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Plant Biology

Transient and sustained increases in inositol 1,4,5-trisphosphate precede the differential growth response in gravistimulated maize pulvini

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ABSTRACT **The internodal maize pulvinus responds to gravistimulation with differential cell elongation on the lower side. As the site of both graviperception and response, the pulvinus is an ideal system to study how organisms sense changes in orientation. We observed a transient 5-fold increase in inositol 1,4,5-trisphosphate (IP_3) within 10 s of gravistimulation in the lower half of the pulvinus, indicating that the positional change was sensed immediately. Over the first 30 min, rapid IP_3 fluctuations were observed between the upper and lower halves. Maize plants require a presentation time of between 2 and 4 h before the cells on the lower side of the pulvinus are committed to elongation. After 2 h of gravistimulation, the lower half consistently had higher IP_3, and IP_3 levels on the lower side continued to increase up to ≈5-fold over basal levels before visible growth. As bending became visible after 8–10 h, IP_3 levels returned to basal values. Additionally, phosphatidylinositol 4-phosphate 5-kinase activity in the lower pulvinus half increased transiently within 10 min of gravistimulation, suggesting that the increased IP_3 production was accompanied by an up-regulation of phosphatidylinositol 4,5-bisphosphate biosynthesis. Neither IP_3 levels nor phosphatidylinositol 4-phosphate 5-kinase activity changed in pulvini halves from vertical control plants. Our data indicate the involvement of IP_3 and inositol phospholipids in both short- and long-term responses to gravistimulation. As a diffusible second messenger, IP_3 provides a mechanism to transmit and amplify the signal from the perceiving to the responding cells in the pulvinus, coordinating a synchronized growth response.**

The constant and continuous vector of gravitational force is an important environmental cue governing the orientation of plant growth. In response to changes in their spatial orientation, plants exhibit differential growth to reorient relative to the gravity vector. When a plant is placed horizontally, roots and shoots exhibit asymmetric growth resulting in downward and upward curvature, respectively. Gravitropic responses of plants are mediated by a cascade of biophysical and biochemical events. The sedimentation of amyloplasts in starch-containing cells is the earliest event recorded so far and can occur within seconds to minutes of gravistimulation (1, 2). The settling of amyloplasts is thought to trigger intra- and intercellular signaling, initiating downstream metabolic changes and involving an asymmetric distribution of auxin, which results in asymmetric growth (2, 3). Although the gravitropic response of plants has been investigated in detail, the biochemical components of the gravity signal transduction cascade are not well characterized.

The role of Ca^{2+} in gravitropic signaling is the subject of much debate (for review, see refs. 4 and 5). Ca^{2+} and calmodulin are discussed as important mediators of gravitropic signaling (6, 7); however, the direct measurement of rapid changes in cytosolic Ca^{2+} during gravistimulation is technically challenging and only a few studies have been carried out. Gehring *et al.* (8) reported an increase in the intracellular concentration of Ca ($[Ca]_i$) within the first 10 min of gravistimulation in maize coleoptiles. In contrast, Legué *et al.* (9) were unable to detect changes in $[Ca]_i$ within the first minute or during the first few hours of gravistimulation of intact *Arabidopsis* roots.

The redistribution of cell-wall Ca^{2+} has been implicated as a crucial step in the plant gravitropic response (10, 11). In addition, animal studies have shown Ca^{2+} mobilization and loss of Ca^{2+} from bone in response to microgravity during space flight (12). Clearly, Ca^{2+} homeostasis in both plants and animals is profoundly affected during gravistimulation, but it has been difficult to delineate the sequence of events and the role of Ca^{2+}.

Changes in Ca^{2+} homeostasis can be mediated by upstream signaling molecules such as the phospholipid-derived second messenger inositol 1,4,5-trisphosphate (IP_3). IP_3 is one of the best-characterized affectors of intracellular Ca^{2+} release (13, 14). The involvement of IP_3 in a gravisignaling cascade would support a role for Ca^{2+} in the transduction or amplification of the signal. The ability of a cell to produce IP_3 signals is linked to the metabolism of phosphatidylinositol polyphosphates. The inositol phospholipid (PI) pathway has been implicated in the early responses of plants to external stimuli (for review, see ref. 15) such as light (16), osmotic stress (17), and fungal elicitors (18). However, to date, the involvement of phosphatidylinositol polyphosphates in the perception of gravity has not been established.

We have investigated the involvement of the PI pathway in gravisignaling by using the maize internodal pulvinus as a model system. By comparing the "upper" and "lower" pulvinus halves of gravistimulated plants, we have attempted to determine whether there are rapid changes in PI metabolism that could be indicative of initial signaling events. Furthermore, because the gravitropic bending response is elicited only after a critical period of gravistimulation, we wanted to determine whether there was evidence for persisting or repetitive biochemical changes in response to gravistimulation. Using the maize pulvinus, we show both transient and prolonged changes in IP_3 before visible growth, along with accompanying changes in phosphatidylinositol 4,5-bisphosphate (PIP_2) biosynthesis. Our data indicate a role for PI metabolism and PIP_2-mediated signal transduction in both graviperception and response.

