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Do Starch Statoliths Act as the Gravisensors in Cereal Grass Pulvini?[1]

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ABSTRACT

To determine if starch statoliths do, in fact, act as gravisensors in cereal grass shoots, starch was removed from the starch statoliths by placing 45-day-old intact barley plants (*Hordeum vulgare* cv 'Larker') in the dark at 25°C for 5 days. Evidence from staining with I_2-KI, scanning electron microscopy, and transmission electron microscopy indicated that starch grains were no longer present in plastids in the pulvini of plants placed in the dark for 5 days. Furthermore, gravitropic curvature response in these pulvini was reduced to zero, even though pulvini from vertically oriented plants were still capable of elongating in response to applied auxin plus gibberellic acid. However, when 0.1 molar sucrose was fed to the dark pretreated, starch statolith-free pulvini during gravistimulation in the dark, they not only reformed starch grains in the starch-depleted plastids in the pulvini, but they also showed an upward bending response. Starch grain reformation appeared to precede reappearance of the gravi-response in these sucrose-fed pulvini. These results strongly support the view that starch statoliths do indeed serve as the gravisensors in cereal grass shoots.

Recent studies in our laboratory (19–21) have been concerned with the cereal grass leaf-sheath pulvinus as a graviresponsive organ system and whether or not the starch-containing plastids in pulvinus cells of this system are indeed the gravisensors (statoliths). The young, undifferentiated grass shoot pulvinus does not possess any starch-containing statoliths, and it shows no upward bending curvature response when oriented horizontally (9, 21, 29). However, when the pulvinus is mature, starch statoliths are present in statenchyma cells along the inside of each of the longitudinally oriented vascular bundles, as seen in transverse sections of the pulvinus (Fig. 1). The structure of starch statoliths in grass shoots as examined by means of LM,[2] SEM, and TEM (9, 19–21, 23, 26), as well as their purported function as gravisensor organelles (1, 14, 17, 22, 25, 27), have been studied extensively. Furthermore, it has been shown that these organelles descend within the presentation time (1–2 min) for initiation of an upward bending graviresponse in the grass shoot pulvinus (21). Even so, and in spite of all previous work, we still have no definitive proof that starch statoliths are the gravisensors that trigger an upward bending response in prostrated cereal grass shoots. In other graviresponsive systems, such as in rootcaps of starch-free *Arabidopsis* mutants, they are not required (7, 25); that is, the roots respond to gravistimulation to the same extent as do nonmutant plants, with starch statoliths present in the root caps.

In light of the above, we set out to determine whether or not gravitropic curvature could occur in barley shoots in the absence of starch in the pulvinus plastids. And, if not, would a gravitropic response occur in pulvini when the starch was resynthesized in starch-depleted plastids? In this paper, we present evidence which supports the hypothesis that starch statoliths are the gravisensors in leaf sheath pulvini of cereal grass shoots. We also discuss their possible role as gravisensors which lead to transduction and asymmetric growth.

MATERIALS AND METHODS

Plant Material. Barley (*Hordeum vulgare* cv 'Larker') plants were grown in the greenhouse under natural daylength conditions of March to May 1986 and 1987 for 45 d.

Dark and Gravistimulation Treatments. Flats of intact 45-d-old plants were placed in a dark room for 5 d at 25°C. During such dark treatments, freehand sections of pulvini from these plants were stained with I_2-KI and examined with a light microscope to determine whether or not starch was present in the pulvinus plastids. When starch grains had completely disappeared from the plastids (usually after 5 d of dark treatment of the barley plants), stem segments were excised from the shoot 6 cm above the p-1 pulvinus (located at the next-to-last node of the shoot) and 3 cm below it. These stem segments were then oriented horizontally with the 3 cm stem portion placed between two 0.5 cm thick glass plates with absorbent paper towels saturated either with distilled water or 0.1 M sucrose. The glass plates were then taped together and placed in a humid Plexiglas chamber in the dark at 25°C. After 24 and 48 h, the upward bending response in the p-1 pulvinus was recorded with a protractor. Sections of pulvini were prepared for LM, SEM, and TEM at 12 and 24 h after treatment. These same procedures were employed for light-grown control plants which were also gravistimulated as above.

