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What's New in Plant Physiology

Role of calcium ions in phytochrome responses: an update

Stanley J. Roux, Randy O. Wayne and Neeraj Datta

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S. J. Roux (reprint requests) et al., Dept of Botany, The Univ. of Texas at Austin, Austin, TX 78713, USA.

Introduction

A decade ago, plant developmental biologists were greatly interested in the discovery that red light, by photoactivating the regulator pigment phytochrome, could induce altered ion fluxes in plant cells. Many believed that these ion fluxes could help promote phytochrome-mediated photomorphogenesis. At this time among animal physiologists there was fresh excitement over the discovery that Ca^{2+} and Ca^{2+}-binding proteins were of central importance in modulating the enhanced activity response of enzymes to growth regulators.

Inspired by these findings, Haupt and Weisenseel (1976) proposed that some phytochrome effects on cellular enzyme activities and on cell growth and development could be mediated through Ca^{2+} and Ca^{2+}-dependent regulator proteins. There were no such Ca^{2+}-dependent proteins known in plants at that time, but a major one was soon discovered, one that was already well known in animal cells: calmodulin.

Calmodulin has now been found in many species throughout the plant kingdom. It occurs both in the cytosol and in organelles of cells, and it can be activated by only small increases in the free Ca^{2+} concentration ($[Ca^{2+}]$) of its subcellular environment. In its Ca^{2+}-activated form it can stimulate the activity of several key enzymes that regulate major metabolic functions.

There is reason to believe that there may be many different Ca^{2+}-binding proteins in plants, but calmodulin is by far the best known. Using calmodulin as a specific example of a Ca^{2+}-dependent regulator protein, the hypothesis of Haupt and Weisenseel would postulate that the photoactivation of phytochrome rapidly leads to an increase in the $[Ca^{2+}]$ in certain subcellular compartments, and that this, in turn, would activate calmodulin and calmodulin-dependent enzymes in these compartments. Some of these enzymes could then catalyze activities that would result in photomorphogenesis. This hypothetical sequence of events is outlined in Fig. 1. There (and throughout this text) the two photointerconvertible forms of phytochrome are referred to by their conventional abbreviations, Pr (the red-light absorbing form of phytochrome) and Pfr (the far-red-light absorbing form of phytochrome). Pfr, which is transfor-

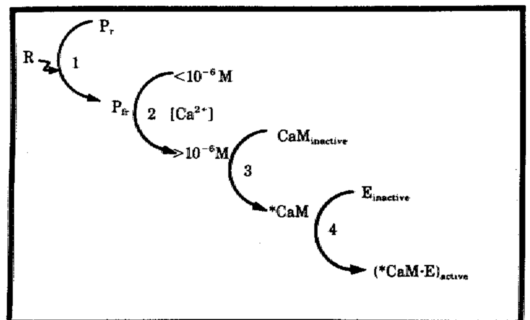

Fig. 1. A proposed model describing a hypothetical cascade of events initiated by the photoactivation of phytochrome. Events: 1, Phytochrome activated by red-light (R) irradiation; 2, Pfr-mediated increase in the $[Ca^{2+}]$ of an intracellular environment (cytosolic or organellar) containing calmodulin (CaM); 3, activation of CaM, i.e., formation of a Ca^{2+}-CaM complex (*CaM); 4, binding of *CaM to *CaM-dependent enzymes (E), thus activating them.

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med from Pr by red light, is considered to be the physiologically active, or response-inducing, form of phytochrome.

Between 1976 and 1983, a half-dozen laboratories published data which were supportive of the hypothesis outlined in Fig. 1 (Roux 1983). In general, these data describe either light-regulated Ca^{2+} transport in cells that would be likely to activate calmodulin, or calmodulin-activated enzyme activities that are also stimulated by light. Since 1983 many additional tests of the hypothesis have been carried out. The purpose of this review is to summarize the recent findings, discuss their significance, and point out key issues about the hypothesis that remain unresolved.

Evidence that Ca^{2+} helps regulate phytochrome responses

One of the most explicit predictions of the response pathway proposed in Fig. 1 is that a phytochrome-induced increase in intracellular $[Ca^{2+}]$ is necessary for the promotion of some phytochrome responses. There are as yet no published reports that provide direct data on this point, due mainly to the technical difficulty of accurately measuring the $[Ca^{2+}]$ in plant cells. However, there are indirect ways of testing this prediction, and several recent papers have reported this. Below we present recent evidence that Ca^{2+} helps to regulate two particularly well-characterized phytochrome responses: red-light stimulated spore germination in the fern, *Onoclea,* and chloroplast rotation in the alga, *Mougeotia.*

Calcium requirement for photoinduced fern spore germination

Red light induces the germination response in spores of *Onoclea*. This response will not occur unless the spores are provided with external Ca^{2+}. The threshold concentration of external Ca^{2+} needed for the response is submicromolar, while 3 μM supports a half-maximal response. These are the same levels required for Ca^{2+} modulation of hormone-stimulated responses in animal cells.