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Abbreviations: IP_3, inositol 1,4,5-trisphosphate; PIP, phosphatidylinositol 4-phosphate; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI, inositol phospholipid.

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MATERIALS AND METHODS

Plant Material. Maize (*Zea mays* L. cv. Pioneer 3183) plants were grown in soil in 20-cm pots (four plants per pot) in a greenhouse and fertilized with a modified Hoagland's solution three times weekly. Experiments were carried out with the P2 pulvinus (the first pulvinus above the soil line) of 6-week-old maize plants. Care was taken to minimize handling and movement of the plants. Control plants were kept vertical and the pulvinus was harvested by cutting the tissue into halves along a random plane. For gravistimulation experiments, plants were gravistimulated for the indicated times by placing the pots horizontally. The pulvini were dissected into upper and lower halves while maintaining a horizontal orientation. For plasma membrane preparations, eight pulvini were harvested for each time point and the tissue was pooled. For IP_3 assays, a single pulvinus was harvested for each time point and the dissected halves were frozen immediately in liquid N_2 until assayed. To eliminate differences in irradiance between the upper and lower sides of the plants, gravistimulation time courses were carried out at night without artificial white light. A green safe light was used during the harvesting.

Isolation of Plasma Membranes. Pulvinus tissue was chopped on ice and then ground in 3 vol of ice-cold buffer (250 mM sucrose/3 mM EDTA/2 mM EGTA/14 mM 2-mercaptoethanol/2 mM DTT/30 mM Tris·HCl, pH 7.4) with 0.1 g of cross-linked polyvinyl polypyrrolidone for four 20-s periods in a Virtis homogenizer. The homogenate was filtered through four layers of cheesecloth and centrifuged at 5,000 × *g* for 10 min to clarify the extract. The supernatant was centrifuged at 40,000 × *g* for 60 min to pellet the microsomes. The microsomal pellet was resuspended in 1.5 ml of grinding buffer and layered on a 6.3% PEG/dextran polymer two-phase gradient to separate the plasma membranes (19). The gradients were mixed by inversion (80 times) and centrifuged at ≈600 × *g* in the cold for 10 min. The upper phase was removed, diluted to 30 ml with grinding buffer, and centrifuged at 40,000 × *g* for 1 h at 4°C. The pellet was washed in 30 mM Tris·HCl, pH 7.4/15 mM $MgCl_2$, centrifuged at 40,000 × *g* for 45 min, and washed twice with the same buffer. The membranes were resuspended in 30 mM Tris·HCl, pH 7.4/15 mM $MgCl_2$. Protein concentrations were determined by the Bradford method (Bio-Rad) with BSA as a standard.

Lipid Kinase Assays. Phosphatidylinositol 4-phosphate (PIP) 5-kinase activity was assayed as described (20) by using 1.5 μg of plasma membrane protein per assay in a phosphorylation reaction mixture containing 30 mM Tris·HCl, pH 7.5/7.5 mM $MgCl_2$/1 mM sodium molybdate/0.01% Triton X-100/0.9 mM [γ-^{32}P]ATP (0.2 μCi/nmol; 1 Ci = 37 GBq). Reactions were incubated for 10 min at room temperature. For assays containing exogenous substrate, PIP presolubilized in 2% Triton X-100 was added to a final concentration of 25 μg of lipid in 0.2% Triton X-100 per reaction. After incubation the PIs were extracted by using an acidic $CHCl_3$/MeOH extraction method (21). Lipids were separated by TLC on LK5D silica gel plates (Whatman) using a $CHCl_3$/MeOH/NH_4OH/H_2O, 86:76:6:16 (vol/vol) solvent system. The ^{32}P-labeled phospholipids were quantified with a Bioscan System 500 Imaging scanner.

IP_3 Assays. After gravistimulation for the indicated times, maize pulvini (upper and lower halves) were harvested and immediately frozen in liquid N_2. The tissue was ground to a fine powder in liquid N_2 and added to a preweighed tube containing 500 μl of ice-cold 20% perchloric acid. After a 20-min incubation on ice, precipitated proteins were pelleted by centrifugation at 4°C for 15 min at 2,000 × *g*. The supernatant was transferred to a clean tube and adjusted to pH 7.5 with 1.5 M KOH/60 mM Hepes buffer containing universal pH indicator dye (0.5 ml/10 ml of buffer; Fisher). The neutralized samples were assayed for IP_3 content with the [^{3}H]IP_3 receptor binding assay system (Amersham Life Science). Assays were carried out along with controls for complete and nonspecific binding according to the manufacturer's instructions by using 50 μl of sample per assay. The IP_3 content of each sample was determined by interpolation from a standard curve generated with commercial IP_3.