Short-Term Responses to Exogenous Hormones or Gravitismulation. The kinetics of responses to hormonally treated vertical segments were compared with those of gravistimulated segments using an angular recording position transducer. Segments were excised from 5 d dark-treated plants, abraded at the pulvinus with jeweler's rouge, and mounted individually in glass vials with 0.1 M sucrose for 20 to 30 min in the dark at 25°C. Segments were either gravistimulated, or left vertical and treated unilaterally with 100 μM IAA plus 10 μM GA_3 in 1% agar (pH 5.5). Lag to first negative gravitropic curvature, and steady state bending response within 10 h after first negative response, were calculated from continuous printouts. Results are presented as means from three independent experiments.

Microscopy. For TEM, small pieces of pulvinus tissue were fixed in 3% gluteraldehyde in 50 mM cacodylate buffer (pH 7.0) for 2 h at room temperature, then washed in three changes (10 min each) of cacodylate buffer. They were postfixed with 1.5%

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[2] Abbreviations: LM, light-microscopy; SEM, scanning electron microscopy; TEM, transmission electron microscopy.

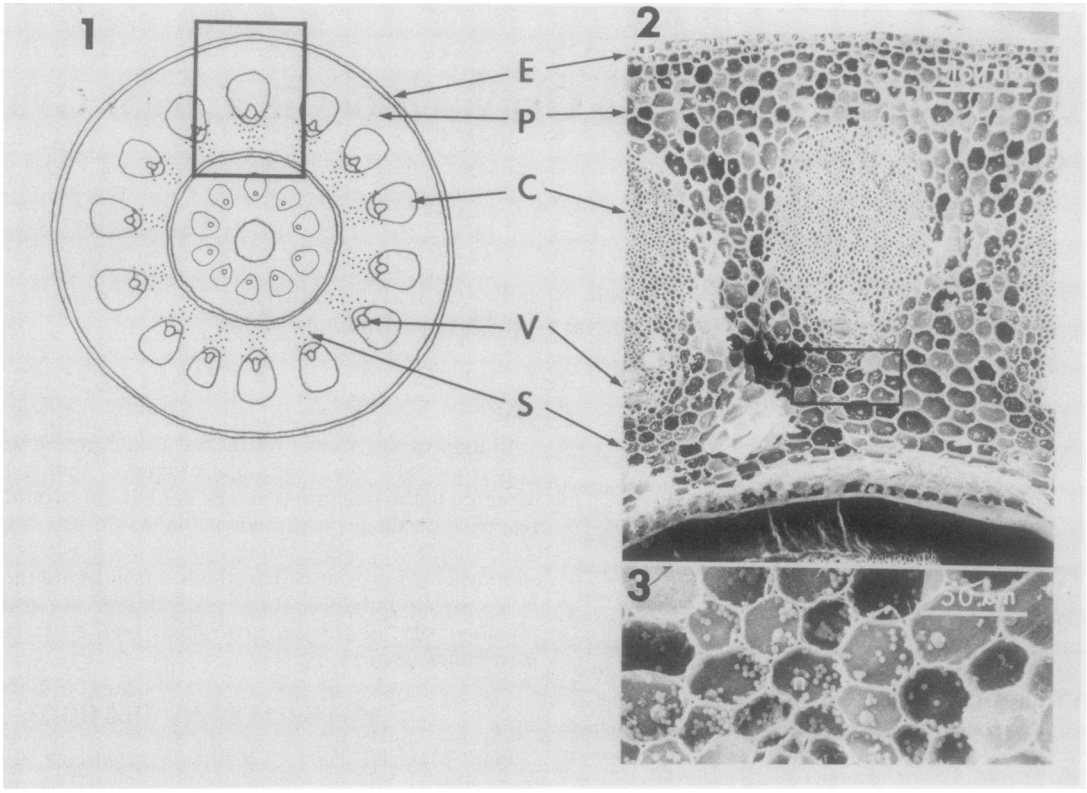

FIG. 1. Illustrations showing the location of statenchyma in the leaf-sheath pulvinus of a barley (*H. vulgare* cv 'Larker') shoot. 1, Cross-sectional diagram depicts the midportion of a barley leaf-sheath pulvinus and an internode base within it. The stippled regions denote the location of regions of statenchyma (S) in the pulvinus. Such regions are located to the inside of each vascular bundle. 2, Scanning electron micrograph shows a single vascular bundle with its associated statenchyma. 3, An enlarged view of this statenchyma tissue. Note that the statenchyma cells contain prominent starch statoliths which appear as white spheres. E, outer epidermis P, parenchyma tissue; C, collenchyma tissue; V, vascular bundle; S, statenchyma (from 20).

