

Antagonists and inhibitors of calcium accumulation do not impair gravity perception though they adversely affect the gravitropic responses of *Coprinus cinereus* stipes

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Control of cell calcium accumulation has been implicated in the gravity perception mechanism of plants, but no attempt has ever been made to assess its role in fungal gravitropism. The possible role of Ca^{2+} in the gravitropic perception and/or response mechanism of *Coprinus cinereus* was examined by treating stipes with a Ca^{2+} channel blocker, verapamil; a Ca^{2+} ionophore, A23187; a Ca^{2+} chelator, BAPTA; or calmidazolium, an inhibitor of calmodulin-mediated Ca^{2+} uptake using concentrations and treatments known to eliminate gravitropism and other tropisms in plant organs. The inhibitors had no effect on gravity perception, but the ionophore (which enhanced extension growth), the chelator and the calmodulin antagonist (which had no effect on the progress of stipe extension) all caused significantly diminished gravitropism. Verapamil caused both diminished gravitropism and decreased growth of the stipe. It is concluded that, under the conditions tested, Ca^{2+} is not involved in gravity perception by the *Coprinus* stipe, but does contribute to transduction of the gravitropic impulse. The results would be consistent with regulation of the gravitropic bending process requiring sequestration of Ca^{2+} within a membrane-bound compartment. The fact that the ionophore, A23187, enhanced stipe extension growth rate (by 30%) but decreased (by 43%) the rate of response, i.e. rate of bending, suggests that tropic bending may not result from a simple redistribution of the normal growth potential of the stipe.

Gravitropism is the bending response which, in plants and fungi alike, is usually assumed to be due to asymmetrical redistribution of growth potential in response to a change in the direction of the gravity vector (Barlow, 1989). Though representing different Kingdoms, comparison of gravitropism in the mushroom stipe with that which occurs in plant roots or stems is worthwhile, because the superficial structural similarities between such cylindrical organs may have led to parallel evolution of growth control strategies.

How gravity is perceived by plants is still controversial. The most commonly held theory is that perception results from displacement of a starch-containing organelle, the statolith, in perceptive plant tissues (Audus, 1962, 1979; Larsen, 1962, 1973; Wilkins, 1966, 1984; Evans, Moore & Hasenstein, 1986), but calcium seems to be central in coupling the initial gravitational stimulus to a cellular response. Several studies have revealed a polar calcium redistribution upon gravistimulation in plant roots and shoots (Acharya Goswami & Audus, 1976; Lee, Mulkey & Evans, 1983*a*; Roux, Biro & Hale, 1983; Slocum & Roux, 1983; Dauwalder, Roux & Rabenberg, 1985; Lee & Evans, 1985; Moore, 1985, 1986*a, b*; Migliaccio & Galston, 1987; Moore, Cameron & Smith, 1989; Björkman & Cleland, 1991) and calcium chelators reversibly eliminate gravitropism (Lee, Mulkey & Evans, 1983*b*; Daye, Biro & Roux, 1984; Poovaiah, McFadden & Reddy, 1987;

Migliaccio & Galston, 1989; Friedmann & Poovaiah, 1991). Direct measurements of calcium changes within plant cells have been impeded by the impermeability of plant cell walls, and only one study has demonstrated that cellular calcium actually increases upon gravistimulation (Gehring *et al.*, 1990). Several studies have shown that gravitropism in roots and shoots is also inhibited by calmodulin antagonists and antagonists to calmodulin-dependent enzymes (Biro *et al.*, 1982; Björkman & Leopold, 1987; Evans *et al.*, 1987; Poovaiah *et al.*, 1987; Stinemetz *et al.*, 1987). It has been suggested that calmodulin plays an intrinsic role specifically in gravity sensing (Björkman & Leopold, 1987) because of its influence on the gravisensing-dependent ion current in maize roots. Overall, it seems that gravity-stimulated redistribution of Ca^{2+} precedes tropic bending in plants and, at least in roots, the crucial step in transduction of the physical stimulus into a biochemical stimulus may be opening of stretch-activated ion channels to permit flow of Ca^{2+} across the plasmalemma (Evans *et al.*, 1986).

