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FINAL REPORT

Enhanced labeling techniques to study the cytoskeleton during root growth and gravitropism

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I. Introduction

Gravity affects the growth and development of all living organisms. One of the most obvious manifestations of gravity's effects on biological systems lies in the ability of plants to direct their growth along a path that is dictated by the gravity vector (called gravitropism). When positioned horizontally, inflorescence stems and hypocotyls in dicots, and pulvini in monocots, respond by bending upward whereas roots typically bend downward. Gravitropism allows plants to readjust their growth to maximize light absorption for photosynthesis and to more efficiently acquire water and nutrients from the soil (Blancaflor and Masson, 2003). Despite its significance for plant survival, there are still major gaps in understanding the cellular and molecular processes by which plants respond to gravity. The major aim of this proposal was to develop improved fluorescence labeling techniques to aid in understanding how the cytoskeleton modulates plant responses to gravity.

II. Significance of the Research

To achieve the space exploration agenda of NASA, basic research in plant biology, including research on mechanisms by which plants respond to gravity, will continue to be essential. This is because plants are a key component of an advanced life support system not only for the (re)generation of resources but also for ensuring the psychological well-being of astronauts during long-term space missions, which are risk areas identified in the Bioastronautics Critical Path Roadmap. The cytoskeleton in particular is an attractive cellular system to investigate within the context of the space exploration program because of its importance in several fundamental cellular processes including the establishment of plant form and orientation. These traits are critical for the targeted improvement of plants not only to increase yields for food production but also to modify plant stature (i.e. generating dwarf plants) to reduce biomass in an advance life support system needed for extended space missions.

III. Research Accomplishments

This NASA funded project led to the publication of (10) peer reviewed papers (see **section V** below for a detailed list of publications) with three of the ten papers featured on the journal cover (e.g. Blancaflor, 2002; Mitra et al., 2003; Hou et al., 2004). The highlights of these publications are summarized in the following sections.

A. Disruption of the actin cytoskeleton enhances the sensitivity and response of roots to gravity

From this research we have shown that the actin cytoskeleton is likely to have a role in regulating plant gravitropism that is different from the predictions of current models. For instance, a popular explanation for actin's involvement in plant gravity sensing and signaling is that sedimenting amyloplasts (statoliths) pull on actin filaments that are linked to mechanosensitive ion channels in the plasma membrane. The activation of these channels then results in the influx of ions into the cytoplasm initiating a chain of events leading to organ bending (Baluska and Hasenstein, 1997; Blancaflor, 2002; Blancaflor and Masson, 2003). However, studies spanning several years indicate that the above model may need revision since plants with a disrupted actin cytoskeleton (Blancaflor and Hasenstein, 1997) or knockouts to actin genes (Gilliland et al., 2003) are still capable of initiating a gravitropic response (i.e. organs still bend upon horizontal reorientation). Most significant to this project were reports showing that disrupting the actin cytoskeleton with pharmacological agents induced a promotive rather

than an inhibitory effect on shoot gravitropism (Yamamoto and Kiss, 2002; Yamamoto at al., 2002). These observations are therefore contrary to models proposing that the actin cytoskeleton is involved in 'turning on' gravity signaling and response in plants.

Funding from NASA has allowed us to show that the promotive effect of actin disruption on gravitropism is even more dramatic in roots. By conducting a detailed analysis of maize root gravitropism after altering the actin cytoskeleton with the potent actin disrupting drug