Purification of Recombinant Inositol Polyphosphate 5-Phosphatase Type I. To rule out the possibility that soluble inositol phosphates other than IP_3 were interfering with the IP_3 receptor binding assay, maize samples and commercial IP_3 were pretreated with a recombinant inositol polyphosphate 5-phosphatase. The inositol polyphosphate 5-phosphatase enzymes hydrolyze D-*myo*-inositol 1,4,5-trisphosphate in a signal-terminating reaction (22). A recombinant human inositol polyphosphate 5-phosphatase I clone was a gift from Phil Majerus (23). The recombinant protein was induced for 3 h by the addition of isopropyl β-D-thiogalactoside (0.5 mM, final concentration). Bacterial cells expressing the His-tagged phosphatase were lysed by sonication and resuspended in 50 mM sodium phosphate/300 mM NaCl, pH 8. The recombinant protein was purified by metal affinity chromatography on a nickel nitrilotriacetate resin (Qiagen, Chatsworth, CA) and eluted from the column with the same buffer containing 50–500 mM imidazole. The activity of the purified column fractions was tested on commercially available IP_3. IP_3-containing samples from maize pulvinus tissue (lower half, gravistimulated for 10 s) and commercial IP_3 were preincubated for various times at room temperature with a recombinant inositol polyphosphate 5-phosphatase I or with a phosphatase sample that was boiled for 10 min and then assayed for IP_3 content. In the absence of phosphatase, the commercial IP_3 sample contained ≈100 pmol of IP_3 per assay and the maize sample contained 10.5 pmol of IP_3 per assay. After the phosphatase treatment, almost no IP_3 could be detected by the IP_3 receptor binding assay in both maize samples and commercial IP_3 (0.38 pmol per assay and 0.18 pmol per assay, respectively) Pretreatment with the boiled phosphatase enzyme had no effect. The results confirm the specificity of the assay for D-*myo*-inositol 1,4,5-trisphosphate.

RESULTS

The Pulvini of Gravistimulated Maize Stems Reach Maximal Gravitropic Upward Curvature Within 48 h. Gravitropic bending of mature maize plants occurs in the internodal pulvinus, a nongrowing tissue that responds to gravistimulation with differential cell elongation on the lower side. The internodal or stem pulvinus is a disc-shaped region of cells located at the base of the internode above the node (24, 25). The most responsive pulvini are the first and second above the soil line, denoted P2 and P3 (24). The bending response after gravistimulation was first visible after 8–10 h.

IP_3 Levels in the Nonstimulated Pulvinus. To elucidate the role of phosphatidylinositol polyphosphates in gravisensing, we first measured *in vivo* levels of IP_3 in control maize stems and pulvini. IP_3 levels in nonstimulated pulvinus tissue were consistently 2- to 3-fold higher than those of internodal stem tissue (Table 1), indicating that basal PIP_2 turnover was more rapid in the pulvinus tissue. In nonstimulated plants, basal IP_3 was uniformly distributed in the tissue and there were no significant differences in IP_3 levels between randomly cut left and right halves of either pulvinal or internodal tissue (Table 1).

Changes in IP_3 Levels Were Evident Within 10 s of Gravistimulation in the Lower Half of the Pulvinus. A key question of our investigation was, how soon after the application of the gravistimulus did the signaling events occur, and therefore, we measured IP_3 levels in samples taken from upper and lower halves of maize pulvini within the first few minutes of gravistimulation. When maize plants were placed horizontally,

Table 1. IP_3 levels in internode and pulvinus

Location	IP_3 content of vertical control plants pmol/g (fresh weight)	
	Left	Right
Internode	141 ± 10	139 ± 10
Pulvinus (t = 0)	276 ± 20	263 ± 20
Pulvinus (t = 2h)	256 ± 20	245 ± 20

IP_3 levels were measured in internodal tissue and over a 2-h period in nonstimulated pulvinus tissue. The tissue was randomly cut into left and right halves, and the IP_3 levels of both halves were assayed separately. The data are the average values (± range) from three experiments assayed in duplicate.

within 10 s there was a transient 5- to 6-fold increase in IP_3 levels only in the lower half of the gravistimulated pulvinus, indicating that by this time the cells on the lower side were biochemically distinct (Fig. 1). Although the basal levels of IP_3 varied with different sets of plants and between experiments [from 300 ± 75 pmol/g (fresh weight) to 1200 ± 150 pmol/g (fresh weight)], the rapid 5- to 6-fold increase was reproducible in five of five experiments.

When measurements in upper and lower halves were continued at 30-s intervals over the first 2 min of stimulation, the values fluctuated asynchronously, suggesting an oscillatory pattern (Fig. 1). The period of rapid oscillatory changes was difficult to document because we could not harvest the plants at sufficiently short time intervals and the oscillation will be defined in part by the sampling time. Extensive sampling, however, revealed that fluctuations in IP_3 levels similar to those shown in Fig. 1 continued with a phase of ≈90 s in upper and lower halves at least for 30 min, indicating that the horizontal orientation was a continuous stimulus over this time (data not shown). Importantly, IP_3 levels in the left and right halves of the vertical control pulvini did not vary over the experimental time period (Table 1).