One of the mechanisms by which external stimuli can be transduced into a biological response is based on Ca^{2+} as an intracellular messenger (Carafoli, 1987; Poovaiah & Reddy, 1987; O'Day, 1991). Cytoplasmic Ca^{2+} is actively maintained at low levels by high-affinity Ca^{2+} -binding proteins, such as calmodulin (Cheung, 1980; Klee, Crouch & Richman, 1980; Cohen & Klee, 1988). Calcium enters cells by facilitated transport through Ca^{2+} channels (Carafoli, 1987; Janis, Silver & Triggler, 1987; Tsien & Tsien, 1990; O'Day, 1991). Once it

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is within the cytoplasm, Ca^{2+} is bound by calmodulin, whereupon a cascade of reactions may be initiated as the Ca^{2+} -calmodulin complex interacts with enzymes, such as protein kinases, phosphatases and phosphodiesterases (Cheung, 1980; Klee *et al.*, 1980; Cohen & Klee, 1988; O'Day, 1991), to bring about amplification of and response to the original stimulus. Calcium can be stored intracellularly in the endoplasmic reticulum, mitochondria, vacuoles and calciosomes (Koch, 1990; O'Day, 1991) and excess cytoplasmic Ca^{2+} can be removed by Ca^{2+} -ATPases or through $\text{Ca}^{2+}/\text{H}^{+}$ or $\text{Ca}^{2+}/\text{Na}^{+}$ antiports (Carafoli, 1987; Janis *et al.*, 1987; O'Day, 1991).

Many components of the calcium secondary messenger system have been identified in filamentous fungi (Tellez-Inon *et al.*, 1985; Janssens & Van Haastert, 1987; Schmid & Harold, 1988; St. Leger, Roberts & Staples, 1989, 1990; Miller, Vogg & Sanders, 1990; Hanson, 1991; Robson *et al.*, 1991a; Robson, Wiebe & Trinci, 1991b; Ulloa *et al.*, 1991). A few studies have shown that in fungi light may be transduced by a receptor/G-protein complex (Kozak & Ross, 1991) and that mechanosensitive ion channels, which direct mechanical or stress-elicited deformation by changing ion permeabilities, are present in *Phycomyces* and yeasts (Dennison & Roth, 1967; Shropshire & Lafay, 1987; Gustin *et al.*, 1988; Zhou *et al.*, 1991) yet little is known about how gravity is perceived, how the stimulus is transduced at the cellular level or how consequent cellular responses are mediated.

Some of the kinetics and mechanics of gravitropism of *C. cinereus* stipes have been investigated recently (Moore, 1991a, b; Hatton & Moore, 1992; Kher *et al.*, 1992). The gravitropic response is rapid, bending normally being evident within 25–30 min. The bend is initiated near the apex of the stipe and moves backwards towards the base as the stipe curves upwards. Approximately 90% of the bending impulse is compensated for (i.e. effectively reversed) as the response progresses, since the apical portion continually straightens out as it returns to the vertical. Bend compensation is dependent on the apex being free to move (Kher *et al.*, 1992; Greening, Holden & Moore, 1993), and while bending and bend compensation seem to be quite separate processes (Kher *et al.*, 1992), nothing is known about their mechanistic basis or how they are initiated or controlled.

In this study, some inhibitors have been assayed for their ability to disrupt the response to gravity in *C. cinereus*. We have not attempted a comprehensive survey but have used methods similar to those employed in studies done on plants, to test specifically for operation in stipes of sensing systems analogous to those which have been detected in plants, particularly maize roots.

Calmidazolium, an inhibitor of calmodulin-dependent Ca^{2+} uptake and Ca^{2+} -ATPase activity (Gietzen, Wuthrich & Bader, 1981; Tuana & MacLennan, 1984), was used to determine whether calmodulin has a role in gravity perception or response. The gravity-induced ionic current associated with gravity sensing in maize roots was eliminated in seedling root tips dipped in 100 μM calmidazolium for 10 min (Björkman & Leopold, 1987), this treatment blocking gravitropic curvature for 1 h.

BAPTA (1,2-bis(2-amino-5-nitro-phenoxy)ethane *N,N,N',N'*-

tetraacetic acid), a Ca^{2+} chelator which complexes Ca^{2+} into a readily diffusible extracellular complex, was used to determine whether external Ca^{2+} activity gradients were involved in gravitropism. Immersion of maize seedling root caps, but not the growing zone, in 10 mM BAPTA for 1 h prior to reorientation eliminated Ca^{2+} activity gradients associated with gravity transduction and resulted in no gravicurvature (Björkman & Cleland, 1991).

