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Key Personnel Changes/Previous PI:			
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Performance Goal Text:

We propose a multi-omics study using the model plant *Arabidopsis* under both 1g and in microgravity conditions (spaceflight). Our approach spans comprehensive phenomics, metabolomics, transcriptomics, and proteomics strategies, and is incisively and uniquely melded via deployment of an integrated computational biology (ICB) approach we are pioneering. Our plant lines include wild type, various mutants we have generated with different lignin amounts through manipulation of the multigene family encoding arogenate dehydratases, and other lines enhanced in carbon assimilation capacity, and combinations thereof. We consider this places us in an unprecedented position to investigate how plants function in altered gravity environments.

We are very well positioned for incisive spaceflight and definition stage (1g) studies to investigate gene/metabolic network relationships and adaptations resulting from varying lignin and carbon assimilation levels, e.g., on photosynthesis; C allocation; water use efficiency (WUE); vascular plant growth/development; vasculature performance; auxin transport; and gravitational adaptations. Our overarching hypothesis is that a comprehensive interrogation (an integrative omics study) of our *Arabidopsis* lines with varying lignin levels and/or modulated carbon concentrating mechanisms (CCMs) or combination thereof will identify gene/metabolic networks, mechanisms and/or pathways that are differentially modulated at 1g and on exposure to microgravity, i.e., various omics (phenomics, transcriptomics, genomics, proteomics, metabolomics, and ICB) will allow us to study these in a truly unprecedented way.

Overall objectives:

1. Establish multi “omics” effects of modulating lignin and CCM levels i) at 1g and ii) in spaceflight.
2. Compare/contrast data, using an ICB approach, to better define and understand gravity sensing and responses, and if threshold/induction parameters are modified or changed, when lignin and CCM levels are varied.

Task Description:

More specifically, we address distinct hypotheses for our various teams, and integrate, dissect, and incisively analyze them holistically in a manner hitherto not possible. These 5 hypotheses include that: modulating lignin and CCM levels differentially affect carbon assimilation/re-allocation, photosynthesis, and WUE (Team 1); modulating lignin and CCM levels differentially affect secondary and primary metabolite levels (metabolomics) (Team 2); system-wide modification in the transcriptome occurs through a common transcriptional regulatory mechanism, and transcriptome/proteome “discrepancies” result from over-simplification of transcript analyses (Team 3); differential alterations in lignin and CCM levels can often be attributed to overall distinct changes in protein expression and phosphorylation patterns in a defined set of proteins (Team 4); an integrated omics analysis will provide urgently needed new insights into global effects on plant biological processes at both 1g and in microgravity (Teams 1-4). Each hypothesis draws upon the most advanced technologies available for study. We consider that our ICB approach will transform omics analysis through our advanced instrumentation and analytical tools. We will utilize (or design) computational tools/mathematical algorithms for integration and correlation of high resolution phenotype measurements (phenomics) with 'low' resolution global subcellular system measurements (transcriptomics, etc.) through 'nth' dimensional analysis.

Our study aligns with Research Emphasis 1 and 3, and decadal survey elements in Cell, Microbial, and Molecular Biology (CMM-3, CMM-5), Organismal and Comparative Biology (OCB 2-5), Developmental Biology (DEV-4), and Plant and Microbial Biology, chapter 4 (P2). Our data generation will also be seamlessly integrated with various web-based platforms to handle, disseminate, and inter-actively utilize through iPlant and OpenMSI, and thus are made available to NASA as well as being a community resource.

Rationale for HRP Directed Research:**Research Impact/Earth Benefits:**

This research will provide NASA and science in general with the first “big ‘omics’ data” analysis, integration, and assessment – at the gene, protein, and metabolic outcome levels – as to how microgravity alters the basic responses of plants when the influence of gravity is removed/minimized. This will serve as the foundation “omics” analyses in subsequent spaceflight and colonization experiments in space, as well as shedding new insights into the manifold effects of gravity during plant growth and development.

Oral Presentations:

"An integrated omics guided approach to lignification and gravitational responses: Current progress and the ISS APH prototype (PH-01)." NASA Lunch Time Meeting, November 24, 2020 (Virtual Seminar).

Our ongoing investigation consisted of two *Arabidopsis* grow-outs on International Space Station (ISS), with plants grown and monitored in the Advanced Plant Habitat (APH), with frozen specimens returned to Earth for multi-omics analyses. The corresponding Ground control growth experiments were carried out at Kennedy Space Center (KSC) by our KSC-affiliated collaborators in a second APH unit. The overall study had six *Arabidopsis* lines, one wild type (WT) line for the four lines of lignin-reduced arogenate dehydratase mutants (adt), with these encompassing a single mutant (adt5), a double mutant (adt4/5), a quadruple mutant (adt3/4/5/6), as well as a second WT line used to generate a carbon capture mechanism CCM mutant, and an adt3/4/5/6/CCM quintuple mutant. The ADT mutants differed in their levels of lignin reduction, relative to WT, with the largest reduction in lignin content being with the quadruple mutant (adt3/4/5/6).

1. ISS and KSC Grow-outs

- The first grow-out in the APH (APH-01) was initiated by NASA astronaut Ricky Arnold on ISS on June 8, 2018 (Day 0). On June 22, 2018 (Day 14), manual FluorPen photosynthesis measurements were carried out, followed by *Arabidopsis* seedlings thinning to give one viable plant per growth site in the APH. “Thinnings,” to remove “excess” numbers of seedlings, were placed in foil, immediately frozen, and stored in the GLACIER freezer at –160 °C on ISS.