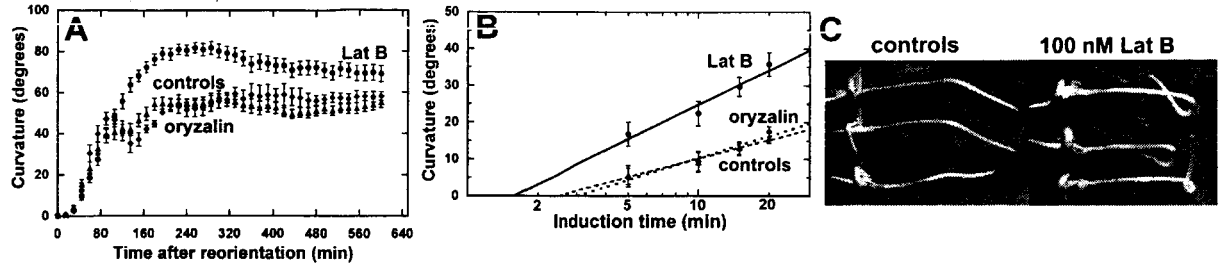

Fig. 1 (A). Time course of curvature of maize roots treated with 1 μM Lat B, 1 μM oryzalin and corresponding DMSO controls after a 90 degree horizontal reorientation. Note the enhanced gravitropic curvature of maize roots treated with Lat B but not with oryzalin. (**B**) Presentation time analysis of maize roots treated with 1 μM Lat B or oryzalin. The intercept of the regression line with the x-axis provided an estimate of the presentation time. Presentation time was 2.45 min for controls, 3.02 min for oryzalin- and 1.58 min for Lat B-treated roots. The smaller presentation time value of Lat B-treated roots indicates increased gravisensitivity. (**C**) Representative images of maize roots showing the extensive curvature response to Lat B after a short 10 min gravistimulus followed by 12 h of rotation on a clinostat. Note that roots treated with Lat B curve past the horizontal while control roots eventually straighten.

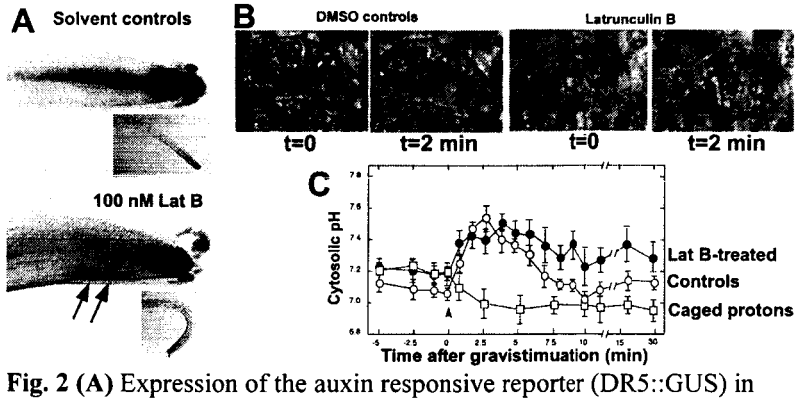

Fig. 2 (A) Expression of the auxin responsive reporter (DR5::GUS) in clinorotated roots treated with 100 nM Lat B. Roots were given a 15 min horizontal stimulus before clinorotation. At 3 h after clinorotation GUS expression on the lower side (arrows) was observed in Lat-B treated roots but not in DMSO controls. Low magnification images (inset) show that Lat-B treated roots curve strongly while control roots would eventually straighten. (**B**) Amyloplasts sediment faster in Lat B-treated *Arabidopsis* roots. Note that immediately after rotating roots 180 degrees, amyloplasts in Lat B-treated roots already begin to move downward (arrow). After 2 min a couple of amyloplasts in LatB-treated columella cells have already reached the bottom of the cell (arrow). (**C**) Lat B treatment extends the cytosolic alkalinization of the columella induced by gravistimulation. Roots were untreated (control), or treated with 100 nM Lat B or 100 nM Lat B + 20 μM caged protons. Note the transient alkalinization upon gravistimulation in the control that is extended by Lat B treatment.

Latrunculin B (Lat B), we have shown that both the sensitivity and response of roots to gravity is significantly increased (Fig. 1A,B; Hou et al., 2003). The enhanced gravity response was most pronounced when Lat B-treated roots were given a brief period of horizontal stimulation (ca. 10 min) followed by extended periods of rotation on a clinostat to randomize the gravity vector. Under such conditions, Lat B-treated roots would continue to curve strongly in the direction of the original gravity vector while control roots would bend weakly and in proportion to time of horizontal stimulation (Fig. 1C; Hou et al., 2003; Blancaflor et al., 2003a). The enhancement of gravitropism

was not observed when the microtubule cytoskeleton was disrupted with oryzalin (Hou et al., 2003). These observations led us to propose that disrupting F-actin may be affecting the reset mechanisms that prevent the root from overshooting the vertical (Hou et al., 2003; Blancaflor and Masson, 2003). Alternatively, these results could indicate that Lat B-treated roots remember the initial gravity stimulus despite randomizing the gravity signal with a clinostat. Although curvature of the root under these conditions is generally considered to be indicative of gravity sensing, this response could reflect the maintenance of the memory of the initial gravity vector. Without an intact and/or dynamic actin cytoskeleton, the initial gravity signal is locked, causing plants to overshoot the vertical.