The calcium **ionophore A23187** was used to release Ca^{2+} from intracellular as well as extracellular stores (Poovaiah & Reddy, 1987; Raghobama *et al.*, 1987; Reddy *et al.*, 1987) and also eliminate the calcium activity gradient. Incubation of maize roots for 2 h in buffer containing 1 μM A23187 eliminated gravicurvature, which was only restored upon supplementation with Ca^{2+} (Poovaiah, *et al.*, 1987).

Verapamil, a Ca^{2+} channel blocker, was used to inhibit Ca^{2+} influx into cells (Janis *et al.*, 1987). Although verapamil has not been tested for its effects on gravitropism, it has been shown to inhibit other tropisms. Negative phototropism to blue light in the coenocytic alga *Vaucheria terrestris* was reversed by 2 μM verapamil in liquid culture (Kataoka, 1990); 10^{-4} – 10^{-6} M verapamil in the medium severely inhibited germination of *Vaucheria longicaulis* aplanospores and growth of germinated filaments (Oliveira, 1990); and 5×10^{-4} M and 10^{-3} M verapamil not only inhibited tip growth and reduced tip cell length in the moss *Funaria hygrometrica* but also caused developmental abnormalities (Wacker & Schnepf, 1990).

MATERIALS AND METHODS

Culture conditions

All experiments were performed with the 'Meathop' dikaryon of *Coprinus cinereus* (Schaeff.: Fr.) S. F. Gray, which was originally isolated from a dung heap in Lower Meathop Hill in Cumbria. The vegetative dikaryon was cultured on complete medium (Moore & Pukkila, 1985) in 9 cm Petri dishes in the dark at 37 °C for 3–4 d. Fruiting bodies were obtained by inoculating the dikaryon onto sterilized horse dung in crystallizing dishes, incubating at 37° for 3–4 d in the dark and then transferring the dung cultures to a 26–28° incubator with a 16 h light/8 h dark illumination cycle (white fluorescent lights, average illuminance 800 lx).

Inhibitor treatments

Fruit bodies of *C. cinereus* become sensitive to gravity after completion of meiosis (Kher *et al.*, 1992), so all tissue used in these experiments was taken from post-meiotic fruit bodies. Mushrooms were removed from culture, the cap tissue discarded and the stipes measured, being kept vertical throughout these procedures. Separation from the mycelium and removal of the cap constitute injuries. Although the stipe is not *dependent* on the presence of the cap and continues to elongate, intact fruit bodies elongate about 25% more than decapitated ones (Hammad *et al.*, 1993). However, excised and decapitated stipes provide for a simplified bioassay, and we consider our system to be analogous to an isolated muscle or nerve preparation a pharmacologist might use.

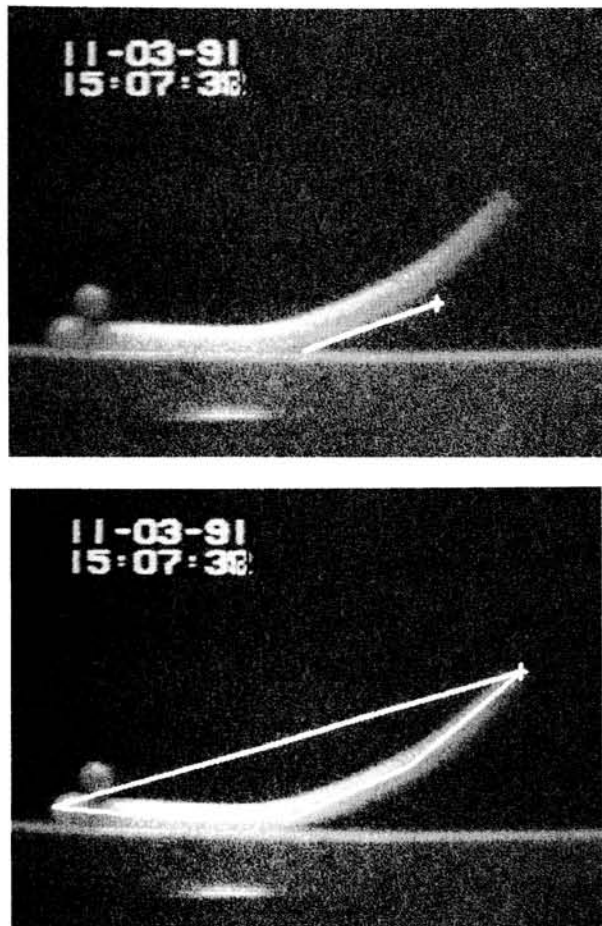


Fig. 1. Photographs of the computer monitor screen showing application of the Skye image analysis program to a digitized image of a gravitropically responding stipe. In the top panel the contact angle is being measured, in the bottom panel the axis length is being measured.