osmium tetroxide for 2 h, then dehydrated in a graded series of ethanol. They were placed in Spurr's resin at 30% (1 h), 50% (1 h), and 100% (overnight), then embedded in 100% Spurr's resin and allowed to polymerize at 60°C overnight. Specimens were sectioned with a diamond knife, stained with uranyl acetate followed by lead citrate, and observed in a Zeiss EM 10-CA transmission electron microscope.

For SEM, the postfixation step in the above procedure was omitted. The tissue was dehydrated as above, then critical-point dried. The specimens were mounted on aluminum stubs with Tube-kote. They were then coated with gold and observed with a Hitachi S-570 scanning electron microscope.

For LM, selected pulvini were hand-sectioned, before and after dark treatment, checked for the presence or absence of starch in the plastids by staining the sections with I_2-KI, and examined with an Olympus Vanox microscope.

RESULTS

After 5 d of pretreatment in the dark, the starch grains in the plastids had been almost completely degraded (Figs. 2 and 4, A and B). Subsequently, after the 5-d dark treatment, excised stem segments, each containing a single pulvinus, were gravistimulated in the dark while incubating the stem bases in either 0.1 M sucrose or distilled water. After 12 h of incubation in 0.1 M sucrose, starch began to reform in starch-depleted plastids in the pulvini (Figs. 2 and 4, C). Similarly treated segments showed a mean lag to first negative gravitropic response of 9.3 h with a mean half-time to a steady state bending response of 5.9 h (Table I). By 24 h, the starch statoliths were more numerous than at 12 h (Fig. 4E). This coincides with maintenance of the graviresponse to 24 and 48 h after gravistimulation (Table II). Stem segments which were incubated in distilled water for 12 and 24 h did not reform starch (Figs. 2 and 4, D and F) and did not respond to gravistimulation (Table II). Such segments were capable of showing a growth response since exogenous hormones produced a rapid and significant response when applied (Table I).

Table I. *Kinetics of Response to Gravistimulated or Hormonally Treated Vertical Pulvini from Dark Pretreated 'Larker' Barley Plants*

Values are means of three individuals ± SE.

Response	Gravistimulated	IAA (100 µM) + GA_3 (10 µM) (Vertical)
Lag to initial bending response (h)	9.3 ± 2.10	1.5 ± 0.25
Steady state bending response (°h^{-1})	2.3 ± 0.80	2.6 ± 0.18
Half-time to steady state response (h)	5.9 ± 0.65	1.9 ± 0.37
Graviresponse (°24 h^{-1})	7.3 ± 0.47	17.7 ± 7.80

Pulvini from excised stem segments from light-grown control plants (without 5-d dark pretreatment), when gravistimulated in the dark while being fed 0.1 M sucrose for 12 and 24 h, maintained or increased the number of starch statoliths and the size of the starch grains in the pulvinus starch statoliths (Figs. 3 and 5, C and E). They also showed a significant upward bending response