To probe further into the cellular mechanisms underlying the enhanced gravity response of roots induced by actin disruption, we conducted studies with seedling roots of *Arabidopsis* so we could exploit the molecular tools available in this model plant. We studied cellular events that are well known to be associated with root gravity responses including the development of asymmetric auxin gradients across opposite flanks of the root (Rashotte et al., 2001), cytoplasmic pH signaling in the gravity sensing columella cells of the root cap (Scott and Allen, 1999; Fasano et al., 2001), and amyloplast dynamics and sedimentation in the columella cells (Blancaflor et al., 1998; MacCleery and Kiss, 1999). Our results show that all three of these events were modified by Lat B-treatment indicating that the actin cytoskeleton is involved in modulating these events (Fig. 2; Hou et al., 2004). For example, indirect visualization of auxin redistribution using DR5::GUS, an auxin responsive reporter (Ulmasov et al., 1997), revealed that the stronger curvature response of roots upon disrupting the actin network was accompanied by a persistent lateral auxin gradient (Fig. 2A; Hou et al., 2004). Actin disruption also increased the rate of amyloplast sedimentation in the columella and extended the duration of the gravity-induced pH changes in the cap, both of which are linked to the initial events of gravity perception and signaling (Fig. 2B,C; Hou et al., 2004). The amplification of these signals when actin was disrupted with Lat B is therefore consistent with actin's role as a down regulator of gravity signaling in roots. Defining how the actin cytoskeleton functions in a structural/motor role to constrain amyloplast motility and how it is involved in regulating the signaling/response elements that contribute to the sustained gravitropic response will be a topic for future research.

B. A novel fluorescent protein reporter allows improved labeling of actin dynamics and organization in living plant cells

Another accomplishment from this NASA funded research was the development of improved methods for imaging the cytoskeleton in fixed and living plant tissue. In addition to plant gravitropism, these improved techniques have allowed the PI's lab to expand into other areas of plant biology including lipid mediated regulation of seedling development (Blancaflor et al., 2003b) and studies of viral cell to cell movement (Mitra et al., 2003). Historically, the PI has primarily utilized immunofluorescence microscopy for his studies on the plant cytoskeleton (e.g. Blancaflor and Hasenstein, 2000; Collings et al., 2001, Blancaflor et al., 2003b). Although these techniques continue to be an integral tool for such studies, the fact that the cytoskeleton is a highly dynamic entity makes it essential that we observe the organization of these structural proteins within the context of the living cell. The discovery of green fluorescent protein (GFP) from the jellyfish *Aequoria victoria* has opened a new dimension in studies of the plant cytoskeleton since it allowed the observation of cytoskeletal dynamics in living, functioning plant cells without the technical difficulties associated with microinjection (Blancaflor and Gilroy, 2000). GFP was first used in studies of the plant cytoskeleton through its fusion with the

microtubule binding domain (MBD) of the mouse microtubule associated protein 4 (GFP-MBD, Marc et al., 1998). Following this pilot report, a series of significant breakthroughs were made toward understanding the dynamic behavior of the microtubule cytoskeleton in plants (Dixit and Cyr, 2002; Dhonukshe and Gadella, 2003; Vos et al., 2004).

In recent years, GFP fusions to talin, a mammalian F-actin binding protein (McCann and Craig, 1997), has been the most widely used reporter to image actin organization in living plant cells (Kost et al., 1998). Although this reporter has been utilized for studies on the actin cytoskeleton in diverse cell types (Fu et. al., 2001, 2002; Jones et al., 2002; Mathur et al., 2003) there have been no reliable reports thus far of its utility for root cell biology. Furthermore, the quality of actin labeling obtained from these talin based reporters particularly for imaging actin in stable plant lines are being questioned (El-Assal et al., 2004). Thus the GFP-talin reporter may not be the best probe for imaging actin *in vivo* because it may not accurately depict the true state of actin organization and dynamics in plant cells.