For exposure to the antagonists, stipes were incubated in the dark at room temperature (22–23°), in a vertical position, totally immersed in the following solutions: 1 μ M calcium ionophore A23187 for 2 h, 10 mM BAPTA for 2 h, 1 mM verapamil for 1 h, 100 μ M calmidazolium for 1 h. All inhibitors were dissolved in distilled water with the exception of BAPTA, which was dissolved in 0.1 N NaOH and adjusted to pH 5.5–6.0 by titration with HCl before use. Controls consisted of stipes incubated in distilled water for either 1 or 2 h. Chemicals were all obtained from Sigma Chemical Co.

Videoanalysis of gravitropism

After treatment, stipes were momentarily washed in distilled water (still kept vertical) and were then taped in a horizontal position to a platform in a humidity chamber (Kher *et al.*, 1992). Stipe responses were videotaped with a domestic-quality VHS camera and VTR. Video images were brought into an Opus PC computer using a Skye SI733 video digitizing card and Skye SI730 image analysis software (Skye Instruments Ltd, Unit 5, Ddole Industrial Estate, Llandrindod Wells, Powys LD1 6DF) (Fig. 1). The technique and the

parameters which are routinely measured were described and illustrated by Kher *et al.* (1992).

RESULTS AND DISCUSSION

A comparison between control and inhibitor-treated stipes revealed that while only verapamil, the Ca^{2+} channel blocker, significantly inhibited stipe extension (by 36%) at the concentration used, the Ca^{2+} ionophore significantly increased stipe extension by about the same amount (Table 1). Generally, stipes elongated by 15–30% during the course of the experiment. The standard deviations of the data in Table 1 at 20–40% were rather high, a feature evident in previous data (Kher *et al.*, 1992). For convenience of analysis, the gravitropic (bending) response of the stipe was divided into three aspects:

- the tip angle, being the angle made between the stipe tip and the horizontal;
- the contact angle, which is the angle between the lower surface of the stipe and the original horizontal support;
- bend movement.

The tip angle is a complex phenotype which represents the accumulated bending integrated with any bend compensation which has been invoked to constrain the tip towards the vertical (Kher *et al.*, 1992). Since the function of the gravitropic response is to restore a disoriented stipe apex to the vertical, the tip angle is a measure of the degree of completion of the biological response. The contact angle (estimated in these observations from digitized images, as illustrated in Fig. 1) is thought to represent a reasonably direct measure of the negatively gravitropic bending effort which underlies the gravitropic response of the stipe. Baseward bend movement is characteristic of the normal response (Kher *et al.*, 1992) and allows accumulation of bending potential to accelerate the response of the organ. Bend movement is always determined by measuring the distance between the base of the stipe and the position of the bend so that it is not complicated by the apical growth of the organ. Other measurements of gravitropism which have been suggested (e.g. Badham, 1984) are inapplicable, as they assume that all growth of the organ is curvature-related and that the gravitropic bend is symmetrical. Neither of these assumptions is supported by observation of our video records of *C. cinereus* (see illustrations in Kher *et al.*, 1992, Greening *et al.*, 1993). In categorizing the

Table 1. Extension rates of *Coprinus cinereus* stipes following treatment with inhibitors of calcium accumulation

Treatment	Stipe extension rate ($\mu\text{m min}^{-1} \pm \text{s.d.}$)
1 h treatments	
Water control	48.8 \pm 17.3 (n = 12)
Calmidazolium [100 μM]	53.8 \pm 23.1 (n = 12)
Verapamil [1 mM]	31.7 \pm 11.9 (n = 12)*
2 h treatments	
Water control	42.8 \pm 13.9 (n = 12)
Ionophore [1 μM A23187]	56.3 \pm 12.4 (n = 11)*
Chelator [10 mM BAPTA]	46.4 \pm 13.9 (n = 11)

* Significantly different ($P = 0.05$, analysis of variance) from corresponding control extension rate.

