

Summary of Research

Title: Cellular bases of light-regulated gravity responses

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This report summarizes the most significant research accomplished in our NAG2-1347 project on the cellular bases of light-regulated gravity responses. It elaborates mainly on our discovery of the role of calcium currents in gravity-directed polar development in single germinating spore cells of the fern *Ceratopteris*, our development of RNA silencing as a viable method of suppressing the expression of specific genes in *Ceratopteris*, and on the structure, expression and distribution of members of the annexin family in flowering plants, especially *Arabidopsis*.

(i) Gravity-directed polarity development in germinating *Ceratopteris* spores

a. Significance of Ca²⁺ currents for gravity-directed polarity development in single spore cells

A model system we are using to further explore the cellular bases of light-regulated gravity responses is the single-celled spore of the fern *Ceratopteris*. Light, through the photoreceptor phytochrome, induces the spore to exit dormancy (Chatterjee and Roux, 2000) and creates the cellular environment needed for gravity to direct the polarity of spore development. The earliest manifestation of the influence of gravity after irradiation is a bottom-to-top calcium current across the cells. This current peaks about 9 h after the light treatment, more than 30 h before the spore coat cracks and the first cell division, and its direction rapidly inverts when the cell is inverted (Chatterjee et al., 2000). It diminishes to near baseline level by 20 h after irradiation, by which time gravity has fixed the polarity of the cell. The next cellular manifestation of this polarity is the downward migration of the nucleus from the cell center, which determines the plane of the first cell division, and the downward emergence and growth of the rhizoid (Chatterjee and Roux, 2000).

At the time we began our project in July of 1999, we had already discovered the gravity-directed calcium current. As part of Aim 1 of our Project we proposed to determine the significance of this current for gravity-induced cell polarity in germinating spores of *Ceratopteris*. We accomplished this aim by blocking the current using a calcium-channel blocker, nifedipine, and then documenting that this inhibition resulted in blocking rhizoid development in about one-third of the cells, and suppressing the influence of gravity on the direction of the rhizoid emergence and growth in the other two-thirds of the cells. These results, which were published in *Planta* (Chatterjee et al. 2000), indicate that the gravity-directed calcium current across germinating fern spores plays a crucial role in the ability of those cells to respond to gravity and in the subsequent establishment of their polarity.

b. Developing tools to discover genes critical for the gravity response in fern spores

Our discovery of a role for calcium currents in the gravity response in single germinating fern spores suggested that calcium channels, calcium pumps, and calcium-binding proteins could be major players in transducing the gravity signal into oriented polarity development in fern spores.

A rigorous way to test this hypothesis would be to test the effects of suppressing the expression of genes encoding proteins likely to be involved in calcium transport and calcium signaling. We have developed a promising approach to achieve this, namely the method of RNA-induced gene silencing. This method is effective in both plants and animals and has been generically referred to as RNA interference (RNAi), although the mechanism of silencing appears to be somewhat different in plants than in animals. We will adopt the RNAi terminology here. According to this method, the introduction of double-stranded (ds) RNA into cells induces a kind of defense response in which cells selectively destroy not only the dsRNA but also single-stranded (ss) mRNA strands containing the same sequence as in the sense strand of the dsRNA. Recently, the laboratory of Stephen Wolniak (U. Maryland) has demonstrated that this method works in the fern *Marsilea*, and, using protocols developed in Wolniak's laboratory, we have obtained strong evidence that the method also works in *Ceratopteris*.

A prerequisite for using the RNAi method to suppress the expression of genes is knowing the sequence of the specific genes of interest. As a step to achieving this, we generated a cDNA library of genes expressed in *Ceratopteris* at 20 h after spore germination was initiated by light; i.e., when most cells were at or near the end of the period during which gravity fixes the polarity of the cell. The clones in this library number 3.8×10^7 cfu, and the average insert size of the cDNAs in this library is 1.56 kb. Single pass sequences of these cDNAs are referred to as Expressed Sequence Tags (ESTs). In collaboration with Purdue University we obtained 500-800 bp of sequence of over 2800 cDNAs randomly selected from this library (Gen Bank accession nos. BE640669-643505). Most of the sequences obtained were only partial sequences, but they were long enough in most cases to identify the protein likely to be encoded by the cDNA.

