Assessing Potential Targets of Calcium Action in Light-modulated Gravitropism

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ABSTRACT

Light, through the mediation of the pigment phytochrome, modulates the gravitropic response of the shoots and roots of many plants. The transduction of both light and gravity stimuli appears to involve Ca$^{2+}$-regulated steps, one or more of which may represent points of intersection between the two transduction chains. To be confident that Ca$^{2+}$ plays a critical role in stimulus-response coupling for gravitropism, it will be important to identify specific targets of Ca$^{2+}$ action whose function can be clearly linked to the regulation of growth. Calcium typically exerts its influence on cell metabolism through binding to and activating key regulatory proteins. The three best characterized of these proteins in plants are the calmodulins, calcium-dependent protein kinases, and annexins. In this review we summarize what is known about the structure and function of these proteins and speculate on how their activation by Ca$^{2+}$ could influence the differential growth response of gravitropism.

INTRODUCTION

The signalling events initiated by various environmental stimuli radiate out into a network of different amplification and transduction steps. Because agents such as Ca$^{2+}$, G-proteins, IP$_3$, and protein kinases and phosphatases are common elements that couple stimulus to response for many distinct stimuli, it is not surprising that networks of transduction steps for different stimuli intersect and affect each other. In the case of gravitropic growth responses initiated by orientation changes, there is a well established literature documenting that these responses can be modulated by red light (R) acting through the pigment phytochrome (Roux and Serlin, 1987). Among many examples of this interaction, certain corn roots can be converted from a diagravitropic mode of growth to an orthogravitropic mode by R (Feldman and Briggs, 1987; Leopold and Wettlaufer, 1988), the coleoptiles of corn seedlings show enhanced gravitropism after an R pretreatment (Wilkins and Goldsmith, 1964), and Arabidopsis hypocotyls are induced to exhibit normal negative gravitropism by the red-absorbing (Pr) form of phytochrome B (Liscum and Hangarter, 1993). These results imply that at least one cellular response initiated by light is the same as, or affects one or more of the gravity-induced cellular responses necessary for gravitropism (Fig. 1).

As summarized in several reviews (Roux, 1990; Poovaiah and Reddy, 1993; Roux, 1994) a number of different laboratories have found strong evidence that a major step in the transduction of both the light and the gravitropic stimulus requires the mediation of Ca$^{2+}$. The evidence includes the important fact that both stimuli alter the concentration of cytosolic free calcium in the responding cells. It seems plausible, then, that a light-induced increase in [Ca$^{2+}$]$_{cyt}$ could play a significant role in promoting graviresponsiveness, and this increase could represent a key intersection where the signal transduction chains for phytochrome and gravity responses meet.

Full confidence in the validity of the Ca$^{2+}$ hypothesis will require that at least one additional piece of information be obtained, namely, the identification of specific targets of calcium action that have a clear and significant impact on growth. However, as yet no specific function of Ca$^{2+}$ in either light- or gravitropically induced growth changes has as yet been identified. Because there is a variety of different Ca$^{2+}$-binding proteins in plants (Roux, 1992), it may be that induced changes in [Ca$^{2+}$]$_{cyt}$ affect cellular physiology in several different ways simultaneously, and that there is no simple connection between Δ [Ca$^{2+}$]$_{cyt}$ and growth changes. Nonetheless, experimental results that would convincingly identify any one Ca$^{2+}$-dependent step in a chain of causality leading to growth changes would represent an important advance.

In most well-described cases, the immediate target of calcium action during the transduction of an environmental stimulus is a calcium-binding protein. Such proteins tend to be highly conserved evolutionarily, with very similar types occurring in both plants and animals. What are the Ca$^{2+}$-binding proteins in plants? Remarkably few have been characterized thus far, though it seems unlikely that this reflects their actual paucity in cells. The three best characterized Ca$^{2+}$-binding proteins in plants thus far are calmodulin (CaM), calcium-dependent protein kinases (CDPKs) and annexins, and these are the ones we will discuss in this chapter.