Table 2. Effect of inhibitor treatment on the angle of the tip of gravitropically stimulated stipes of *Coprinus cinereus*

Treatment	Reaction time (min)	Rate of response (degree min ⁻¹)	Final tip angle (degrees)
1 h treatments			
Water control	54.9 ± 31.1	0.49 ± 0.23	44.1 ± 21.1
Calmidazolium [100 µM]	50.4 ± 42.1	0.45 ± 0.21	35.1 ± 17.5
Verapamil [1 mM]	58.1 ± 38.7	0.39 ± 0.16	36.4 ± 19.2
2 h treatments			
Water control	50.3 ± 34.8	0.49 ± 0.15	37.7 ± 15.8
Ionophore [1 µM A23187]	73.4 ± 31.9	0.28 ± 0.11*	25.5 ± 10.8*
Chelator [10 mM BAPTA]	61.3 ± 36.8	0.21 ± 0.19*	18.8 ± 17.5*

Entries show the mean ± s.d., the number of replicates being the same as shown in Table 1. Asterisks identify values which are significantly different ($P = 0.05$, analysis of variance) from the corresponding water controls.

Table 3. Effect of inhibitor treatment on the contact angle of gravitropically stimulated stipes of *Coprinus cinereus*

Treatment	Reaction time (min)	Rate of response (degree min ⁻¹)	Final contact angle (degrees)
1 h treatments			
Water control	50.2 ± 29.7	0.43 ± 0.12	22.9 ± 4.1
Calmidazolium [100 µM]	52.9 ± 42.8	0.32 ± 0.13*	18.6 ± 3.7*
Verapamil [1 mM]	57.7 ± 37.5	0.31 ± 0.11*	18.4 ± 4.1*
2 h treatments			
Water control	44.7 ± 36.1	0.29 ± 0.13	21.1 ± 4.9
Ionophore [1 µM A23187]	70.8 ± 28.4	0.30 ± 0.13	16.7 ± 4.6*
Chelator [10 mM BAPTA]	58.7 ± 49.6	0.16 ± 0.12*	10.1 ± 6.1*

Entries show the mean ± s.d., the number of replicates being the same as shown in Table 1. Asterisks identify values which are significantly different ($P = 0.05$, analysis of variance) from the corresponding water controls.

effects of applied chemicals, the inhibitors were considered to be able to interfere with gravitropism if they significantly decreased the final tip or contact angle or if they interfered with bend movement. These data are summarized in Tables 2, 3 and 4.

The most important result is that none of these inhibitors eliminated the gravitropic response despite the fact that the treatment concentrations and conditions used were sufficient to eliminate tropisms in plant organs. This suggests that Ca²⁺ is not as directly involved in gravity sensing as it seems to be in plants.

Considering only statistically significant differences, both the Ca²⁺ chelator (BAPTA) and the ionophore A23187 reduced the rate of response of the stipe apex, causing a decrease in the final tip angle (Table 2). Calmidazolium and verapamil had no effect on the final tip angle and none of the inhibitors delayed the onset of bending, i.e. reaction times were unchanged.

The effects of inhibitor treatment on the contact angle are summarized in Table 3. All the inhibitors caused a significant decrease in the final contact angle, and all except the

ionophore caused a significant reduction in the rate of response.

The results of inhibitor treatment on bend movement (Table 4) reveal that only the Ca²⁺ chelator, BAPTA, and the ionophore A23187 significantly restricted bend movement towards the base of the stipe.

Under these experimental conditions, the inhibitors failed to delay or eliminate stipe gravicurvature as they have been reported to do in plant roots, yet they did influence the bending process. This suggests that while Ca²⁺ may not be involved in the initial steps of gravity sensing, an extracellular Ca²⁺ gradient and intracellular sequestration of Ca²⁺ are required for the bending response in *C. cinereus*. The Ca²⁺ chelator, BAPTA, disrupted gravitropic bending most noticeably, and also had a long-term effect on the curvature compensation process which brings stipes back to the vertical. Stipes treated as described here for 2 h with BAPTA before being placed horizontal had curled past the vertical after 18 h, while control stipes had returned to and remained at the vertical. Thus Ca²⁺ may also have a role in the curvature compensation mechanism and/or may be required to maintain the apical polarity which is so clearly expressed by this phenomenon (Kher *et al.*, 1992). Regulation of apical dominance by Ca²⁺ has been shown in filamentous hyphal growth of other fungi (Robson *et al.*, 1991*b*; Schmid & Harold, 1988; Yuan & Heath, 1991*a, b*), but this is the first report describing the possibility of its being involved in maintaining apical dominance in a differentiated multihyphal structure. The Ca²⁺ ionophore, which releases Ca²⁺ from extra-, inter- and intracellular stores (Poovaiah & Reddy, 1987), also caused reduced curvature by decreasing the rate of response. For both chelator treatment (expected to restrict availability of Ca²⁺ to stipe cells) and ionophore treatment (expected to increase Ca²⁺ concentrations from intra- and intercellular stores) to reduce the gravitropic response suggests that the phenotype we have measured is a complex one, with steps dependent on both high and low Ca²⁺ concentrations. Another important observation of ionophore treatment on stipe extension and stipe gravitropism is that even though the ionophore significantly increased stipe extension (Table 1) it caused a reduction in the gravitropic response, suggesting that tropic bending does not simply result from redistribution of the normal growth potential of the stipe. Generation of gravitropic curvature may depend on a mechanism which is different from that which promotes vertical stipe growth.