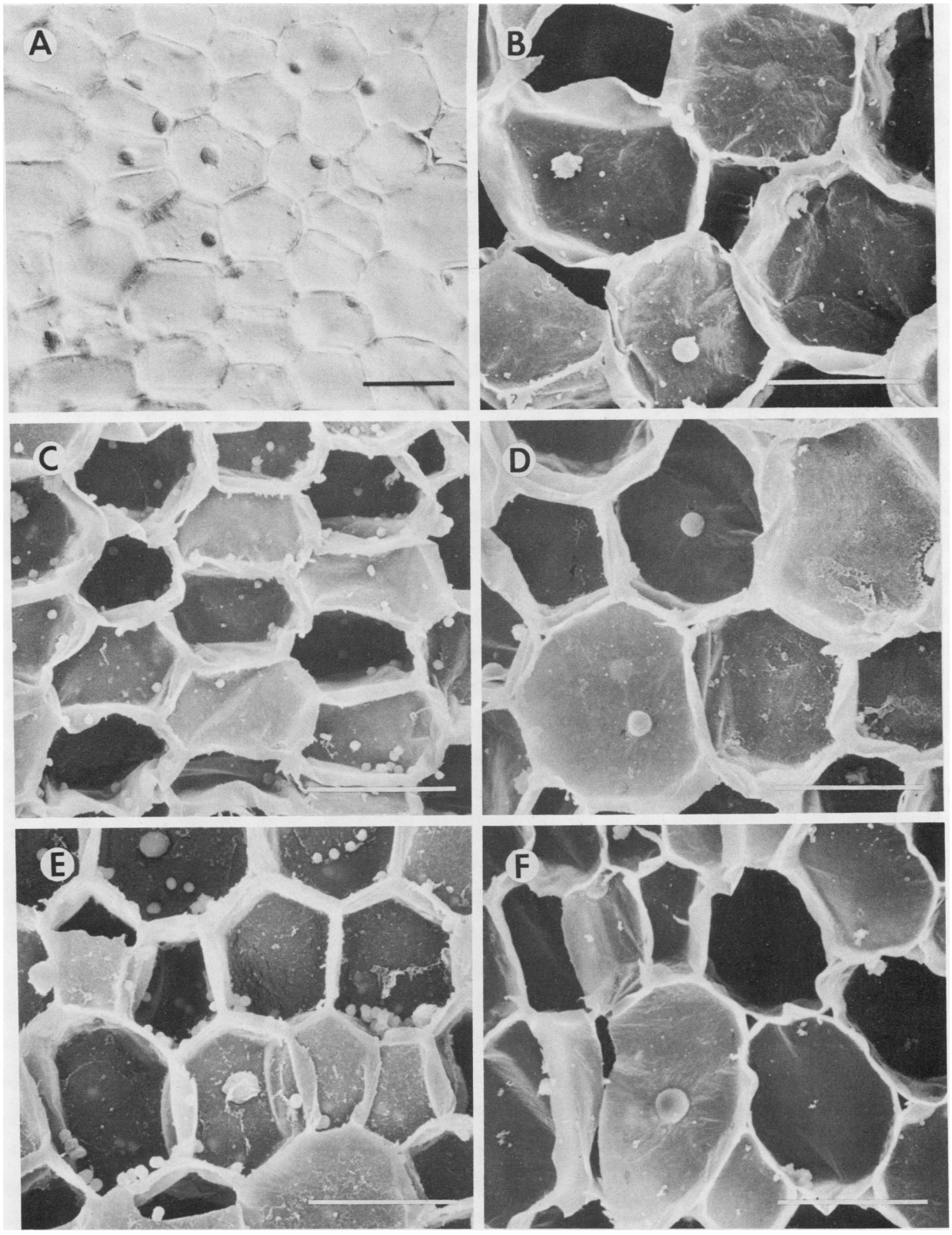

FIG. 2. Light micrograph, Nomarski optics (A), and scanning electron micrographs (B–F) of statenchyma cells in p-1 leaf-sheath pulvini of dark pretreated barley plants. A and B illustrate the effect of 5 d of dark pretreatment on the occurrence of starch statoliths in the statenchyma cells. Note that starch statoliths have completely disappeared (compare with Fig. 3, A and B, light-grown control). Excised segments from these same dark pretreated plants were then gravistimulated and incubated in 0.1 M sucrose (C and E) or distilled water (D and F) in the dark at room temperature for 12 and 24 h, respectively. Bars = 30 μm.