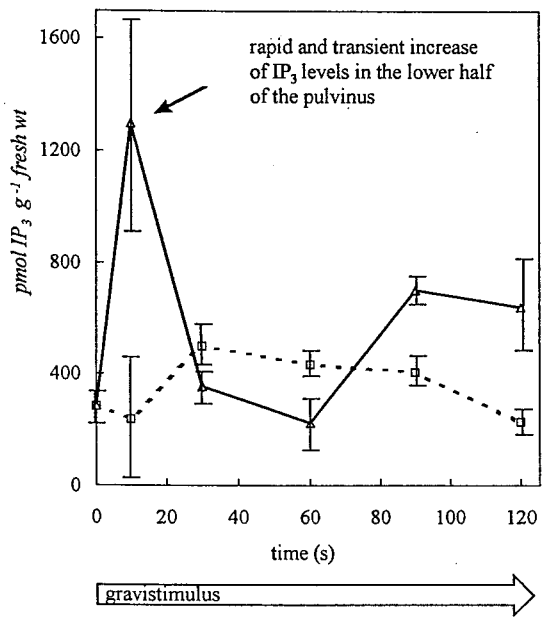

FIG. 1. Within 10 s of gravistimulation, IP_3 levels increased 5-fold in the lower half of the pulvinus. IP_3 levels were measured in upper (dashed lines) and lower halves (solid lines) of gravistimulated maize pulvini at the times indicated. No significant difference in IP_3 levels could be detected between pulvinus halves from vertical control plants, as indicated in Table 1. The points represent the average of values from three experiments, assayed in duplicate. Vertical bars indicate the range. Basal IP_3 values were between 250 and 300 pmol/g (fresh weight).

PIP_2 Biosynthesis Changes During Gravistimulation. To determine whether there was a change in PI biosynthesis during gravistimulation, we examined the *in vitro* phosphorylation rates of PIP to PIP_2 in plasma membranes of vertical and gravistimulated maize pulvini. We measured the activity of PIP 5-kinase in plasma membranes prepared from upper and lower halves of maize pulvini over the first 2 h of gravistimulation. The assays were performed in the absence and in the presence of excess phosphorylation substrate to elucidate changes in specific enzyme activities. Representative data from one experiment where plants were gravistimulated and harvested in the dark are shown in Fig. 2. In five of five experiments, after 10 min of gravistimulation there was 30% more PIP_2 formed by plasma membranes isolated from the lower half of the pulvinus than from the upper. Increases and decreases in PIP 5-kinase activity fluctuated between upper and lower halves of the pulvinus over the first 2 h of gravistimulation (Fig. 2*A*). When assays were carried out in the

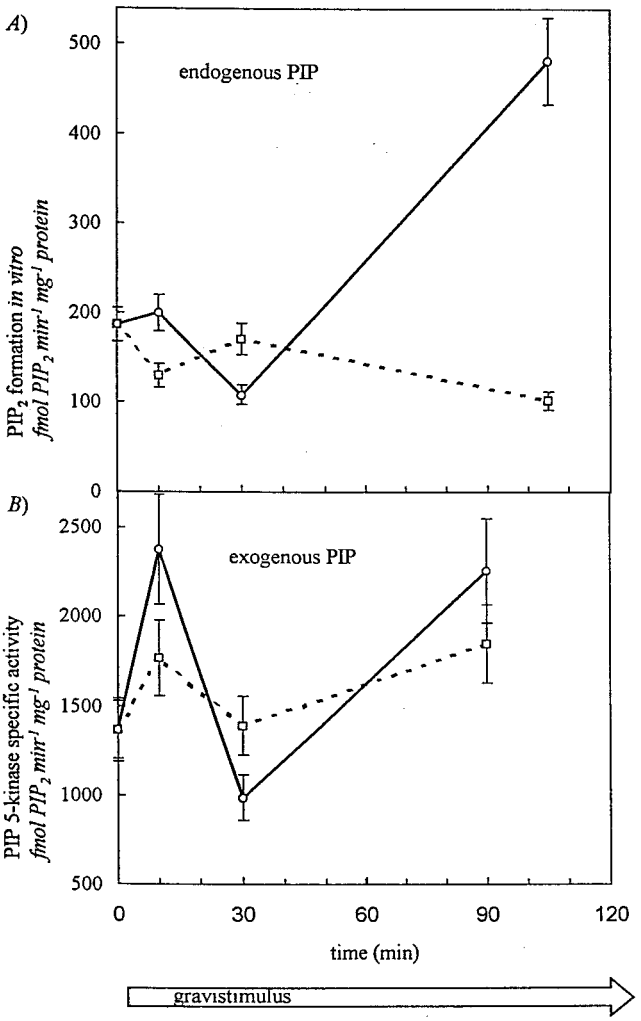

FIG. 2. Synthesis of PIP_2 and the specific activity of PIP 5-kinase changed during gravistimulation. Plasma membranes were prepared from upper and lower halves of pulvinus tissue at the indicated times of gravistimulation and were assayed *in vitro* for PIP_2 formation from endogenous (*A*) or exogenous (*B*) substrate. Data are from one representative experiment where plants were stimulated and harvested in the dark. Similar fluctuations were obtained when plants were stimulated and harvested in the light. Dotted line, upper half of pulvinus; solid line, lower half of pulvinus. Each data point represents the average from duplicate values.

presence of added PIP, similar changes in phosphorylation of PIP, as shown in Fig. 2*A,* were detected (Fig. 2*B*), indicating that the PIP 5-kinase specific activity in the plasma membrane was changing. The described changes in PIP 5-kinase activity between upper and lower half of the pulvinus were observed irrespective of whether plants were gravistimulated and harvested in the light or in the dark. Importantly, we could not observe significant differences in PIP 5-kinase activities between pulvinus halves from vertical control plants, either with endogenous or exogenous substrate and taken at different times, ruling out the possibility of internal fluctuations during a 2-h experiment (Table 2).