CALMODULIN

Calmodulin is a highly-conserved protein, with animal and plant versions typically showing about 90% identity. Most species tested have several CaM isoforms encoded by different genes, and they also have CaM-like proteins that typically show 60-70% amino acid sequence identity to CaM. The physiological significance of these multiple variants is unknown, but initial results suggest that different isoforms are regulated differently and/or have different subcellular locales (Gawienowski et al., 1993; Takezawa et al., 1995), and thus could participate in different transduction chains. Calmodulin itself can be
TARGETS OF CALCIUM ACTION IN GRAVITROPISM

GRAVITY

↓

RECEPTOR RESPONSE
(e.g. mechanoreceptor activation)

↓

Cellular Changes, Set A → Cellular Changes, Set B, C, etc. → GRAVITROPISM

LIGHT

↓

RECEPTOR RESPONSE
(e.g. phytochrome activation)

↓

OTHER PHOTOMORPHOGENIC RESPONSES

Figure 1. A model to account for the observation that light regulates gravitropism. It illustrates the postulate that the two transduction pathways intersect at some common step.

post-translationally modified in a number of ways, and, in the case of its methylation at position 115, this modification has biochemical and physiological consequences (Roberts and Harmon, 1992).

That CaM may play a role in coupling the gravity stimulus to gravitropic growth is suggested by the results of Biro et al. (1984), who showed that antagonists of CaM action can selectively block gravitropic growth under conditions in which they do not inhibit growth. That CaM may be an actual target of Ca\(^{2+}\) action in phytochrome responses is indicated by the experiments of Neuhaus et al. (1993). They found that microinjection of Ca\(^{2+}\)-activated CaM could induce the expression of a phytochrome-regulated gene (Cab reporter gene) in mutant cells that did not contain the PhyA protein known to control that gene. However, even identifying CaM as a participant in light- (or gravity-) regulated signalling chains does not clarify what cellular activity it is modulating, for CaM exerts its influence through the enzymes to which it binds, and CaM has multiple potential binding-partners in cells (Roberts and Harmon, 1992). Here we have chosen to discuss only three CaM-regulated enzymes whose activities may be plausibly related to responses known to be induced by gravitropic and red-light stimuli, a CaM-dependent protein kinase, a nuclear NTPase, and a plasma-membrane associated Ca\(^{2+}\)-ATPase.

CaM-dependent Protein Kinase

Protein phosphorylation and dephosphorylation are common events in the signal transduction chains initiated by virtually all stimuli, including those of gravity (Friedmann and Poovaiah, 1991) and light (Roux, 1992). Until recently the only characterized protein kinases regulated by Ca\(^{2+}\) in plants were members of the CDPK family, all of which contain a CaM-like domain, but are independent of exogenous CaM for their activity (Harmon et al., 1987; Harper et al., 1991). Now, however, there are reports from three different laboratories which indicate that there are calmodulin-dependent protein kinases in plants. Watillon et al. (1993) and Patil et al. (1995) have isolated and sequenced cDNA clones that code for Ca\(^{2+}\)/CaM-dependent protein kinases, and Lu et al. (1993) have characterized a Ca\(^{2+}\)/CaM-dependent protein kinase II homologue from maize roots.

The report of Lu et al. (1993) is of particular interest in the context of this review because they inhibited the activity of the CaMK II homologue in corn roots (called MCK1), they also abolished the light-regulated gravitropism of these roots. In subsequent work they used a \(^{35}\)S-CaM gel overlay assay to monitor the CaM-binding activity of the MCK1 protein and found that binding was inhibited by 50 \(\mu\)M KN-93 (an inhibitor of mammalian CaMK II), but not by 5 \(\mu\)M. In parallel with these results, 50 \(\mu\)M but not 5 \(\mu\)M KN-93 blocked the gravitropic response of the roots (Lu and Feldman, personal communication). Their results strongly indicate that a CaMK II homologue in corn roots plays an important role in transducing the stimuli of light and gravity into gravitropic growth.

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There is abundant CaM in the nucleus (Dauwalder et al., 1986), so the pea nuclear NTPase can be considered as one of the enzymes potentially regulated by CaM in this organelle. Its function is as yet unknown (there are more than a half-dozen nuclear enzymes known to have NTPase activity), but its association with the nuclear envelope and similarity to the rat liver NTPase make it plausible to speculate that it could have a role in the control of RNA transport out of the nucleus, which is a major step in the control of gene expression. Although the 47 kD NTPase seems to be controlled by phytochrome and is definitely regulated by Ca$^{2+}$-activated CaM, it would be premature to postulate any role for it in light-regulated gravitropism. The main rationale for highlighting it here is to call attention to the fact that some key targets of Ca$^{2+}$ action are nuclear. Since gravitropic stimuli critically affect nuclear metabolism, such as differential gene expression (McClure and Guilfoyle, 1989), the transduction chains they initiate will include steps that occur in the nucleus. Given the role of Ca$^{2+}$ in gravitropism, it will be useful to identify and study Ca$^{2+}$-regulated nuclear enzymes such as the 47 kD NTPase, and to assess how (whether) their function could influence cell growth.