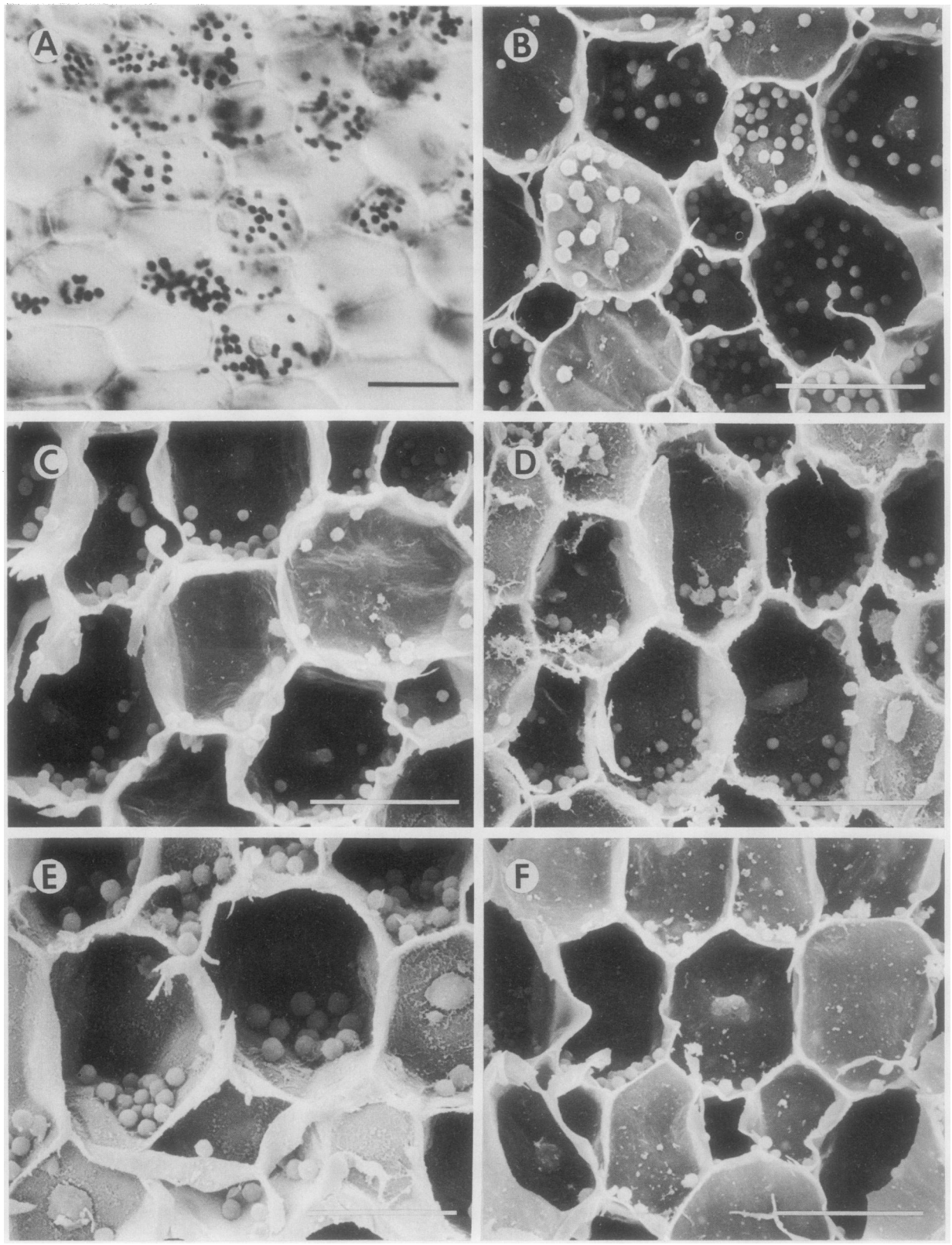

FIG. 3. Light micrograph, Nomarski optics (A), and scanning electron micrographs (B–F) of statenchyma cells in p-1 leaf sheath pulvini of light-grown barley plants, showing numerous starch statoliths in statenchyma cells (A and B). Excised stem segments from these plants were then gravistimulated while being incubated in 0.1 M sucrose (C and E) or in distilled water (D and F) in the dark at room temperature for 12 and 24 h, respectively. Bars = 30 μm.