A Presentation Time Between 2 and 4 h of Gravistimulation Is Necessary to Induce a Bending Response in Maize Plants. To investigate whether there was a correlation between the stimulation time needed for the cells to make a commitment to elongate and IP_3 levels, we first analyzed the time of gravistimulation necessary to induce bending (presentation time). Plants were gravistimulated for 1, 2, 4, and 6 h and then placed back upright. After 48 h, the angle of bending was measured in these plants and in both continuously vertical and continuously gravistimulated plants. Plants placed horizontally for only 1 or 2 h and then returned to vertical did not show a bending response. Plants stimulated for 4 h or longer exhibited significant gravitropic bending after 48 h (Fig. 3). The minimum time of gravistimulation required to trigger the bending response was thus determined to be between 2 and 4 h. Within this critical time period the lower side of the pulvinus had committed to elongate and would start to extend even when the plants were returned to vertical.

A Gradual Sustained Increase in IP_3 Levels Is Evident in the Lower Pulvinus Half Before Visible Elongation. Because the minimum time of gravistimulation necessary to invoke a bending response was between 2 and 4 h (Fig. 3), we investigated whether changes in IP_3 levels occurred in this time period in correlation with the establishment of differential growth. Plants were gravistimulated for 1 h, 4.5 h, or continuously, and pulvinus tissue was harvested at 2, 3, 5.5, 7, and 10 h. Plants stimulated for only 1 h showed an initial increase in IP_3; however, by 3 h IP_3 levels had dropped and the levels of IP_3 between the upper and lower halves in these plants did not differ significantly from each other or from the vertical controls (Fig. 4). For plants gravistimulated for 4.5 h or continuously, IP_3 levels on the lower halves of the pulvinus gradually increased during gravistimulation to 5- to 6-fold over the basal levels (Fig. 4). IP_3 levels in the upper halves of the pulvini did not increase more than 2-fold during stimulation, indicating a distinctly different response in the upper and lower half. When plants were returned to vertical after 4.5 h of stimulation, IP_3 levels in the lower pulvinus half dropped to basal levels within another 2 h. Interestingly, IP_3 levels decreased after 7 h (before visible bending) even in the lower pulvinus halves of plants that remained horizontal. The sustained increase in IP_3 was only observed in the lower half when the cells were committed to elongate and IP_3 levels decreased before growth. In addition to the changes in IP_3, the production of PIP_2 on the lower pulvinus half was increased between 4 and 8 h of gravistimulation (Fig. 5). Assays performed with (data not shown) and without added lipid phosphorylation substrate (Fig. 5) showed this increase, indicating that both phosphatidylinositol polyphosphate levels and the specific activity of PIP 5-kinase were up-regulated in the lower half after 4 h of gravistimulation. The lipid kinase assays were carried out *in vitro,* and therefore, it is difficult to directly compare increases in PIP_2 with the increases in IP_3 measured *in vivo*. However, the up-regulation of PIP_2 synthesis, and its timing, are consistent with and could contribute to the sustained increase in IP_3.

Table 2. PIP_2 formation in plasma membranes from vertical control plants

Time, min	Ratio of PIP_2 formed in left and right halves	
	From endogenous substrate	From exogenous substrate
0	1.08 ± 0.07	1.01 ± 0.05
45	1.00 ± 0.05	0.97 ± 0.06
130	1.05 ± 0.07	0.96 ± 0.08

Plasma membranes were isolated and analyzed for *in vitro* PIP_2 formation from endogenous and exogenous substrate. Pulvinus tissue was harvested from vertical control plants as indicated over a period of about 2 h. Pulvini from eight plants were cut in halves on a random plane and designated left and right. The ratio of the enzyme activities from the left and right halves was calculated. Basal PIP_2 formed in a representative experiment was 3.9 pmol per min per mg of protein, 3.7 pmol per min per mg of protein, and 4.1 pmol per min per mg of protein for 0, 45, and 130 min, respectively. Data are the average ± range.