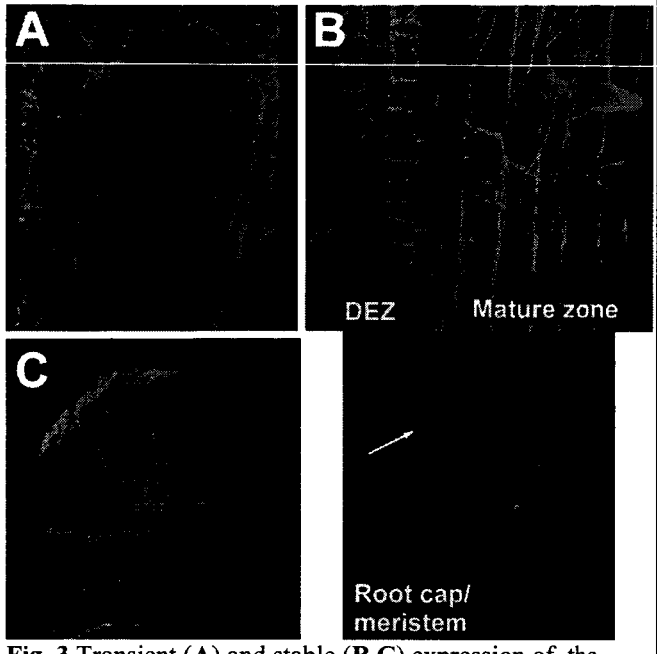

Fig. 3 Transient (**A**) and stable (**B,C**) expression of the ABD2-GFP fusion in an onion epidermal cell (**A**), *Arabidopsis* roots (**B**) and tobacco BY2 cells (**C**). In roots, one could observe filamentous actin in different developmental regions including the distal elongation zone (DEZ), mature zone and root cap/meristem area. Structures not revealed by the more popular GFP-talin such as phragmoplasts (arrow) can also be observed (Wang et al., 2004).

Through funding from NASA, we developed a set of novel *in vivo* F-actin reporters that are likely to further advance research on the plant actin cytoskeleton, particularly as it pertains to root development and gravitropism (Fig. 3; Wang et al., 2004). These reporters are based on GFP fusions to *Arabidopsis* fimbrin 1 (AtFim1), an F-actin cross-linking protein that contains two actin binding domains (ABDs) arranged in tandem within a single polypeptide chain (McCurdy and Staiger, 2000). We showed that GFP fusions to truncated variants of the AtFim1 protein, specifically ABD2, can decorate F-actin in living cells and allow the visualization of a finer and more dynamic F-actin network compared to the more popular Talin-GFP (Fig. 3A; Wang et al., 2004). More importantly, stable expression of these Fimbrin based GFP reporters in *Arabidopsis* allowed imaging of F-actin in different developmental regions of the root (Fig. 3B) including the root cap, which is important for our studies on gravitropism. Also, we have generated tobacco BY2 cells (Fig. 3C) and hairy roots of *Medicago truncatula* (data not shown) stably expressing these newly developed actin markers paving the way for detailed studies of actin turn over and dynamics in other important plant biological systems. Since we first published on this new F-actin reporter gene (Wang et. al., 2004), we have had numerous requests for this construct from various researchers in the plant science community.