**Plasma-membrane associated Ca-ATPase**

The pumping of Ca$^{2+}$ from the cytoplasm to the extracellular matrix of plant cells is carried out by a Mg-ATP-dependent Ca$^{2+}$-ATPase localized on the plasma membrane (PM). In animals a major Ca$^{2+}$-ATPase of the PM is regulated by CaM, but in plants evidence for a PM-localized Ca$^{2+}$ pump that is regulated by CaM has been weak and controversial. Recently, however, Rasi-Caldogno et al. (1993) have reported on the properties of a CaM activated Ca$^{2+}$-ATPase in radish PM preparations. They found that 1 μM CaM could stimulate a PM-associated Ca$^{2+}$-ATPase activity over 3-fold compared to the effects of Ca$^{2+}$ alone. However, to get this result consistently, they first had to deplete the PM of endogenous CaM by extensive treatment with EDTA. Like the CaM-activated Ca$^{2+}$ pump of erythrocyte membranes, the radish Ca$^{2+}$-ATPase could also be activated by protease treatment that was thought to remove an autoinhibitory domain of the pump. This work suggests that previous attempts by others to demonstrate a CaM activation of PM-associated Ca$^{2+}$ pumps may have been foiled by the presence of tightly bound endogenous CaM which could saturate the activity of the pump and render it insensitive to exogenously added CaM.

Assuming that a CaM-activated Ca$^{2+}$ pump is a feature of many plant cells, then a gravity- or light-induced rise in the cytosolic free Ca$^{2+}$ concentration ([Ca$^{2+}]_{cyt}$) could activate the pump and result in a transient increase flux of Ca$^{2+}$ into the wall. Red light induces Ca$^{2+}$ extrusion in oat protoplasts (Hale and Roux, 1980).
The question of whether a transient increase of \([\text{Ca}^{2+}]_{\text{wall}}\) would affect wall extension properties is not clearly answerable by available data. On the one hand, removing Ca\(^{2+}\) from the wall by chelation or acidification increases wall plastic extensibility (Virk and Cleland, 1988); on the other hand, wall expansion properties seem to be relatively insensitive to bulk changes in total wall calcium (Bagshaw and Cleland, 1993). Perhaps the key parameter to measure is not bulk wall calcium, but the concentration of free Ca\(^{2+}\) in the wall (= \([\text{Ca}^{2+}]_{\text{wall}}\)). Using a Ca\(^{2+}\)-selective microelectrode, Bjorkman and Cleland (1991) showed that there was a rapid differential in the wall Ca\(^{2+}\) activity between the upper and lower sides of gravisimulated maize root tips, and that this gradient was necessary for root gravitropism. Presumably this \(\Delta[\text{Ca}^{2+}]_{\text{wall}}\) would be most apparent in the wall space immediately adjacent to the plasma membrane (so called "inner" wall), which is the region most critical for determining the directionality of growth (Richmond et al., 1980). There is no information on how much of an increase in \([\text{Ca}^{2+}]_{\text{wall}}\) would be needed to induce a growth change, because the mechanism by which \(\Delta[\text{Ca}^{2+}]_{\text{wall}}\) affects wall extension properties is unknown. In this regard, the reports of wall CaM and CaM-binding proteins (Ye et al., 1989) raises the possibility that \(\Delta[\text{Ca}^{2+}]_{\text{wall}}\) in the low micromolar range might suffice to stimulate wall enzymatic changes, some of which could be relevant to growth.