Over 2000 different genes are represented among the almost 3000 ESTs sequenced. The cDNAs are of high quality, many of them near full length. They have been successfully used as starting templates to produce dsRNA constructs for our RNAi studies. We have also used standard PCR methods to obtain full-length versions of several of these cDNAs, which we have then overexpressed in bacterial cells. This has allowed us to purify and characterize proteins encoded by these genes, and we plan to use some of these proteins to raise antibodies.

Included among the different cDNAs in the library are several that encode proteins whose functions would make them likely mediators or regulators of calcium currents, including calmodulin (CaM), calcium-dependent protein kinase (CDPK), two different annexin proteins, whose functions in animals appear to include calcium channel activity (required for the influx phase of the current), and a plasma membrane Ca^{2+} -ATPase pump (needed for the efflux phase of the current). We used the RNAi approach to successfully suppress the expression of four of these genes. This breakthrough, which was published this year in *Plant Physiology* (Stout et al., 2003),

In collaboration with Dr. Gloria Muday, we documented that two zucchini annexins bind tightly to plant F-actin along with the naphthylphthalamic acid (NPA)-binding protein involved in polar auxin transport (Hu et al., 2000). The NPA-binding protein is asymmetrically localized to the basal ends of some plant cells. Polar localization of annexins is well documented in certain animal and plant cells and is presumed to indicate an important signaling role for annexin in polar activities of these cells.

c. Annexins exhibit a gravity-dependent polar localization in pea cells

To test our hypothesis that annexins may be involved in gravity responses, we used immunocytochemistry to investigate the effects of gravistimulation on annexin localization in etiolated pea shoots. An asymmetric annexin immunostaining pattern was observed in a defined group of cells in the downward-pointing hook region, located just below the growing apex in an area classically referred to as a leaf gap. Prior to gravistimulation, the highest concentration of annexin was oriented toward the direction of gravity along the apical end of the immunostained cells. Within 15 min. after gravistimulation there was a redistribution of the originally polar and diffuse annexin immunostain, resulting in dissipation of the annexin asymmetry (Clark et al., 2000).

The immunostained cells were positioned such that they could facilitate the transport of materials needed for cell division toward the apical meristem under normal growth conditions. After gravistimulation one could expect that the flow of materials would be partially diverted to areas of differential growth and would be less strongly oriented toward the apical meristem. This explanation helps account for both the apical localization pattern observed prior to gravistimulation and the dissipation of this pattern after gravistimulation, and it is consistent with the currently favored role for annexins in the secretory activity of cells.

The gravity-dependent, apically polar localization of annexins in the leaf gap region of pea cells demonstrates that annexin localization is responsive to the gravity vector and suggests that annexins participate in the diverse processes that connect the gravity signal to growth responses in pea shoots (Clark et al., 2000). These results were among the first to show that the gravity signal can alter the distribution of a particular calcium-binding protein. There is little or no information to implicate leaf gap cells in auxin transport in shoots, so whether or not these data relate to postulates on the relationship of annexin-F-actin associations to polar transport remains to be determined.

d. Acquisition of T-DNA insertional knockouts of all 8 annexin genes in *Arabidopsis*

We recently initiated a collaboration with Syngenta to characterize T-DNA knockouts in all eight of the *Arabidopsis* annexins. We searched the GARLIC database of Syngenta for the *Arabidopsis* annexins and found T-DNA insertions in all eight genes that could lead to gene disruption and “knockouts” for these annexins. We recently received the seeds harboring the T-DNA insertional knockouts of all the annexins. Assuming that the knockout mutation for each annexin can be confirmed, these mutants will be invaluable tools for assessing the role of annexins in plant growth generally, and in gravity responses specifically. As described in the experimental section below, we are particularly interested to learn whether any one or combination of annexin knockouts alters the kinetics of auxin transport or of root or shoot gravitropism.

will permit us to now test the importance in directing or maintaining the calcium current needed for gravity-directed polarity development.

c. Reducing expression of Ca²⁺-binding proteins by RNAi alters fern spore development