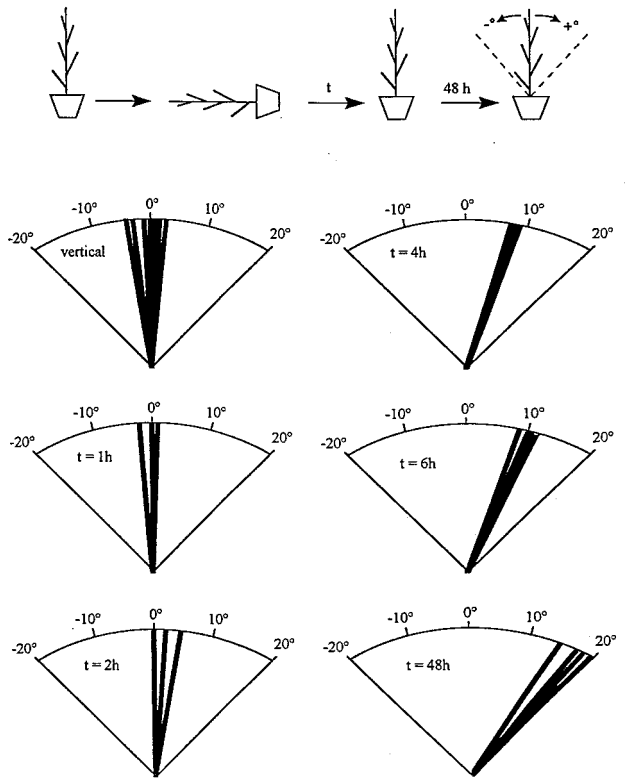

FIG. 3. The gravistimulus had to be presented for 2–4 h to initiate the bending response. Vertically grown plants were gravistimulated horizontally for the times indicated and then returned to a vertical orientation. After 48 h, the bending response was measured as the angle deviated from the vertical. Significant bending from the vertical occurred in plants that had been gravistimulated for 4 h or more. Positive angles indicate negative gravitropic bending. Data are from five plants for each treatment; where values overlap only a single line is drawn.

DISCUSSION

All cells in a plant are subject to the same gravitational force. Therefore, to induce a differential growth response, mechanisms are needed in a tissue to establish an initial asymmetry in graviperception and in the transduction of the signal. Our data suggest that changes in the metabolism of phosphatidylinositol polyphosphates are involved in the initial signaling events and in the ensuing process of commitment to cell elongation.

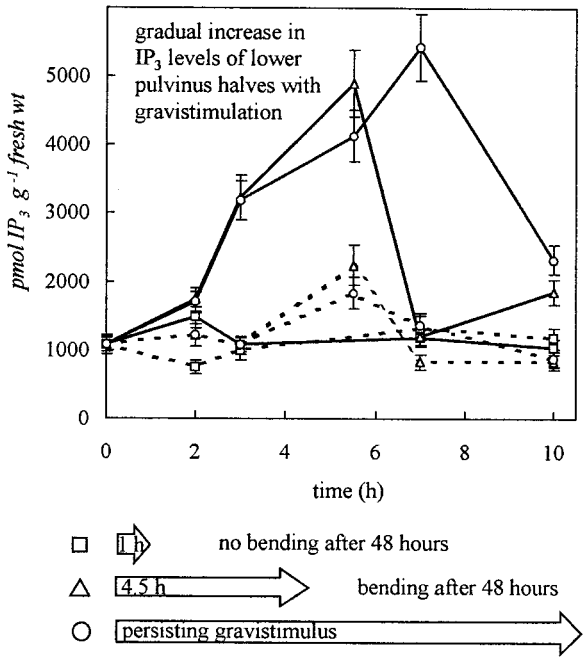

FIG. 4. Levels of IP_3 gradually increased in the lower half of the maize pulvinus between 3 and 7 h of gravistimulation. IP_3 levels were determined in upper and lower halves of pulvini from plants gravistimulated for 1 h (squares), 4.5 h (triangles), or permanently (circles). Only plants gravistimulated for longer than 4 h showed a bending response after 48 h. IP_3 levels in vertical control plants were 1,200 ± 150 pmol/g (fresh weight), and there were no significant differences between randomly cut pulvinus halves. The values plotted are the average of two experiments assayed in duplicate. Vertical bars indicate the range.

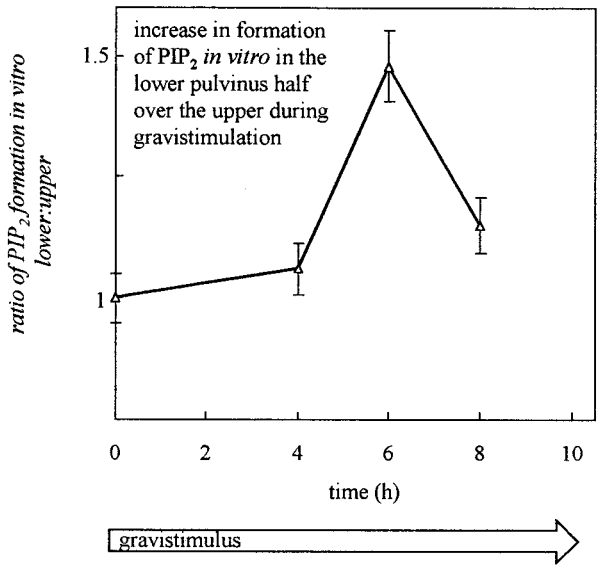

FIG. 5. Formation of PIP_2, the lipid precursor of IP_3, increased in plasma membranes of lower halves of maize pulvini with the time of gravistimulation. Phosphorylation of endogenous PIP was measured in plasma membranes prepared from upper and lower halves of gravistimulated maize pulvini at the times indicated. The ratio of lower value/upper value for each time point was plotted. Basal PIP_2 formation in vertical plants was 680 ± 30 fmol per min per mg of protein. The data are from one representative experiment assayed in duplicate. Three experiments gave similar results.