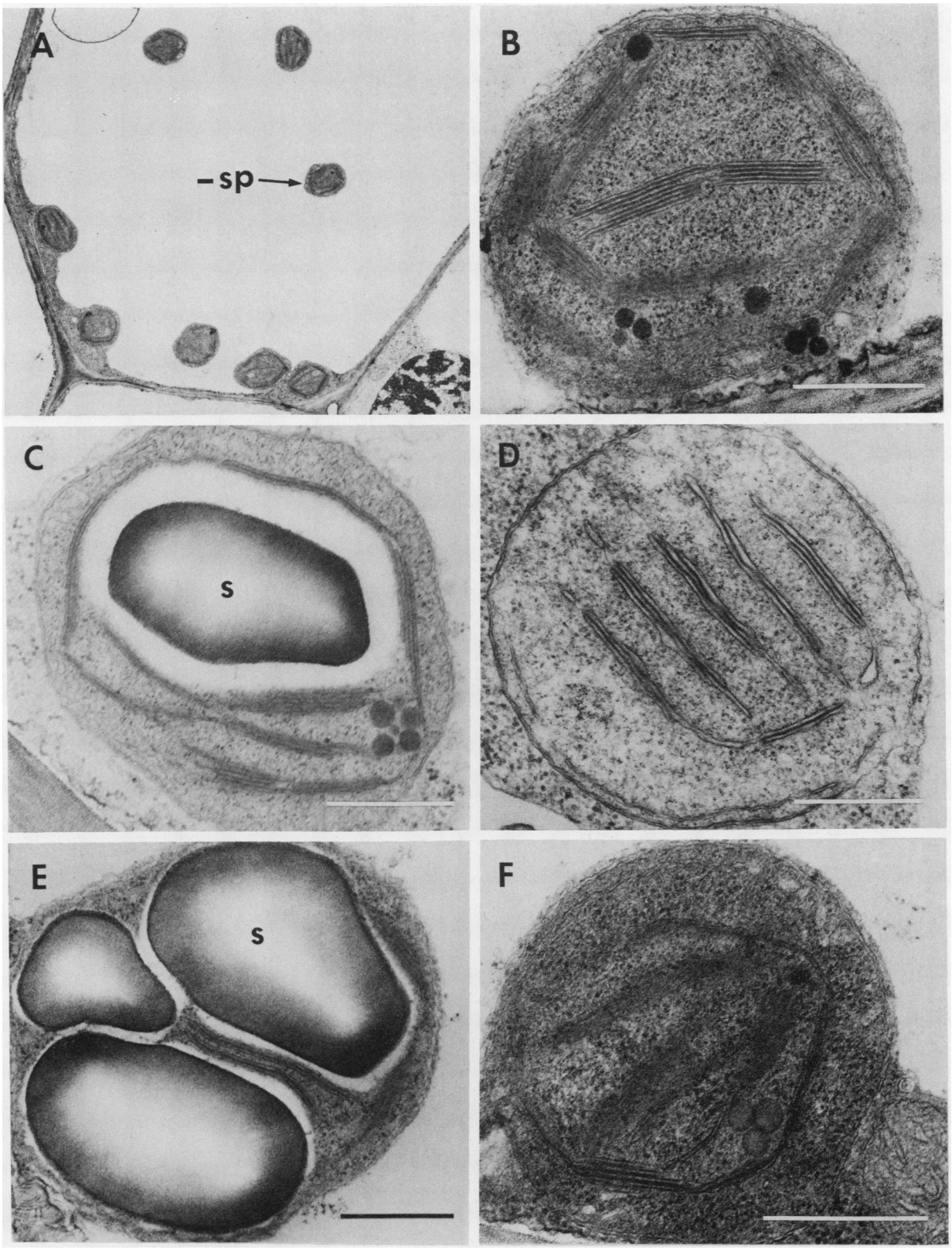

FIG. 4. Transmission electron micrographs of starch-depleted plastids in statenchyma cells of the p-1 leaf-sheath pulvini of dark pretreated 'Larker' barley plants. A and B illustrate the effect of 5 d of dark treatment on the occurrence of starch in the plastids. Note that the starch is absent. After the 5 d of dark pretreatment, excised segments from these plants were gravistimulated while being incubated in either 0.1 M sucrose (C and E) or in distilled water (D and F) in the dark at room temperature for 12 and 24 h, respectively. S, starch; −sp, starch-depleted plastid. Bars = 0.5 μm.