Our initial studies on the effects of suppressing specific genes in fern spores tested what phenotypic changes resulted from suppressing the expression of annexins. In preparation for the annexin RNAi studies we isolated and sequenced two different full-length cDNAs for annexin genes that are expressed in *Ceratopteris* spores during the first 24 h of germination, *AnnCr1* and *AnnCr2*. These annexins are 81% identical at the amino acid level. Using quantitative RT-PCR we confirmed that both of these annexins are expressed very early during initiation of germination, though with slightly different kinetics. We found that the application of 0.03 mg/mL dsRNA for *AnnCr1*, and 0.01 mg/mL for *AnnCr2* induces mutant phenotypes: delayed germination, shortened rhizoids, and poorly developed or no prothallus. Raising the dsRNA to 0.1 mg/mL for *AnnCr1* and 0.14 mg/mL for *AnnCr2* completely blocks germination. As is characteristic of RNAi-mediated responses, ssRNA applied in the lower range of effective concentrations of the dsRNA is ineffective. It takes a 5-fold greater concentration of ss RNA to achieve the same phenotypes induced by dsRNA. Initial RT-PCR results show that the dsRNA for annexins is significantly and selectively reducing the level of both annexin mRNAs and not other messages. thus the effects of the dsRNA treatments appear relatively specific.

(ii) Annexin and differential growth studies in *Arabidopsis* and other higher plants

A key aim of the NAG2-1347 NASA project was to identify different sites of function of Ca²⁺-binding proteins that have been implicated in growth control, with a focus on understanding the role of these proteins in gravity responses. In this regard, we have been investigating the annexin family of Ca²⁺-binding proteins, because of their demonstrated role in the delivery to plant walls of secretory vesicles containing materials needed for growth. We have now identified and sequenced 8 different annexin genes expressed in *Arabidopsis* (Clark et al., 2001)

a. Different members of the annexin family show different expression patterns

We documented the differential expression of 7 of the 8 annexins in *Arabidopsis* by quantitative RT-PCR, then examined the tissue- and cell-specific expression patterns of two of these annexins, *AnnAt1* and *AnnAt2*, during early development using Northern analysis and *in situ* RNA localization. We found that these two different annexins have a mostly non-overlapping pattern. This comprehensive study was published in *Plant Physiology* (Clark et al., 2001). Since then we have confirmed the individual RNA localization patterns for *AnnAt1* and *AnnAt2* in immunolocalization studies using monospecific antibodies (data not shown).

The expression and immunolocalization data for these two *Arabidopsis* annexins provide additional support for the idea that annexins are found in highly secretory plant cell types. Two of these cell types, pollen tubes and root cap cells, require Ca²⁺ for this secretion, and annexin appears to be one of the targets for this Ca²⁺ signal (Clark, Thompson and Roux, 2001). Root hairs also exhibit polar secretion and growth that is directed by calcium. We found that *AnnAt1*, but not *AnnAt2*, was expressed in these structures (Clark et al., 2001), and we have since confirmed this specificity by immunolocalization (Clark et al., ms. in prep.).

b. Annexins bind tightly and specifically to F-actin

(iii) Other genes potentially involved in gravitropic growth

a. Effects of extracellular ATP on gravitropic growth suggest involvement of ectoapyrases

Plants may use the steep ATP gradient that exists across the plasma membrane in a symport process to help power the efflux of various compounds that are exported by the ATP-Binding Cassette (ABC) transporter P-glycoprotein, also called the Multi-Drug Resistance 1 (MDR1) transporter (Roux et al., 2001). Raising the level of extracellular ATP (xATP), either by adding ATP to the growth medium or by inhibiting the activity of ATP-hydrolyzing enzymes such as ectoapyrase, would be expected to decrease the steepness of this gradient and thus decrease the transport efficiency of MDR1 and any other ABC transporters that could use ATP gradients for symport (Roux et al., 2001). As reported by Noh et al. (2001; *Plant Cell* **13**: 2441-2454), MDR-like genes appear to be required for auxin transport, and we recently found that the list of compounds whose transport out of cells can be blocked by increasing [xATP] includes the hormone auxin. Adding mM ATP to the growth medium can mimic NPA in inhibiting auxin distribution in DR5-GUS transgenic plants, and increases the retention of ³H-IAA in corn root tips. In corn root segments, significantly less ³H-IAA is polarly transported from apex to base when 5 mM ATP is supplied in the receiver block. These ATP effects, which have been published in *Plant Physiology* (Tang et al., 2003), cannot be mimicked by equivalent concentrations of ADP or inorganic phosphate. They predict that genetically modifying the expression of ectoapyrases could alter the rate of auxin export from cells, which, in turn, should alter the kinetics of gravitropism. This prediction is currently being tested.