Within 10 s of gravistimulation, there was a rapid and transient increase in IP_3 in the lower half of the maize pulvinus. To our knowledge, this is one of the earliest metabolic events in plant gravitropic signaling documented thus far. Repetitive fluctuations in IP_3 levels with a phase of 60–90 s were documented for the first 2 min and persisted after 30 min of gravistimulation, suggesting ongoing oscillations of IP_3.

In response to gravistimulation, metabolite levels of the PI pathway changed as well as apparent specific enzyme activities in the plasma membrane. We could detect alternating fluctuations in PIP 5-kinase activity in the upper and the lower pulvinus halves that persisted for at least 2 h. The changes in PIP 5-kinase-specific activity may be mediated by phosphorylation (26, 27) and/or translocation to the membrane (28). Winter *et al.* (29) have shown that in maize pulvini the membrane association of sucrose synthase changes in response to gravistimulation possibly by changes in the phosphorylation state of the enzyme. Our data indicate that the metabolite flux through the PI pathway (i.e., PIP_2 turnover) is altered. Repeated depletion and replenishing of the PIP_2 signaling pool could account for the changes in IP_3 and PIs that we observe. Because of the time required to harvest sufficient tissue to isolate plasma membranes, we could not determine, whether PIP 5-kinase activity changes preceded the 10-s changes in IP_3 upon stimulation, which would suggest a low PIP_2 signaling pool as described for some model systems (30, 31), or whether the changes in PIP 5-kinase activity were a consequence of a depletion of the PIP_2 signaling pools.

In addition to the rapid initial transient changes in IP_3, we measured a gradual sustained increase in IP_3 up to 6-fold over control values in lower halves of pulvini over 3–7 h of gravistimulation. This sustained increase in IP_3 was only detected in plants gravistimulated for 4 h or more. In plants gravistimulated for 1 h, there was no significant sustained increase in IP_3 levels. Because the time period between 2 and 4 h appears to be crucial for establishing the differential growth, these data suggest that IP_3 levels have to build up to a certain threshold intensity before the cells are committed to elongate. Consistent with the increase in IP_3, we measured an up-regulation of PIP_2 synthesis *in vitro* over this time.

The rapidity of the initial increase in IP_3 seen only in the lower half of the pulvinus suggests a preexisting asymmetry in the gravisensing or the transduction of the signal as part of a mechanism to induce differential growth. Asymmetries in the sensory machinery could be established on a cellular level by the vectorial distribution of ion channels or an asymmetric localization or opening state of plasmodesmata. Although the cellular sensors of the statolith signal have not yet been identified, the fact that IP_3 levels decreased when gravistimulated plants were returned to vertical before growth implies that the asymmetry of the sensors was maintained during gravistimulation.

Although starch-containing cells in the maize stem are restricted to the pulvinus (24, 25), not all cells in the pulvinus contain amyloplasts and would be able to sense gravity. However, all cells in the tissue respond to gravistimulation with a concerted cell elongation. This suggests the need for a mechanism of cell–cell communication to synchronize and coordinate the growth response. IP_3 could be part of both the initiation and propagation of a signal from perceiving to responding cells (32). If IP_3 from each cell was transmitted vectorially from the upper to the lower pulvinus half, this could contribute to the initial increase in IP_3 in the lower half. With time, the constant stimulus imparted on the horizontal tissue would lead to an up-regulation of PIP_2 biosynthesis and a sustained increase in IP_3 on the lower side that could initiate or coordinate the events preceding cell elongation.

Ca^{2+} signals are pivotal in initiating many developmental processes in plant and animal cells (33), and several recent studies highlight the role of IP_3-mediated Ca^{2+} pulses in the regulation of downstream targets (34, 35). Key events in the animal cell division cycle are triggered by Ca^{2+} transients,

which in turn are preceded by cyclic changes in IP_3 levels (36). In *Xenopus* (37) and zebrafish (38) embryos, the IP_3–Ca^{2+} signaling system has been implicated in transducing signals between the dorsal and ventral side during dorsoventral specification. Similarly in plants pollen tube growth is regulated by a slow wave of Ca^{2+}, which is initiated and propagated in part by IP_3 (39). Furthermore, microinjected IP_3 has been shown to cause an increase in cytoplasmic Ca^{2+} in all plant cells studied (32, 39, 40). In light of these reports, our discovery of short- and long-term changes in IP_3 has profound implications on the role of Ca^{2+} in graviperception and response.

In summary, we suggest a short- and long-term involvement of IP_3 signaling in the graviperception of maize. A combined mechanism like this would enable the plant to distinguish between short or transient movements caused by wind, on one hand, and complete dislodging, as might be caused by heavy rain, on the other. The initial IP_3 spike would serve as an initiation signal and the gradual increase in IP_3 levels over several hours could be part of the fixation process for the orientation of differential growth. In the described scenario, cytosolic IP_3 oscillations could activate multiple metabolic processes, which in the maize pulvinus may mediate the shift in the metabolic state from a resting to an elongating cell.