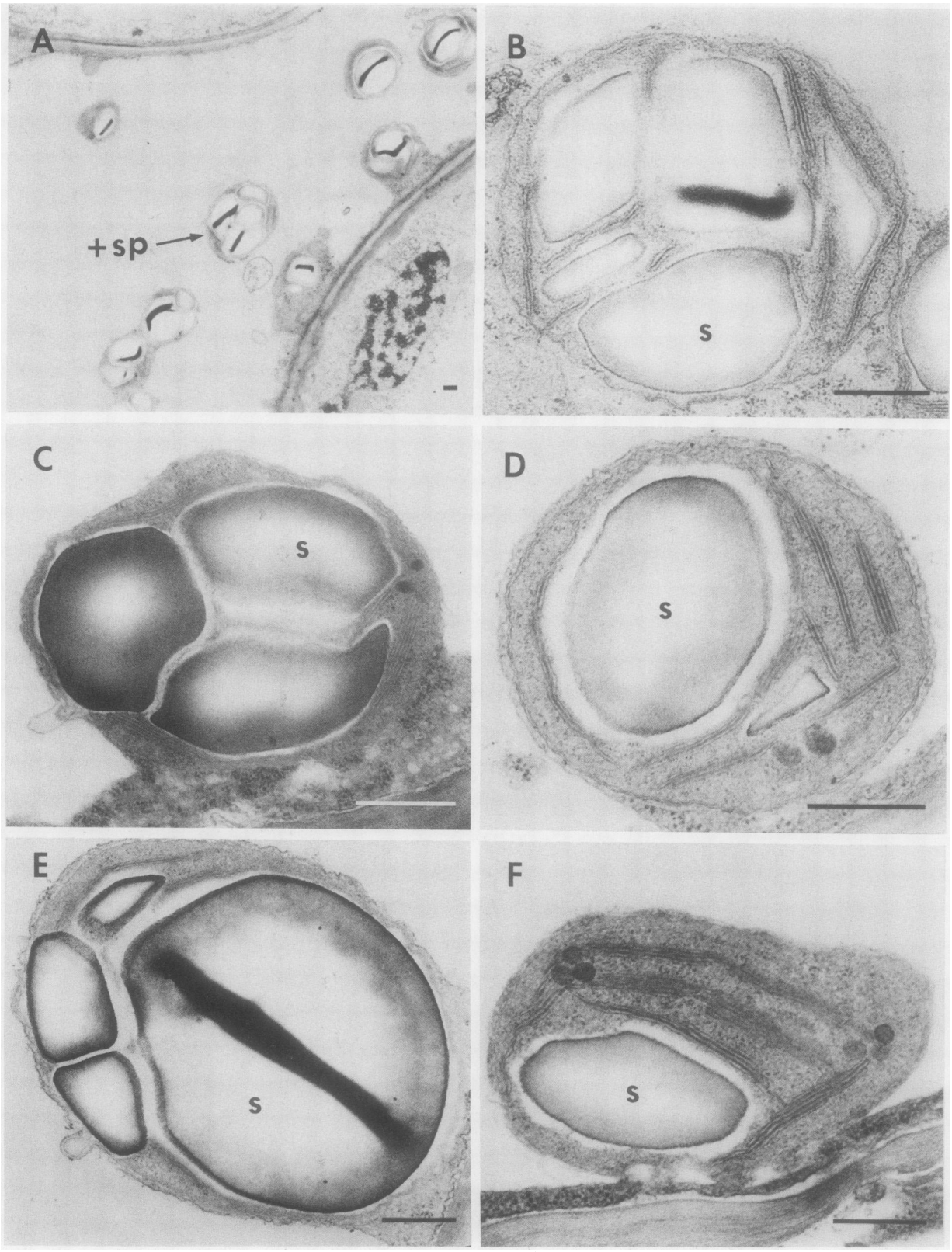

FIG. 5. Transmission electron micrographs of starch statoliths in statenchyma cells of the p-1 leaf-sheath pulvini of light-grown 'Larker' barley plants. Note the prominent starch grains in the starch statoliths in A and B. Excised segments from these plants were gravistimulated while being incubated in either 0.1 M sucrose (C and E) or in distilled water (D and F) in the dark at room temperature for 12 and 24 h, respectively. S, starch; +sp, starch-containing plastid (starch statolith). Bars = 0.5 μm.