Moving maize coleoptiles from a vertical to a horizontal position results in an increased level of Ca\(^{2+}\) available for pyroantimonate precipitation (= free Ca\(^{2+}\)) in walls along the upper side of the coleoptile before gravitropic curvature begins (Slocum and Roux, 1982). Integrating this result with the discussion above, we speculate that a plausible role for CaM in gravitropism would be to transduce an increased \([\text{Ca}^{2+}]_{\text{o}}\) (resulting from the signal-induced release of Ca\(^{2+}\) from internal stores) into an increased \([\text{Ca}^{2+}]_{\text{wall}}\) by activating a CaM-dependent Ca\(^{2+}\) pump in the PM. To the extent that Ca\(^{2+}\) pump activation was asymmetric, and \(\Delta[\text{Ca}^{2+}]_{\text{wall}}\) can alter wall extensibility, this change could contribute to the observed asymmetries in wall growth and subsequent coleoptile curvature.

CALCIUM-DEPENDENT PROTEIN KINASES

Biochemically, CDPKs rank as second only to CaM as the best characterized Ca\(^{2+}\)-binding proteins in plants. Biochemical characterization of the soybean CDPK (Putnam-Evans et al., 1990) was soon followed by the isolation and sequence of the cDNA that encoded it (Harper et al., 1991). Now multiple members of this family have been identified in Arabidopsis (M. Sussman, personal communication). The properties common to all of them are summarized in Fig. 3. As speculated for the multiple calmodulins, the many different CDPKs may be specialized to operate in many different cellular micro-environments on many different specific substrates.

To understand the significance of CDPKs in signal transduction for light, gravity, or any other input stimulus, it will be necessary to begin identifying the substrates phosphorylated by them. Recent progress on this question has been reported. Weaver et al. (1991) showed that nodulin 26, which is an ion channel in symbosome membranes (Weaver et al., 1994), is phosphorylated by a CDPK in vivo and in vitro. Since the role of nodulin 26 in symbosome membrane transport is not yet defined, the significance of the phosphorylation is unclear. However, phosphorylation of ion channels typically alters their transport properties, and so the phosphorylation of nodulin 26 by a CDPK is expected to have a functional impact on it.

Yang and Boss (1994) have shown that a CDPK in carrot cells phosphorylates and thus activates a protein called PIK-A49. When activated, PIK-A49 stimulates the activity of the enzyme phosphatidylinositol 4-kinase, which converts phosphatidyl inositol (PI) to phosphatidylinositol-4-monophosphate (PIP). PIP serves as a precursor for phosphatidylinositol 4, 5-bisphosphate (PIP2), which, in turn is the precursor source of IP3, an agent well known to induce Ca\(^{2+}\) release from internal cellular stores such as vacuoles and the ER (Coté and Crain, 1993). Thus in this case of PIK-A49 phosphorylation, there is a clear potential connection between CDPK activity and the regulation of Ca\(^{2+}\) transport by IP3 in cells. There are as yet no results implicating IP3 in gravitropism, although there is good evidence that IP3 can substitute for R in some phytochrome responses (Roux, 1994).

Phytochrome regulates the phosphorylation of proteins in pea nuclei in a Ca\(^{2+}\)-dependent manner (Datta et al., 1985), and a CDPK from pea nuclei has been partially purified and characterized (Li et al., 1991). Current studies to identify the phosphoprotein substrates of the nuclear CDPK will help reveal the significance of this phosphorylation in pea nuclear metabolism.

ANNEXINS

Annexins represent a more recent addition to the list of important Ca\(^{2+}\)-binding proteins in plants. Annexins were first discovered in vertebrate cells, where at least a dozen different annexins have been identified. All animal annexins tested to date exhibit ion channel functions in biological membranes, several of them promote the fusion of membranes in cells and in model systems, and annexin VII has voltage-dependent channel activity that is highly selective for Ca\(^{2+}\). The defining in vitro characteristic of purified annexins is that they bind to phospholipids in a Ca\(^{2+}\)-dependent manner. Structurally, they all have a ca. 60 to 70-amino-acid sequence motif that is imperfectly repeated at least four times. Alignment identity among repeats within a given annexin sequence is typically between 30 and 40%.

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CALCIUM-DEPENDENT PROTEIN KINASES

- multi-gene family
- bind and are activated directly by Ca\(^{2+}\)
- have autoinhibitory domain
- have calmodulin-like domain linked to C-terminus of kinase catalytic domain

Schematic representation of domain structure of CDPK\(\alpha\):

![Diagram of CDPK\(\alpha\) domain structure]

- catalytic domain
- calcium-binding domain
- putative autoinhibitory domain

100 aa

Figure 3. Biochemical properties of CDPKs and domain structure of CDPK\(\alpha\) from soybean. Illustration adapted from Roberts and Harmon (1992).