The question of whether light promotes an uptake of Ca^{2+} into *Onoclea* spores has been recently studied by Wayne and Hepler (1985). Using atomic absorption spectroscopy, they show that red light induces an increase in the concentration of total intracellular calcium and that the stimulation is reversed by a subsequent irradiation with far-red light. During a 5 min irradiation, red light causes an increase in the concentration of total intracellular calcium of ca 500 μM. One could suppose that such a large influx would generate an increase in the $[Ca^{2+}]$ of some subcellular environment sufficient to activate the calmodulin there. Though the data do not directly show this, they are certainly consistent with that interpretation.

Additional evidence supporting the importance of the contribution of Ca^{2+} to phytochrome-induced fern spore germination comes from the observations that La^{3+} blocks both Ca^{2+} uptake and germination (Wayne and Hepler 1984, 1985). La^{3+} must be present prior to or during irradiation in order to inhibit germination. If La^{3+} is added 5 min after the start of irradiation, it is no longer effective, indicating that only a transient uptake of external Ca^{2+} is needed for germination.

Using the ionophore A23187 (a compound that transports Ca^{2+} across membranes), it is possible to chemically induce an increase in the intracellular Ca^{2+}. This treatment stimulates the germination of fern spores in the dark, if Ca^{2+} is present in the germination medium (Wayne and Hepler 1984). Thus A23187 treatment and red-light treatment (i.e., the production of Pfr) have equivalent effects on fern spore germination. This finding supports the postulate that phytochrome stimulates germination by inducing an increased transport of Ca^{2+} into fern spores. However, Pfr itself may not *directly* transport Ca^{2+}, since under certain conditions fern spores will take up newly added Ca^{2+} even after Pfr has been photoconverted back to Pr (Wayne and Hepler 1984).

Another indication that there may be intermediate steps between the photoactivation of phytochrome and Ca^{2+} influx into cells comes from the recent observations of Das and Sopory (1985). They found that red light (1 min) stimulates an increase in the rate of $^{45}Ca^{2+}$ uptake into corn protoplasts and subsequent irradiation with far-red light cancels the uptake response, but only if it is given immediately after red irradiation. The stimulation of $^{45}Ca^{2+}$ uptake is enhanced by introducing a 3 min dark period between the red light irradiation and the addition of $^{45}Ca^{2+}$, indicating that reactions were occurring during the dark period that further enhanced $^{45}Ca^{2+}$ uptake. The authors provide some indirect evidence that the intermediary steps include the breakdown of a membrane lipid, phosphatidylinositol-4,5-bisphosphate, into diacylglycerol and inositol triphosphate. This membrane reaction is known to stimulate the uptake of Ca^{2+} in animal cells (Nishizuka 1984).

The phosphotidylinositol pathway has just recently begun to receive significant attention as a key mediator for converting signal stimuli into sensory responses in animal and plant cells (Schäfer et al. 1985). Studies on its possible participation in phytochrome-mediated responses hold great promise for generating valuable new insights.

Ca^{2+} fluxes and phytochrome function in *Mougeotia*

Paralleling the studies showing Ca^{2+} mediation of fern spore germination are demonstrations of Ca^{2+} involvement in the phytochrome-regulated chloroplast rotation in *Mougeotia.* In this response, unilaterally applied red light stimulates the single plate-like chloroplast of *Mougeotia* to rotate until it faces the direction of the light.

Initial investigations revealed that red light induces the uptake of $^{45}Ca^{2+}$ while subsequent exposure to far-red light prevents the response (Dreyer and Weisenseel 1979). In addition to these fluxes across the plasma membrane, there appears to be another light-regulated Ca^{2+} flux into and out of Ca^{2+}-containing vesicles in *Mougeotia* cells (Wagner and Rossbacher 1980). These vesicles are in equilibrium with external Ca^{2+} and lose their Ca^{2+} store when cells are cultured in low external Ca^{2+}. Following this treatment, cells are no longer able to respond to light. When cells are labelled with the fluorescent Ca^{2+}-chelate probe chlorotetracycline, a change in the vesicle fluorescence is observed during light-induced chloroplast rotation, indicating a relationship between the level of Ca^{2+} stored in the vesicles and the light response.