C. Fluorescent protein reporters for auxin gradients and amyloplast dynamics in graviresponding roots

In recent years, there has been accumulating evidence supporting the classical Cholodny-Went theory, which proposes that a lateral gradient of auxin induces organ curvature by promoting differential cellular growth on opposite flanks of a horizontally positioned plant organ (Friml, 2003). In roots for instance, basipetal auxin transport (i.e. from the root tip to the elongation zone) regulates gravitropism and this transport stream appears to be modulated by protein phosphorylation (Rashotte et al., 2001). Through NASA funding we have used the auxin reporter DR5::GUS (Ulmasov et al., 1997) to visualize auxin gradients in graviresponding *Arabidopsis* roots (see Fig. 2A; Hou et al. 2004). Several investigators have also used this reporter as an indicator of auxin response and redistribution during gravistimulation (e.g. Rashotte et al., 2001; Boonsirichai et al., 2003; Buer and Muday, 2004). We have independently created a GFP based auxin reporter (called DR5::mGFP-ER; Fig. 4), which is somewhat similar to the auxin sensor described in Ottenschlager et al. (2003). This reporter has allowed us to better analyze auxin response and (re)distribution patterns in living plant roots. For instance, roots of *Arabidopsis* harboring the DR5::mGFP-ER fusion showed a significant increase in GFP fluorescence in auxin-treated roots compared to roots treated with the solvent control solution (Fig. 4A). More importantly, gravistimulated roots exhibited an increase in fluorescence intensity on the lower side of the root after 1 h of gravistimulation (Fig. 4B), which was faster than the time it took for us to observe these gradients using the DR5::GUS construct (compare Fig. 2A and 4B).

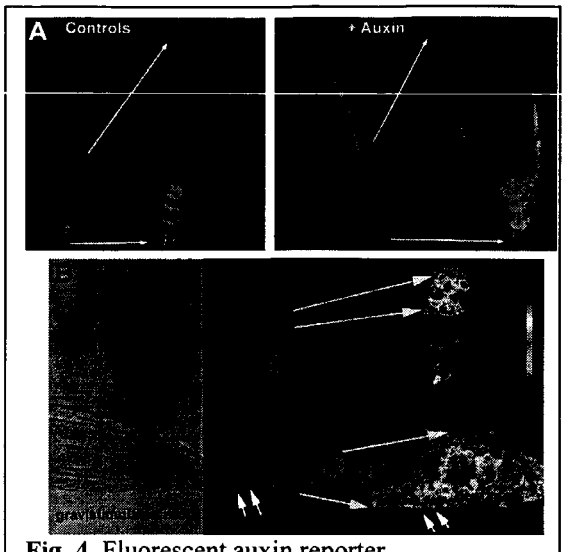

Fig. 4. Fluorescent auxin reporter (DR5::mGFP-ER) expression in roots of *Arabidopsis*. (**A**) In control roots, fluorescence emission is limited to the columella cells of the root cap. Treatment with exogenous auxin (IAA) results in increased fluorescence emission throughout the root. (**B**) Brightfield and corresponding fluorescence/pseudocolored images of vertical and gravistimulated roots harboring the DR5::mGFP-ER construct. One hour after gravistimulation, increased fluorescence on the lower flank of the root was observed (double white arrows). Red on pseudocolored image indicates increased fluorescence.

We have also generated transgenic lines of *Arabidopsis* stably expressing RecA-GFP fusion, which targets to the plastids (Kohler et al., 1997). In our transgenic plants, the RecA-GFP was placed under the control of a root cap specific promoter (RCP1) allowing the fluorescent visualization of amyloplasts in the root caps of *Arabidopsis* seedlings. Time lapse confocal microscopy of these GFP decorated amyloplasts show that they have similar dynamics to amyloplasts visualized by bright field optics (e.g. Fig. 1B; Hou et al., 2004a). With fluorescently tagged amyloplasts it should now be possible to further analyze amyloplast dynamics in relation to the state of actin organization (Fig. 3) or auxin redistribution (Fig. 4) using multicolor fluorescence microscopy.

IV. Peer-reviewed publications resulting from this award (NASA funding acknowledged)

1.Shin H, Shin H-S, Guo Z, Blancaflor EB, Masson PH, Chen R (2005) Complex regulation of Arabidopsis AGR1/PIN2-mediated root gravitropic response and basipetal auxin transport by catharidin-sensitive protein phosphatases. *Plant Journal* 42: 188-200

*2.Hou G, Kramer VL, Wang Y-S, Chen R, Perbal G, Gilroy S, Blancaflor EB (2004) The promotion of gravitropism in Arabidopsis roots upon actin disruption is coupled with the extended alkalinization of the columella cytoplasm and a persistent lateral auxin gradient. *Plant Journal* 31: 113-125 –cover picture

3.Wang Y-S, Motes CM, Mohamalawari DR, Blancaflor EB (2004). Green fluorescent protein fusions to Arabidopsis fimbrin 1 for spatio-temporal imaging of F-actin dynamics in roots. *Cell Motility and the Cytoskeleton* 59: 79-93

