5-s horizontal exposures to produce approx. 4° of curvature while it takes repeated 60-s exposures to produce equivalent curvature in the mutant (Table 4). By this criterion, WT roots are 12 times more sensitive than mutant roots. A 12-fold difference in sensitivity was also found between WT and TC7 *Arabidopsis* roots (Kiss et al. 1989).

Comparison of N. sylvestris and Arabidopsis mutants. Table 5 compares key gravitropic parameters for both WT and mutant roots of *Arabidopsis* and *N. sylvestris*. The first three parameters indicate that, relative to their respective WTs, the *N. sylvestris* mutant may be closer in gravitropic sensitivity than the *Arabidopsis* mutant. However, this differential is not apparent in data from intermittent-

Table 5. Comparison of gravitropism between *Arabidopsis* and *N. sylvestris*. Data for *Arabidopsis* from Kiss et al. (1989)

	Arabidopsis		N. sylvestris	
	WT	Mutant (TC7)	WT	Mutant (NS 458)
Deviation from gravity vector during vertical growth (SD in degrees)	12.8	27.4	10.8	12.8
Time (min) that curvature is first detected in a time course	5	20	10	20
Calculated presentation time (min)	0.4	1.9	4.8	5.1
Perception time (s), i.e. smallest dose tested that resulted in curvature after intermittent stimulation	≤10	≈120	≤5	30–60

stimulation experiments. As discussed above, based upon the amount of intermittent stimulation necessary to produce an equal amount of curvature (within a species), both WT *Arabidopsis* and WT *N. sylvestris* roots are roughly 12 times more sensitive than their respective mutants.

These data indicate that compared to the roots of the *Arabidopsis* mutant and WT, the roots of the *N. sylvestris* mutant are closer in gravitropic performance to their WT when they are gravistimulated for longer periods (≥ the approximate presentation time). At shorter gravistimulation periods (< the approx. presentation time), there is little apparent difference in sensitivity between the roots of the *Arabidopsis* and *N. sylvestris* mutants relative to their respective WTs.

Do mutant plastids function as statoliths? The starch-deficient plastids in the *N. sylvestris* mutant presumably have a higher *buoyant mass* than the starchless *Arabidopsis* plastids since the former contain protein in addition to starch. (Buoyant mass, the force exerted by the plastid on the cytosol, is proportional to the difference between the densities of the plastid and the cytosol, and to plastid volume; Sack and Kiss 1989b). *If* mutant plastids function as statoliths, then the data indicate that a small increase in buoyant mass dramatically decreases the "noisiness" of the gravitropic response; e.g. mutant *N. sylvestris* roots are essentially as oriented around the vertical as WT roots.

Since the starchless *Arabidopsis* plastids are relatively dense and are the most movable component in columella cells (Kiss et al. 1989), and since a presumed relatively small increase in plastid buoyant mass (*N. sylvestris* mutant versus *Arabi-*

dopsis mutant) significantly improves the orientation of *N. sylvestris* mutant roots, we suggest that plastids are the likeliest candidates to be triggering gravity perception in roots of both mutants. If this hypothesis is correct, then the absence of substantial sedimentation of starch-deficient plastids in *N. sylvestris* confirms findings from the starchless *Arabidopsis* mutant (Caspar and Pickard 1989; Kiss et al. 1989) that significant plastid sedimentation is not necessary for a plastid to function as a statolith. If the starch-deficient plastids do *not* contribute to gravity perception, then another unknown system of perception must be present in roots in addition to amyloplasts.

Conclusions

Both the starch-deficient *N. sylvestris* mutant discussed here and the starchless *Arabidopsis* mutant studied by Kiss et al. (1989) are deficient in plastid phosphoglucomutase. Roots of both mutants are impaired in gravitropic sensitivity. Together these results strongly support the conclusions that a full complement of starch is necessary for full gravitropic sensitivity in roots and that amyloplasts function in gravity perception. Furthermore, it is likely that plastids function as statoliths in both mutants.

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