Findings in our laboratory and others have revealed the presence of annexin-like proteins in plant cells (Smallwood et al., 1990; Clark et al., 1992). Criteria for judging the plant proteins to be annexin-like were their sequence similarity to animal annexins and their property of binding to phospholipid affinity columns in a Ca\(^{2+}\)-dependent fashion. Inferences from immunolocalization studies are that annexins may play key roles in Ca\(^{2+}\)-transport and secretory processes important for growth in plant cells (Clark et al., 1992; Blackbourn and Battey, 1993). Below we will review progress on the structure and potential cellular functions of annexins in plants, and discuss their possible roles in gravitropic growth.

Structure of plant annexins

Thus far only one full-length or near full-length cDNA sequence for a plant annexin has been reported (Pirck et al., 1994). The four internal repeats in this clover (Medicago sativa) annexin are not as similar to each other as are the repeats in many animal annexins. If amino acids #10-71 are taken as the first repeat motif, this sequence aligns with repeat 2 (amino acids # 77-141) at 24% identity, with repeat 3 (amino acids # 163-222) at 30% identity, and with repeat 4 (amino acids # 240-301) at 29% identity (Fig. 4A). When the clover sequence is compared with those of previously published partial sequences of annexin-like plant proteins, the similarities are immediately obvious (Fig. 4B). On the other hand annexin sequences are clearly not as conserved as calmodulin sequences.

Given that there are multiple annexins in animal cells, it would not be surprising if this was the case also in plant cells. Antibodies raised to p35, the pea annexin-like
A. ALIGNMENT OF FOUR PUTATIVE REPEAT REGIONS OF CLOVER ANNEXIN

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B. ALIGNMENT OF REGION FROM THE DEDUCED SEQUENCE OF CLOVER ANNEXIN WITH PARTIAL SEQUENCES OF MAIZE, TOMATO AND PEA ANNEXINS

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[CODE: | = identity; : = conservative substitution]

Figure 4. (A) Alignment illustrating the similarity of four different “repeat” regions of annexin from clover (Medicago sativa). (B) Alignment illustrating similarity of clover sequence (deduced from the nucleotide sequence of its cDNA) to amino acid sequences of peptides isolated from tomato, maize (Blackbourn et al., 1992), and pea (Clark et al., 1992) annexins.

protein (Clark et al, 1992), have significant binding affinity for the protein products of two cDNAs selected from a pea library, but the deduced sequence of neither of these proteins contains p35 sequences (S.-H. Kim and S. Roux, unpublished). Both proteins show about 25% sequence identity with the clover annexin. To examine their functional relationship to annexins these proteins will be overexpressed in bacteria, purified and tested to determine whether they bind Ca$^{2+}$ and/or bind to a phospholipid affinity column in a Ca$^{2+}$-dependent fashion. If they do, they should be classified as annexin-like proteins, or, perhaps annexins belonging to a family different from that of the clover annexin.
Annexin localization

Clark et al. (1992, 1993) have localized p35 in ferns and dicots predominately in cells that are rapidly elongating or actively engaged in secretion. Immunolocalization of p35 in pea seedlings shows that it is highly concentrated in rapidly elongating cells undergoing xylogenesis or differentiating into phloem sieve tubes, and in peripheral root cap cells that are actively secreting mucilage. The peripheral pattern of annexin immunostaining is strikingly different from the more central columella cell pattern seen for CaM immunostaining in the root cap (Fig. 5). At the ultrastructural level, annexins are found predominately in association with Golgi vesicles and the plasma membrane.

In the ferns Dryopteris and Anemia, spore germination is initiated by red light through the mediation of phytochrome. In collaboration with the laboratory of R. Scheurlein, we have used antibodies against the pea annexin (p35) in immunocytochemical analyses to find that annexin expression also appears to be induced by light. When annexin immunostaining is first detected after R treatment, it is not homogeneously distributed throughout the spore. Rather, virtually all of it is concentrated in a region that predicts where the developing rhizoid will emerge. As the rhizoid actually emerges and grows out in its typical polar fashion, the immunostain remains concentrated at the apical tips of rhizoids, where all growth is occurring (Clark et al., 1993). We have recently used confocal laser microscopy to more precisely localize the fern rhizoid annexins, and found that they are truly concentrated in the tip 5 μm. We have also confirmed that several proteins extracted from germinating spores both bind to a phospholipid affinity column in a Ca<sup>2+</sup>-dependent fashion, and are recognized by anti-p35 antibodies in a Western blot analysis. To test whether annexin tip localization was coincident with polar growth in other systems, we examined elongating tobacco pollen tubes. Here, too, we found annexin immunostaining concentrated in the growing tip region, a pattern that had earlier been reported for lily pollen tubes (Blackbourn et al., 1992).