Calmidazolium disrupts calmodulin-dependent Ca²⁺ uptake (Gietzen *et al.*, 1981; Tuana & MacLennan, 1984); here it decreased the rate of response of the contact angle, suggesting that not only is Ca²⁺ uptake necessary for normal gravitropism, corroborating the results obtained with the Ca²⁺ chelator, but also that calmodulin is involved in this uptake process. It remains to be investigated whether the role of calmodulin-mediated Ca²⁺ uptake during the gravitropic response in *C. cinereus* is to remove accumulated Ca²⁺ (resulting from gravity-dependent influx) from the cytoplasm via Ca²⁺-ATPases; or to shuttle Ca²⁺ to another destination in the cytoplasm, such as the endoplasmic reticulum or vacuole; or (as a complex) to initiate a cascade of reactions resulting in asymmetrical extension.

Table 4. Effect of inhibitor treatment on movement of the gravitropic bend in stipes of *Coprinus cinereus*

Treatment	Rate of bend of movement towards base ($\mu\text{m min}^{-1}$)	Amount of bend movement (mm)	Final position of bend (% of stipe length measured from base)
1 h treatments			
Water control	57.9 \pm 23.8	7.2 \pm 4.3	56.0 \pm 3.7
Calmidazolium [100 μM]	66.5 \pm 25.6	6.9 \pm 4.5	62.3 \pm 13.5
Verapamil [1 mM]	82.8 \pm 26.0*	7.9 \pm 2.6	55.0 \pm 8.6
2 h treatments			
Water control	60.4 \pm 7.6	7.9 \pm 4.3	48.8 \pm 13.3
Ionophore [1 μM A23187]	29.7 \pm 30.6*	3.7 \pm 3.6*	64.3 \pm 14.5*
Chelator [10 mM BAPTA]	33.2 \pm 45.5	3.8 \pm 3.7*	61.3 \pm 8.5*

Entries show the mean \pm s.d., the number of replicates being the same as shown in Table 1. Asterisks identify values which are significantly different ($P = 0.05$, analysis of variance) from the corresponding water controls.

Verapamil, which blocks Ca^{2+} channels (Janis *et al.*, 1987; Tsien & Tsien, 1990) and thus impedes facilitated transport of Ca^{2+} across membranes, had the least noticeable effects on gravitropism and also significantly reduced stipe extension at the concentration used. The decreased bending seen in stipes treated with verapamil could not be attributed solely to a reduced gravitropic response, as it may have been a consequence of the reduced extension. Although Ca^{2+} uptake seems to be required for the gravitropic response (as suggested by chelator and calmidazolium treatments), Ca^{2+} uptake through Ca^{2+} ion channels seems to be more critical for the portfolio of reactions which are expressed as stipe extension.

Our results suggest that controlled sequestering of intracellular Ca^{2+} is required for normal gravitropic bending, and consequently that lack of a Ca^{2+} activity gradient inhibits gravitropic bending. Bending, bend movement and bend compensation were all affected by loss of the extracellular Ca^{2+} gradient (and therefore lack of Ca^{2+} transport across the membrane), suggesting that Ca^{2+} is essential for the control of these processes. However, the role and destiny of Ca^{2+} needs to be determined to establish what biochemical processes are involved. Most significantly, though, all the treated stipes reacted to gravity, so, in direct contrast to the reactions of plant organs tested under similar conditions, Ca^{2+} activity gradients do not seem to be involved in gravity sensing by *Coprinus* stipes.

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