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1. Sack, F. D. (1991) *Int. Rev. Cytol.* **127,** 193–252.
2. Kaufman, P. B., Wu, L.-L., Brock, T. G. & Kim, D. (1995) in *Plant Hormones,* ed. Davies, P. J. (Kluwer, Boston), pp. 547–571.
3. Lomax, T. L., Muday, G. K. & Rubery, P. H. (1995) in *Plant Hormones,* ed. Davies, P. J. (Kluwer, Boston), pp. 509–530.
4. Belyavskaya, N. A. (1996) *Int. Rev. Cytol.* **168,** 123–185.
5. Sinclair, W. & Trewavas, A. J. (1997) *Planta* **203,** S85–S90.
6. Steinmetz, C. L., Kuzmanoff, K. M., Evans, M. L. & Jarret, A. J. (1987) *Plant Physiol.* **84,** 1337–1342.
7. Sinclair, W., Oliver, I., Maher, P. & Trewavas, A. J. (1996) *Planta* **199,** 343–351.
8. Gehring, C. A., Williams, D. A., Cody, S. H. & Parish, R. W. (1990) *Nature (London)* **345,** 528–530.
9. Legué, V., Blancaflor, E., Wymer, C., Perbal, G., Fantin, D. & Gilroy, S. (1997) *Plant Physiol.* **114,** 789–800.
10. Slocum, R. D. & Roux, S. J. (1983) *Planta* **157,** 481–492.
11. Bjorkman, T. & Cleland, R. E. (1991) *Planta* **185,** 379–384.
12. Leach, C. S., Cintron, N. & Krauhs, J. M. (1991) *J. Clin. Pharmacol.* **31,** 921–927.
13. Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **341,** 197–205.
14. Berridge, M. J. (1993) *Nature (London)* **361,** 315–325.
15. Drøbak, B. K. (1992) *Biochem. J.* **288,** 697–712.
16. Morse, M. J., Crain, R. C. & Satter, R. L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 7075–7078.
17. Cho, M. H., Shears, S. B. & Boss, W. F. (1993) *Plant Physiol.* **103,** 637–647.
18. Kurosaki, F., Tsurusawa, Y. & Nishi, A. (1987) *Plant Physiol.* **85,** 601–604.
19. Wheeler, J. J. & Boss, W. F. (1987) *Plant Physiol.* **85,** 389–392.
20. Cho, M. H. & Boss, W. F. (1995) in *Methods in Cell Biology,* eds. Galbraith, D. W., Bohnert, H. J. & Bourque, D. P. (Academic, San Diego), Vol. 49, pp. 543–554.
21. Cho, M. H., Chen, Q., Okpodu, C. M. & Boss, W. F. (1992) *LC–GC* **10**, 464–468.
22. Laxminarayan, K. M., Chan, B. K., Tetaz, T., Bird, P. I. & Mitchell, C. A. (1994) *J. Biol. Chem.* **269,** 17305–17310.
23. Auethavekiat, V., Abrams, C. S. & Majerus, P. W. (1997) *J. Biol. Chem.* **272,** 1786–1790.
24. Collings, D. A., Winter, H., Wyatt, S. E. & Allen, N. S. (1998) *Planta* **207,** 246–258.
25. Dayanandan, P., Hebard, F. V., Baldwin, V. D. & Kaufman, P. B. (1977) *Am. J. Bot.* **64,** 1189–1199.
26. Hinchliffe, K. A., Irvine, R. F. & Divecha, N. (1998) *Biochem. J.* **329,** 115–119.
27. Itoh, T., Ijuin, T. & Takenawa, T. (1998*) J. Biol. Chem.* **273**, 20292–20299.
28. Oancea, E., Teruel, M. N., Quest, A. F. G. & Meyer, T. (1998) *J. Cell Biol.* **140,** 485–498.
29. Winter, H., Huber, J. L. & Huber, S. C. (1997) *FEBS Lett.* **420,** 151–155.
30. Lassing, I. & Lindberg, U. (1990) *FEBS Lett.* **262,** 231–233.
31. Heilmann, I., Perera, I. Y., Gross, W. & Boss, W. F. (1999) *Plant Physiol.,* in press.
32. Tucker, E. B. & Boss, W. F. (1996) *Plant Physiol.* **111,** 459–467.
33. Berridge, M. J., Bootman, M. D. & Lipp, P. (1998) *Nature (London)* **395,** 645–648.
34. Li, W., Llopis, J., Whitney, M., Zlokarnik, G. & Tsien, R. Y. (1998) *Nature (London)* **392,** 936–941.
35. Dolmetsch, R. E., Lewis, R. S., Goodnow, C. C. & Healy, J. I. (1997) *Nature (London)* **386,** 855–858.
36. Clapa, B., Pesando, D., Wilding, M. & Whitaker, M. (1994) *Nature (London)* **368,** 875–878.
37. Kume, S., Muto, A., Inoue, T., Suga, K., Okano, H. & Mikoshiba, K. (1997) *Science* **278,** 1940–1943.
38. Ault, K. T., Durmowicz, G., Galione, A., Harger, P. L. & Busa, W. B. (1996) *Development (Cambridge, U.K.)* **122,** 2033–2041.
39. Franklin-Tong, V. E., Drøbak, B. K., Allan, A. C., Watkins, P. A. C. & Trewavas, A. J. (1996) *Plant Cell* **8,** 1305–1321.
40. Gilroy, S., Read, N. D. & Trewavas, A. J. (1990) *Nature (London)* **346,** 769–771.