b. Suppressing the expression of Ran-binding protein makes *Arabidopsis* hypersensitive to auxin

Our studies of Ca²⁺ signaling led us to characterize a protein that binds to and regulates the activity of Ran, the small nuclear GTPase. We found that antisense expression of this *Arabidopsis* protein, AtRanBP1c, renders roots hypersensitive to auxin and alters auxin-induced root growth and development (Kim et al., 2001). These data suggest that AtRanBP1c plays a key role in the nuclear delivery of proteins that suppress auxin action. Plants hypersensitive to auxin could be useful tools in the ongoing analysis of the role of auxin in gravitropism.

(iv) Summary: Significance of Work Completed

The nifedipine experiments carried out in *Ceratopteris* indicate that the gravity-directed calcium current in single germinating spore cells is a key intermediate in the signaling steps that connect the gravity signal to an oriented response. To critically test this hypothesis it will be important to selectively disrupt the molecular machinery that drives this current and converts the calcium signal into polarity development. Such tests are greatly facilitated by our production of an extensive EST library and development of the RNAi method of suppressing gene expression. These are significant advances that should make it possible for us to learn the effects of blocking the expression of specific genes whose products would be expected to help drive the current or transduce its effects into growth responses.

Our molecular characterization of the *Arabidopsis* annexins and their expression pattern gives us critical leads on which annexins are most likely to be involved in growth control. Recent studies support the conclusion that annexins play a major role in the secretory activities needed for growth, and having knockouts of each annexin gene will allow a rigorous evaluation of that

prediction. The different expression patterns of annexins and their significantly different structures suggest that they will not have completely overlapping functions. Our acquisition of knockout mutants of each annexin gene will allow us to test this hypothesis by analyzing the phenotypes of each mutant.

Because of the central role of auxin transport in gravitropism, any clarification of the molecular mechanisms that control this transport will advance an understanding of gravity signaling in vascular plants. The recent discoveries that MDR-like transporters help to mediate auxin export from cells (Noh et al., 2001, *Plant Cell* **13**: 2441-2454), and that transmembrane ATP gradients can influence the efficiency of these transporters (Roux et al., 2001) provide potentially important insights on how auxin transport is controlled, and they help to rationalize the observation that significantly lowering the transmembrane ATP gradient severely disrupts both polar auxin transport and gravitropic growth (Tang et al., 2003). Experiments that clarify the interpretation of these novel discoveries and test predictions of the MDR-ATP gradient-auxin export model will reveal their value for an improved understanding of gravitropism, and such experiments are in progress.

Taken together, the results obtained during the term of this project provide both a data base and a rationale for further investigating the significance of the gravity-directed calcium current for gravity-directed growth in fern spores, the role of annexins in mediating gravity-directed secretory activity during gravitropic growth, and the role of annexins and ATP gradients in mediating the transport of auxin out of cells. This next series of experiments, which are in progress now with NASA support, will more clearly define the significance of what was accomplished in the NAG2-1347 project.

