Summary of Research

1) Title of the Grant: Calcium/Calmodulin-mediated Gravitropic Response in Plants

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Summary of Research

The goal of this project was to gain a fundamental understanding of how calcium/calmodulin-mediated signaling is involved in gravity signal transduction in plants. During the period of support, significant progress was made in elucidating the role of calmodulin and its target proteins in gravitropism. This laboratory has made breakthroughs by cloning and characterizing genes that are involved in calcium/calmodulin-mediated signaling. Some of these genes show altered expression under hypergravity and simulated microgravity conditions. A major advance was made in our attempts to understand gravity signal transduction by cloning and characterizing a catalase which requires calcium/calmodulin for its activation. Our results suggest that calcium/calmodulin have dual roles in regulating the level of hydrogen peroxide (H_{2}O_{2}), a signal molecule that plays a major role in gravitropism. It is well established that auxin plays a major role in gravitropism. Our results indicate that there is a “cross-talk” between calcium/calmodulin-mediated signaling and auxin-mediated signal transduction. Auxin-regulated SAUR proteins that are involved in gravitropism bind to calmodulin in a calcium-dependent manner. A novel chimeric calcium/calmodulin-dependent protein kinase was cloned and characterized and its role in gravity signal transduction was investigated. These studies have provided some answers to the fundamental questions about how signal molecules such as calcium, H_{2}O_{2}, and hormones such as auxin bring about the ultimate gravitropic response and the integral role of calmodulin in gravity signal transduction. This NASA-funded study has led to some spinoffs that have applications in solving agricultural problems. The Washington State University Research Foundation has obtained several patents related to this work.

Specific Accomplishments

1. **Plant catalase is a Ca^{2+}/CaM-binding protein and it plays a role in gravitropism by controlling hydrogen peroxide homeostasis:** Hydrogen peroxide (H_{2}O_{2}) is one of the toxic reactive oxygen species present in all aerobic organisms. Higher levels of H_{2}O_{2} accumulation in the cell causes significant cell damage unless dealt with properly. Recent studies, however, also support that H_{2}O_{2} acts as an intracellular signal to activate physiological responses to different stimuli. To allow for these different roles, cellular levels of H_{2}O_{2} must be tightly controlled. Catalase is one of the major anti-oxidant enzymes which breaks down H_{2}O_{2} into water and oxygen. By screening an *Arabidopsis* seedling cDNA expression library using ^{35}S-labeled CaM, catalase3 (AtCat3) was isolated and identified as a CaM-binding protein. The CaM-binding region was mapped (415 to 451 in the C-terminus) where high homology exists among plant catalases. However, there is no homology in this region between plant, bacterial and animal catalases, suggesting that CaM binds to plant catalases, but not to bacterial and animal catalases. To study the role of Ca^{2+}/CaM in regulating catalase activity, plant catalase was purified and used. In addition, the effects of Ca^{2+}/CaM in regulating human, bovine and *Aspergillus* catalase were studied. *In vitro* activity assays revealed that Ca^{2+}/CaM increased plant catalase activity by
~ 2.5 fold. Ca^{2+} or CaM alone had no effect on enzyme activity. Ca^{2+}/CaM did not activate human, bovine or Aspergillus catalases. Our results demonstrated that Ca^{2+}/CaM controls the breakdown of cellular H_{2}O_{2} by binding to and activating plant catalases. Other reports suggest that Ca^{2+}/CaM may indirectly regulate NADPH oxidase leading to the production of H_{2}O_{2} via modulation of NAD kinase activity. Based on these findings, we have proposed a model for a dual regulation of the level of cellular H_{2}O_{2} by Ca^{2+}/CaM and the significance of this dual regulation in gravitropism.

Calcium is known to alter directional growth in plants and plays a critical role in gravitropism. To test the effect of H_{2}O_{2} on gravitropic response in maize roots, 3-day-old seedlings were dipped in the H_{2}O_{2} solution at different concentrations for varying periods. They were then rinsed in distilled water and the seedlings were arranged horizontally on the plain agar medium. 1 mM H_{2}O_{2} treatment blocked the gravitropic bending. To test whether the interaction between Ca^{2+} signaling and H_{2}O_{2} signaling exists, we studied root gravitropic responses after placing the agar block containing both 10 mM CaCl_{2} and 10 mM H_{2}O_{2} on the upper surface of the root tip. The agar plates were then arranged so that the roots were in a horizontal position. Surprisingly, the root oriented towards the gravity (downward) instead of towards the Ca^{2+} or H_{2}O_{2} source. For controls, plain agar blocks were placed on the upper side of the root tips. Photographs were taken to study the root orientation. Our results suggest that there is an interrelationship between Ca^{2+}/CaM and H_{2}O_{2}, which triggers a series of events leading to the gravitropic response. A model describing the interaction between Ca^{2+}/CaM/H_{2}O_{2} in triggering gravitropic response is shown below.