Another key observation supporting the hypothesis that Ca^{2+} helps to mediate the induction of chloroplast rotation by red light comes from the experiments of Serlin and Roux (1984). They describe a procedure for artificially inducing an influx of the ion into selected regions of *Mougeotia* cells using microtips of glass rods coated with the ionophore A23187. When they apply the drug-coated microtips on opposite sides of a cell near the edges of the chloroplast, they observe rotation of the chloroplast in the dark, just as if the edges had been irradiated with red light. External Ca^{2+} has to be present in order for the ionophore-stimulated rotation to take place. These results indicate that the chloroplast edge moves away from localized zones of Ca^{2+} influx, analogous to its movement away from localized regions of relatively higher Pfr concentrations (Haupt 1982).

External Ca^{2+} is required for A23187 to induce chloroplast rotation, but the same induction by Pfr requires only that the internal Ca^{2+}-storing vesicles contain Ca^{2+} (Wagner and Rossbacher 1980). These contrasting results indicate that, though light may promote Ca^{2+} uptake into *Mougeotia* (Dreyer and Weisenseel 1979), this uptake may not be essential for chloroplast turning if sufficient internal stores of Ca^{2+} are available for release by Pfr. They also imply that light may more efficiently release Ca^{2+} from internal stores than A23187. The recent data of Serlin *et al.* (1984) indicate that Pfr also promotes Ca^{2+} efflux from intracellular stores of Ca^{2+}, namely, mitochondria, in higher plant cells.

Calmodulin antagonists block the red-light stimulated chloroplast rotation

Calmodulin has been isolated from filaments of *Mougeotia* (Wagner et al. 1984) and may help regulate the chloroplast rotation response. The calmodulin antagonists trifluoperazine (20–50 μM) and W-7 (100 μM) inhibit light-induced chloroplast turning (Wagner et al. 1984). These drugs may have non-specific detergent effect on membranes. In order to interpret better whether W-7 was affecting chloroplast rotation non-specifically or through calmodulin inhibition, Serlin and Roux (1984) compared its ability to block the response to that of W-5, an analogue that has similar detergent effects, but is a much weaker calmodulin inhibitor. They found that W-7 inhibits the light response much more effectively than W-5, indicating that it probably exerts its effects through blocking calmodulin action.

Phosphorylation as a key step in calmodulin-regulated responses

A recent observation that may prove to be importantly related to the phytochrome and calmodulin effects in *Mougeotia* indicates that Ca^{2+} stimulates the phosphorylation of several proteins from *Mougeotia*. The Ca^{2+}-stimulated protein kinase is also able to phosphorylate rabbit myosin light chains added to *Mougeotia* homogenates (Atmüller et al. 1985). The phosphorylation of an endogenous myosin light chain may regulate the force-generating mechanism for the red-light stimulation of chloroplast rotation. This hypothesis seems all the more attractive in light of recent information on phytochrome-regulated phosphorylation responses in pea nuclei (Datta et al. 1985). Before discussing this result, it will be useful to briefly review the importance of phosphorylation as a regulatory mechanism.

The activity of many enzymes is regulated post-translationally by the controlled addition or removal of phosphate groups. In animal cells, external physiological stimuli, such as hormones and growth factors, have been shown to modulate the phosphorylation level of key regulatory enzymes, often through Ca^{2+} and calmodulin-dependent mechanisms. The effects of these stimuli on cell growth and development appear to require the mediation of enzyme phosphorylation (Cohen 1982).

Hints that phosphorylation may be an important regulatory mechanism in plants have come from recent reports that Ca^{2+}-activated calmodulin promotes the phosphorylation of plant cell proteins. Veluthambi and Poovaiah (1984) have shown that the phosphorylation of several membrane and soluble proteins from corn coleoptiles is promoted by exogenous additions of Ca^{2+} and calmodulin to crude preparations of these proteins. Their results also show that calmodulin inhibitors reduce the extent of phosphorylation, but the high concentrations used raise questions about the specificity of the inhibition.

The most likely biochemical site of action of calmodulin in its regulation of phosphorylation would be protein kinases and/or protein phosphatases. These two enzymes control the phosphorylation (kinase) and dephosphorylation (phosphatase) of proteins (Fig. 2). In animal cells, the activity of some of these kinases and phosphatases is regulated by calmodulin.