4.Hou G, Hill JP, Blancaflor EB (2004) Developmental anatomy and auxin response of lateral root formation in *Ceratopteris richardii*. *Journal of Experimental Botany* 397: 685-693

5.Blancaflor EB, Hou G, Chapman KD (2003) Elevated levels of *N*-Lauroylethanolamine, an endogenous constituent of desiccated seeds, disrupt normal root development in *Arabidopsis thaliana* seedlings. *Planta* 217: 206-217

6.Hou G, Mohamalawari DR, Blancaflor EB (2003) Enhanced gravitropism of roots with a disrupted cap actin cytoskeleton. *Plant Physiology* 131:1360-1373

7.Blancaflor EB, Hou G, Mohamalawari DR (2003) The promotive effect of latrunculin B on gravitropism of maize roots is concentration dependent. *Advances in Space Research* 31:2215-2220

*8.Mitra R, Krishnamurthy K, Blancaflor EB, Payton M, Nelson RS, Verchot-Lubicz J (2003) The potato virus *X* TGBp2 protein association with the endoplasmic reticulum plays a role in but is not sufficient for viral cell-to-cell movement. *Virology* 312: 35-48

9.Blancaflor EB, Masson PH (2003) Update on Plant gravitropism. Unraveling the ups and downs of a complex process. *Plant Physiology* 113: 1677-1690

*10.Blancaflor EB (2002) The cytoskeleton and gravitropism in higher plants. *Journal of Plant Growth Regulation* 21: 120-136

*Featured on the journal cover

V. Subject Inventions

Various fluorescent probes for in vivo imaging of F-actin in living plant cells

VI. Scientific meetings/invited seminars where NASA funded research was presented and acknowledged

A. Invited seminars

1. Biology Department seminar series, University of North Texas, Denton, TX, October, 2001

2. 34th Committee on Space Research (COSPAR) Scientific assembly, Houston, TX, October, 2002

3. Biology Department seminar series, Texas Woman's University, Denton, TX, November, 2002
4. Gordon Research Conference: Mechanotransduction and Gravity Signaling in Biological Systems, Connecticut College, New London, CT, July 2003

5. Biology Department seminar series, Texas A.M. University, College Station TX, December, 2003

6.Botany Department seminar series, Oklahoma State University, Stillwater, OK, March, 2004

7. 15th Symposium in Plant Physiology, Pennsylvania State University, State College, PA, May, 2004

8. Botany Department seminar series, University of Oklahoma, Norman, OK, September, 2004

9. Department of Environmental and Plant Biology seminar series, Ohio University, Athens, OH, October 2004

10. School of Biological Sciences seminar series, Washington State University, Pullman, WA, November, 2004

11.Department of Biology seminar series, Oklahoma Christian University, Oklahoma City, OK, January, 2005

B. Participation at scientific meetings

Results from this were presented at various national meetings throughout the funding period. Poster and oral presentations were given at the American Society of Plant Biologists (ASPB), American Society for Gravitational and Space Biology (ASGSB) and American Society of Cell Biology (ASCB) annual meetings. Moreover, presentations were made to the Oklahoma and Texas Society for Microscopy.

C. Presentations to general public/K-12/Outreach

Several presentations about this NASA funded research were made to the general public and K-12 students/teachers. For example, the PI has given presentations about new cellular imaging technology to diverse groups including 5th grade students at the Ardmore Plainview School, Ardmore high school chemistry students, the Ardmore adult day care center, Noble Foundation retirees and the Astronomy science academy for Oklahoma high school students. The PI's projects that were funded through this NASA project were always featured in his presentations. Also, the Foundation routinely hosts visits by local K-12 students. The stunning visual impact from images produced by modern bio-imaging technology made the PI's lab and his NASA funded research one of the highlights of these visits. For instance, the Noble Foundation sponsors a public lectures in science series and one of the lectures in this series, entitled "modern microscopes and the marvels of plant motion", was given by the PI. This lecture provided the Ardmore community an overview of how modern light microscopes are revolutionizing the way in which scientists are able to study cells and how these techniques impact space biological research. This NASA funded research was also featured on local and state newspapers increasing further the public's awareness of space research particularly in a region that is in need of improved science education.

VII. References

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