![Model diagram](image)

2. Evidence for the involvement of Ca^{2+}/CaM-mediated signaling in auxin-induced gravitropism: The use of ^{35}S-labeled CaM to screen a corn root cDNA expression library has led to the isolation of a CaM-binding protein, encoded by a cDNA with sequence similarity to
small auxin up RNAs (SAURs), a class of early auxin-responsive genes. The cDNA designated as ZmSAUR1 (Zea mays SAURs) was expressed in E. coli and the recombinant protein was purified by CaM affinity chromatography. The CaM-binding assay revealed that the recombinant protein binds to CaM in a Ca$^{2+}$-dependent manner. Deletion analysis revealed that the CaM-binding site was located at the N-terminal domain. A synthetic peptide of amino acids 20-45, corresponding to the potential CaM-binding region, was used for Ca$^{2+}$-dependent mobility shift assays. The synthetic peptide formed a stable complex with CaM only in the presence of Ca$^{2+}$. The CaM affinity assay indicated that ZmSAUR1 binds to CaM with high affinity ($K_d$, 15 nM) in a Ca$^{2+}$-dependent manner. Comparison of the N-terminal portions of all the characterized SAURs revealed that they all contain a stretch of the basic $\alpha$-amphiphilic helix similar to the CaM-binding region of ZmSAUR1. CaM binds to the two synthetic peptides from the N-terminal regions of Arabidopsis SAUR-AC1 and soybean 10A5, suggesting that this is a general phenomenon for all SAURs. Northern analysis was carried out using the total RNA isolated from auxin-treated corn coleoptile segments. The ZmSAUR1 gene expression began within 10 min, increased rapidly between 10-60 min, and peaked around 60 min following 10 $\mu$M NAA ($\alpha$-naphthaleneacetic acid) treatment. These results indicate that ZmSAUR1 is an early auxin-responsive gene. The CaM antagonist, W-7, inhibited auxin-induced cell elongation, but not auxin-induced expression of ZmSAUR1. This suggests that Ca$^{2+}$/CaM do not regulate ZmSAUR1 at the transcriptional level. CaM binding to ZmSAUR1 in a Ca$^{2+}$-dependent manner suggests that Ca$^{2+}$/CaM regulate ZmSAUR1 at the post-translational level. Our data provide the first direct evidence for the involvement of Ca$^{2+}$/CaM-mediated signaling in auxin-mediated signal transduction. There are more than 20 SAURs in the Arabidopsis genome. However, we observed that only five of them share high homology with ZmSAUR1. Since roots also respond to auxin, we expected that SAURs will be induced during root gravitropism.

3. Effects of hypergravity and simulated microgravity on Ca$^{2+}$/CaM-regulated genes: We have performed ground-based studies to provide evidence that Ca$^{2+}$/CaM play a central role in gravity signal transduction using facilities at the Dutch Experiment Support Center which specializes in acceleration research. We have studied the effects of hypergravity and simulated microgravity on genes that are involved in Ca$^{2+}$/CaM-mediated signaling. To study the effects of hypergravity on gene expression, plants were subjected to 10 x earth gravity for varying periods (5 hrs to 5 days) using the MidiCAR centrifuge. To study the effects of simulated microgravity on gene expression, the Random Positioning Machine (RPM) was used (ranging from 5 hrs to 5 days). To study these effects of simulated microgravity and hypergravity on the expression pattern of catalase, SAURs and AtSRs, RT-PCR analysis was performed using gene-specific primers, which were designed from the least conserved regions of each gene. The Arabidopsis actin 8 gene (ACT8) was used as a positive internal control. PCR primers for detection of ACT8 mRNAs were 5'- ATGAAGATTAAGGTCGTGGC-3' and 5'-TCCGAGTTTGAAGAGGCTAC-3'. Total RNA from two week old entire seedlings was treated with RNase-free DNase (GIBCO-BRL). Both cDNA synthesis and PCR amplification were performed (25 ng total RNA) using gene specific primers (SuperScript One-Step RT-PCR, GIBCO-BRL). To maintain the amplification of the internal control and our genes within the exponential phase, the number of
PCR cycles was adjusted to 25 cycles for ACT8, catalase and SAUR and 35 cycles for all AtSR genes (see also Final Summary of Research for NAG5-11364). The amplified PCR products (9 μl) were electrophoresed on a 1.5 % (w/v) agarose gel, stained with ethidium bromide, and scanned using an image analyzer. Preliminary results revealed an altered expression pattern of these genes regulated by simulated microgravity and hypergravity (see Fig. 5). For example, among three catalases, only one (AtCat1) showed a positive response to simulated microgravity treatment. Among six AtSRs, only four (AtSR1,2,5,6) showed detectable changes. Interestingly, expression of the SAURs (AtSAUR1,2) was not clearly detected when seedlings were exposed to microgravity, suggesting that simulated microgravity has altered auxin redistribution/transport. The PI recently presented a report on some of the recent findings at the joint meeting of the European Space Agency (ESA) and the International Society for Gravitational Physiology (ISGP) meetings in Stockholm, Sweden in June 2002. A manuscript summarizing these results is currently in press.

Publications resulting from this support:


**Book Chapter:**


**Published Abstracts:**


**Patent received during this grant period:**

Assignee: Washington State University Research Foundation, Inc., Pullman, WA.