In plants, a number of Ca^{2+}/calmodulin-dependent protein kinases have been identified, including some

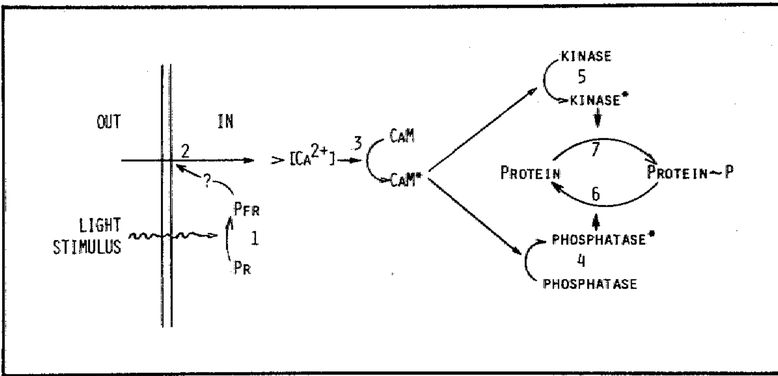

Fig. 2. A proposed expansion of the model described in Fig. 1, incorporating the results of recent findings. This model leads from the photoactivation of phytochrome (1) to modulation of protein phosphorylation by the addition (7) or removal (6) of phosphates (~P) to or from regulator proteins. Pfr, directly or ("?") indirectly induces the uptake of Ca^{2+} into cells (2); this leads to an increase in intracellular [Ca^{2+}] and the activation of CaM (3). *CaM may activate either one or more phosphatases (4) or one or more kinases (5). An asterisk (*) designates the relatively more active form of a protein or enzyme.

that have been purified and partially characterized (Ranjeva et al. 1984). However, except for the protein quinate: NAD^+ 3-oxidoreductase (Ranjeva et al. 1983), it is not known which proteins these kinases phosphorylate. Thus far there have been no demonstrations of calmodulin-dependent protein phosphatases in plants.

Phytochrome regulation of nuclear protein phosphorylation

Kleinsmith et al. (1975) reviewed the early evidence that there could be a causal link between the phosphorylation of nuclear proteins and the regulation of gene expression in animal cells. More recently, Ranjeva et al. (1984) summarized results that indicate this model may apply also to plant cells. The data on this question are thus far mainly correlative only, e.g., treatment "X" promotes both the phosphorylation of a specific chromatin-associated protein and RNA synthesis, but the number and variety of these correlations provide a strong rationale for further testing the hypothesis.

Paralleling the increased interest in phosphorylation as a plausible mechanism of gene control, there has been an explosion of new data demonstrating the selective regulation of gene expression by phytochrome (Tobin and Silverthorne 1985). Data on *how* phytochrome up- or down-regulates the transcription of certain genes, however, have been non-existant. Given the context of information reviewed in this section, a model (such as proposed in the preceding section) postulating phosphorylation as an intermediate step in transducing light effects on transcription would certainly be worthy of investigation.

A first step toward testing this model has been recently reported by Datta et al. (1985). They studied the effects of red and far-red light and of Ca^{2+} and calmodulin on protein phosphorylation in isolated pea nuclei. Their results show that red light and micromolar levels of Ca^{2+} stimulate the phosphorylation of the same nuclear proteins, and that far-red light given immediately after red light negates this response. Calcium chelation by ethyleneglycol-bis (β-aminoethylether)-N, N, N′, N′-tetraacetic acid and calmodulin antagonists (chlorpromazine and compound 48/80) also negate the response. Although these authors do not report any effects of exogenous calmodulin, nuclei are known to contain significant endogenous levels of it (Biro et al. 1984). In addition to providing an impetus to investigations on the role of phosphorylation in the chain of events leading to photomodulated gene expression, these data further support the hypothesis that Ca^{2+} and calmodulin are key mediators of phytochrome responses.

Concluding remarks

Two recent sets of results have greatly strengthened the hypothesis that Ca^{2+} mediates some phytochrome responses: the photomorphogenic effects of red light can be mimicked in darkness by the chemical induction of Ca^{2+} uptake into cells, and these same effects can be blocked by calmodulin antagonists. When these results are considered together with both recent and older evidence that Pfr promotes the uptake of Ca^{2+} into cells, the data favoring the hypothesis become even more convincing.

A critical missing result needed for demonstrating the validity of the hypothesis is a direct measurement of a Pfr-promoted increase in cytosolic [Ca^{2+}] up to $10^{-6}M$ or higher. Such an increase would be required to activate most of the cellular calmodulin. If, as proposed by Das and Sopory (1985), phytochrome affects inositol phospholipid turnover, more subtle increases in the cytosolic [Ca^{2+}] might suffice to activate enzymes that could regulate key cellular activities.

There is now experimental evidence that Ca^{2+}, calmodulin and Pfr all play a role in the regulation of nuclear protein phosphorylation. It remains to be seen

what nuclear functions this affects, but an exciting and *testable* possibility is that the phosphorylation of nuclear proteins may be an intermediate step linking the photoactivation of phytochrome with the photoregulation of gene expression. The key unanswered questions here are what are the nuclear proteins whose phosphorylation level is altered by light, what is the effect of phosphorylation on them, and what is their functional relationship, if any, to the control of mRNA transcription or processing. Resolving these issues will contribute greatly to an understanding of cellular mechanisms of photomorphogenesis.

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