PROJECT SUPPORTED PUBLICATIONS

a. Refereed original research articles

- Chatterjee, A., Porterfield, D. M., Smith, P. and Roux, S. J.. 2000. Gravity-directed calcium current in germinating spores of *Ceratopteris*. *Planta* **210** : 607 -610.
- Clark, G., Rafati, D., Bolton, R., Dauwalder, M., and Roux, S. J. 2000. Redistribution of annexin in gravistimulated pea plumules. *Plant Physiol. Biochem.* **38**: 937-947.
- Hu, S., Brady, S., Kovar, D., Staiger, C., Clark, G., Roux, S. J. and Muday, G. 2000. Identification of plant actin-binding proteins by F-actin affinity chromatography. *Plant J.* **24**: 127-137.
- Clark, G., Sessions, A., Eastburn, D. and Roux, S. J. 2001. Differential expression of members of the annexin multigene family in Arabidopsis. *Plant Physiol.* **126**: 1072-1084.
- Kim, S.-H., Arnold, D., Lloyd, A. and Roux, S. J. 2001. Antisense expression of an Arabidopsis Ran-binding protein renders transgenic roots hypersensitive to auxin and alters auxin-induced root-growth and development by arresting mitotic progress. *Plant Cell* **13**: 2619-2630.
- Tang, W, Brady, S, Sun, Y, Muday, G, and Roux, S. J. 2003. Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. *Plant Physiol.* **131**: 147-154.
- Stout, S, Clark, G, Archer-Evans, S, and Roux, S.J. 2003. Rapid and efficient suppression of gene expression in a single-cell model system. *Plant Physiol.* **131**: 1165-1168.

- Kim, S.-H. and Roux, S. J.. 2003. An *Arabidopsis* Ran-binding protein, AtRanBP1c, is a co-activator of Ran GTPase-activating protein and requires the C-terminus for its cytoplasmic localization. *Planta* **216**: 1047-1052.

b. Review chapters

- Chatterjee, A. and Roux, S. J. 2000. *Ceratopteris richardii*: A productive model for revealing secrets of signaling and development. *J. Plant Growth Regulation* **19**: 284-289.
- Clark, G., Thompson, G. Jr., and Roux, S. J. 2001. Signal transduction mechanisms in plants: An overview. *Current Science* **80**: 181-188.
- Roux, S.J., Thomas, C. and Rajagopal, A. 2001. A role for ectoapyrases and extracellular ATP in plant growth and development. **In: *Signal Transduction in Plants. Current Advances.*** (Eds. S. K. Sopory, R. Oelmueller, and S. C. Maheshwari). Kluwer Academic / Plenum Publishers, New York.

c. Meeting abstracts

- Clark, G., Bolton, R., Dauwalder, M., and Roux, S. J. 2000. Biochemical characterization and immunolocalization of Arabidopsis annexins. Plant Biology '00. url: <http://www.rycomusa.com/aspp2000/public/P21/0838.html>
- Salmi, M., Bushart, T., Stout, S., and Roux, S. J. 2001. Analysis of gene expression during gravity-directed polarity fixation in *Ceratopteris richardii* spores. Program Abstracts, NASA Cell Science Conference, March 2001.
- Stout, S. , Salmi, M., Bushart T, Clark, G., Roux, S. 2001. Use of RNAi to characterize calcium signaling genes expressed in *ceratopteris richardii* during spore germination (Abstract). *Gravitational and Space Biology Bulletin* 15: 21.
- Roux, S., Tang, W., and Sun, S. 2001. Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport (Abstract). *Gravitational and Space Biology Bulletin* 15: 31.
- Tang, W., Sun, Y., and Roux, S. J. 2001. Extracellular ATP inhibits root gravitropism at concentrations that inhibit polar auxin transport. Abstract # 286, Plant Biology '01. url: <http://www.rycomusa.com/aspp2001/public/P30/0700.html>
- Clark, G., Dauwalder, M., Sessions, A., and Roux, S. J. 2001. Isolation and characterization of seven Arabidopsis annexin cDNAs. Abstract #49, Plant Biology '01. url: <http://www.rycomusa.com/aspp2001/public/P20/0691.html>
- Tang, W., Windsor, J., Lloyd, A., Roux, S. 2002. Transmembrane ATP gradients influence the ABC transporter-mediated transport of both auxin and xenobiotics out of cells (Abstract). *Comparative Biochemistry and Physiology* 132/A, Supplement 1.
- Archer-Evans, S., Clark, G., Stout, S., and Roux, S. J. 2002. Biochemical and molecular characterization of two different annexins in the fern *Ceratopteris* during early development. *Plant Biology* 2002. Abstract # 838. url: <http://abstracts.aspb.org/pb2002/public/P71/0691.html>
- Stout, S. and Roux, S. J. 2002. Use of RNA silencing to characterize calcium signaling genes expressed in *Ceratopteris richardii* during spore germination. *Plant Biology* 2002. Abstract # 833 url: <http://abstracts.aspb.org/pb2002/public/P71/0630.html>