Table II. *Effect of Dark Pretreatments on the Gravitropic Response in Leaf-Sheath Pulvini of 'Larker' Barley Plants*

Pretreatment before Gravistimulation	Gravistimulation[a] in the Dark at 25°C	Gravitropic Response[b] 24 h	Gravitropic Response[b] 48 h
Dark [c]	Stem segments fed 0.1 M sucrose	8° ± 0.69	39° ± 1.97
	Stem segments fed distilled water	0°	0°
Light[d]	Stem segments fed 0.1 M sucrose	32° ± 1.82	67° ± 3.99
	Stem segments fed distilled water	16° ± 1.59	25° ± 2.15

[a] For gravistimulation treatments, we used excised stem segments from barley shoots. Each contained a single pulvinus. See "Materials and Methods" for details on stem segment preparation and conditions employed for gravistimulation of these segments. [b] SE are indicated after each set of curvature response data. Twenty pulvinus-containing stem segments were used for each treatment in each experiment. If starch fails to be reformed for any reason (probably plastid membrane system damage during the long dark pretreatment), then no upward bending response occurs. [c] For dark treatments, flats of intact 45-d-old barley plants were kept in the dark for 5 d at 25°C. [d] For control, comparable-aged plants were maintained in the greenhouse under natural d length conditions of March to May, 1986 and 1987 at 25°C.

when gravistimulated (Table II). Pulvini in stem segments from these same light-grown barley plants, which were gravistimulated while being fed distilled water, showed a significant reduction in number of starch statoliths and size of starch grains in the pulvinus starch statoliths (Figs. 3 and 5, D and F) and a reduced amount of upward bending response in the pulvini after gravistimulation (Table II).

DISCUSSION

Once starch (density = 1.5) in the starch statoliths has accumulated in sufficient amounts to cause the starch statoliths to sediment in statocyte cells, an upward bending response occurs in pulvini of cereal grass plants that are oriented horizontally. Starchless plastids are less dense (Fig. 4, A and B), and therefore, do not sediment in response to gravistimulation of the pulvini. These pulvini do not manifest any upward bending response. However, when such starch-depleted plastids are induced to resynthesize starch by sucrose feeding, the pulvini are capable of responding to gravistimulation. Such results strongly suggest that starch statoliths of sufficient density are required for gravisensing and for the gravitropic response in barley leaf-sheath pulvini.

It has been proposed that any charged particles (*e.g.* starch statoliths) normally moving in a bioelectric field (*e.g.* ER, microtubules, plasmalemma) can act as gravisensors by generating a small current or by perturbing the bioelectric field of the cell, after displacement of the particles, resulting in a graviresponse (3). However, in some organisms starch statoliths do not appear to be the gravisensing organelles. These include *Phycomyces, Neurospora,* and starch-free *Arabidopsis* mutants, which respond to gravity in a normal manner. How can we account for gravitational responses that occur in these organisms that are devoid of starch statoliths? In these organisms, the cell(s) that detect gravity may do so as a result of conformational changes in the bioelectric field which alter the flow of oxidant to the cell's cathode (3).

What roles might starch statoliths play as gravisensors in the grass pulvinus system? Two possibilities are: (a) they could act as information carriers, bringing key enzymes to substrates that result in the release of free, active hormones from their inactive conjugates (2, 18–20, 24); (b) these relatively dense bodies could act as 'pressure probes' to alter the physical characteristics of cell components such as microtubules and/or microfilaments. This altered physical state could make possible the lateral transport of hormones, such as IAA or GAs, or allow for intake or outflow of ions, such as K^+ and Ca^{2+} (8, 10, 11, 13, 15, 16, 25, 28). While processes (a) and/or (b) occur, a third possible role (c) is that starch in the statoliths may also serve as a source of substrate (D-glucose) that is used in cell growth metabolism, particularly in the the synthesis of new cell wall polysaccharides (4, 5, 19, 20).

Possibility (a) is currently under investigation in our laboratory through time-course analyses of amounts of native gibberellins and free IAA and their respective conjugates in graviresponding pulvini as compared with upright, control pulvini. Possibility (b) is more difficult to approach, but it may be worthwhile to try the patch-clamp technique (12, 16) to investigate possible changes in ion currents across the plasmalemma of pulvinus protoplasts induced by sedimentation of starch statoliths. To test possibility (c), starch levels in pulvini are currently being analyzed in our laboratory using an enzymic assay (6) during dark pretreatment (0–5 d), as well as during the time starch-depleted pulvini are fed sucrose while being gravistimulated. These experiments will give us an insight into the possible role of starch as a source of substrate (D-glucose) for ATP production and cell wall synthesis. It will also indicate more precisely how and when starch disappearance and resynthesis occur and how these events are correlated with the kinetics for the gravitropic response in cereal grass pulvini.

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