Postulated cellular functions of annexins

The results reviewed above, plus in vitro data showing that purified plant annexins promote liposome aggregation, have led to the postulate that one cellular role of annexins is to promote Ca<sup>2+</sup>-mediated membrane fusion during vesicle-mediated secretion (Clark et al., 1992; Blackbourn and Battey, 1993). There are a number of ways this function could be important for gravitropism.
Presumably, gravitropic growth requires the asymmetrically directed transport of wall materials to expanding cell walls. Localized Ca\(^{2+}\) gradients set up by asymmetric opening of Ca\(^{2+}\) channels could result in localized, annexin-mediated secretion of wall materials needed for asymmetric growth. According to this model annexins would be more involved in downstream events closer to the growth response than in earlier sensing and signalling events in gravitropism.

Calcium concentration gradients parallel growth and secretion gradients in growing pollen tubes (Miller et al., 1992) and in fern rhizoids (Scheuerlein, Poenie and Roux, unpublished), and thus parallel annexin gradients. This coincidence of high concentrations of both cytosolic free Ca\(^{2+}\) and annexin may be related to Ca\(^{2+}\) channel activity that has been observed for a number of the animal annexins. There are several reports of animal annexins serving directly as Ca\(^{2+}\) channels (e.g., Pollard et al., 1990), but thus far this function has not been tested for plant annexins. Maintenance of the Ca\(^{2+}\) gradient is essential for polarized secretion and growth, so it may be that the role of annexin in secretion may be related to its Ca\(^{2+}\) channel activity. As functional regions of annexins begin to be mapped (Huber et al., 1992) it should be possible to test whether genetically modified annexins with impaired channel activity are also impaired in promoting vesicle fusion and secretion.

**SUMMARY AND CONCLUSION**

There is good evidence to support the hypothesis that Ca\(^{2+}\) plays a role in stimulus-response coupling for both gravitropic and phytochrome responses in plants, but knowledge of the cellular targets of Ca\(^{2+}\) action in signalling remains rudimentary. The three targets highlighted in this review, CaMs, CDPKs, and annexins, probably represent only a fraction of the potential targets in cells. None of them are the end points of transduction chains, so only by identifying their down-stream effectors will it be possible to certify them as participants in any specific response to gravity or light.

Because there are multiple CaMs, CDPKs, and annexins it may seem an overwhelmingly complex task to specify what any one of them specifically is doing. One possible solution to this problem would be to "divide and conquer"; i.e., to ask in which subcellular domain a particular Ca\(^{2+}\)-binding protein is functioning, or at what particular phase of development is this Ca\(^{2+}\)-binding protein operative. Do CaM-binding partners on the plasma membrane interact with only one of the cellular CaMs? Is nuclear CaM or cell wall-localized CaM different from other cellular CaMs? Does every cell type in every stage of its development express all of the CDPKs or all of the annexins? Just as it has been possible to use mutants to selectively link the absence of one type of phytochrome with the loss of a particular light response (Roux, 1994), it should be possible to link specific CaMs, CDPKs and annexins to particular cellular functions and thus specify their role in a particular signalling chain.

At this early stage in the characterization of Ca\(^{2+}\)-binding proteins in plants, any role proposed for CaM, CDPK and annexin participation in promoting gravitropism is necessarily speculative. The speculations proposed in this review are testable, however, and if they serve as starting points and catalysts for experimental designs that more narrowly define how Ca\(^{2+}\) pushes the light- and gravity-initiated transduction chain forward toward gravitropism, they will have served a useful purpose.

**Acknowledgements**—The research from the author's laboratory described in this review was supported by grants from the National Aeronautics and Space Administration (NAGW 1519) and National Science Foundation (DCB 91-06245).

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