- Spaceflight environment effects were observed with the remaining plant lines growing in APH-01. Following the thinning operation, several plants unexpectedly died, and others grew somewhat smaller in size. The impact on our experiment was that only one harvest at 45 days was possible in order to ensure sufficient plant material availability for our multi-omics analysis. FluorPen photosynthesis measurements were, however, again conducted after 31 days (4 weeks and 3 days) of growth (July 9, 2018). All remaining plants were finally harvested on ISS on July 23, 2018, and immediately frozen and stored in the GLACIER freezer at –160 °C (as for the thinnings). The frozen ISS-grown plant specimens were returned to Earth on January 13, 2019, and transported/delivered frozen to the Institute of Biological

Chemistry (IBC), Washington State University (WSU), on January 15, 2019.

- The corresponding Ground control APH-01 study, at Kennedy Space Center (KSC), was initiated a week later (June 15, 2018; Day 0), with FluorPen photosynthesis measurements/thinnings done on June 29, 2018 (Day 14). FluorPen photosynthesis measurements were again carried out on July 13, 2018 (Day 28), with final harvest on July 27, 2018 (Day 42). Ground control plants were transported to Institute of Biological Chemistry (IBC), Washington State University (WSU) on January 25, 2019.

- The second ISS grow-out was initiated by astronaut Serena Auñón-Chancellor on September 18, 2018. Following FluorPen photosynthesis measurements, the subsequent thinning operation on ISS (October 2, 2018) removed the “excess” plants that were immediately frozen and stored in the GLACIER freezer at -160°C . The thinning procedure may have adversely affected growth/development of some Arabidopsis plants in APH-01 with several again dying after thinnings had been harvested. FluorPen measurements were carried out on ISS on October 19, 2018 (Day 31). Slightly slower Arabidopsis growth was observed in the ISS APH facility, relative to our Ground control expectations, with only a single harvest at ~6 weeks (November 2, 2018; Day 45) being possible, in order to obtain the required plant material for our multi-omics analysis. The 6 week old harvested plants were immediately frozen and stored in the GLACIER freezer at -160°C . All frozen specimens were returned to Earth on January 7, 2020, and transported/delivered frozen to IBC/WSU on January 09, 2020.

- The corresponding Ground control was initiated at KSC a week later (September 25, 2018; Day 0), with FluorPen photosynthesis measurements/thinning occurring on October 9, 2018 (Day 14), FluorPen photosynthesis measurements were again conducted on October 26, 2018 (Day 31), with the final harvest being on November 9, 2018 (Day 45). The Ground control plants were transported/delivered to IBC/WSU on January 09, 2020.

- Covid-19 Impacts: The Progress Report herein for this reporting period focuses upon the continued analyses of Arabidopsis plants on ISS and KSC (Ground controls) from both Grow-out #1 and Grow-out #2. Progress was, however, significantly delayed due to Covid-19. Disruptions included personnel being unable to work for extended periods of time, high-end instrumentation required for our multi-omics analyses being inaccessible due to restrictions on working, and manufacturer delays in providing materials and supplies.

2. Consortium Member David Hanson (University of New Mexico, UNM) – Morphology

Estimated colorimetric lignified vasculature distribution analyses in inflorescence “stem” cross-sections using both staining and microscopy were completed. Samples were processed by standard microscopy sectioning protocols with Toluidine Blue (TBO) staining, which stains the lignified vasculature blue and other non-lignified tissue pinkish red. These different staining patterns were used to estimate the ratio of lignified vasculature to non-lignified tissue in images.

Grow-out #2

The tendency of growing stems in the ADT genotypes to turn toward the Science Carrier base was similar to Grow-out #1. ADT knockout mutants with less stained vasculature had stems touching the base of the Science Carrier faster than WT or WT-CCM lines. Stem cross-sectional area was also variable between both Ground and ISS samples, but both showed expected patterns of colorimetric lignified vasculature to non-lignified tissue ratio with ADT disruptions. There was a lower colorimetric lignified vasculature to non-lignified tissue ratio in knockout mutants (lowest in the quadruple knockouts) and more in WT lines. Lignin modified ADT knockout mutants grown aboard ISS again showed non-uniform distribution of stained lignified vasculature around the lignified vasculature.

3. Consortium Members Laurence Davin and Norman Lewis (WSU/IBC) – Arabidopsis Multi-omics Sample Preparation

ISS and KSC (Ground control) samples from Grow-out #2 were processed at WSU/IBC as described below in order to obtain: • RNAs for transcriptomics, • Proteins for proteomics, • Lipids for lipidomics, • Metabolites for metabolomics, • Intact stem tissues for microscopy analysis, • Ground stem tissue for lignin and stable isotope analyses.

Samples from both ISS and Ground controls (KSC), stored individually in double-pocketed foil bags (one pocket containing rosette leaves, and the other pocket containing stems with flowers, siliques, and cauline leaves), were each removed from the -80°C freezer and placed in dry ice. Each sample was first weighed in its foil bag, and then placed into liquid nitrogen. Following sample removal for processing, each foil bag was re-weighed in order to obtain the fresh (frozen) weight of each sample.

Arabidopsis rosette leaf tissue sample DNA screening: Individual Arabidopsis ADT mutant and WT and WT-CCM lines in their respective positions in the APH Science Carrier were analyzed using a REDExtract-N-Amp™ Plant PCR Kit (SIGMA) protocol to confirm each genotype was in the assigned position.

4. Arabidopsis Multi-omics Analyses

In addition to the ongoing analysis of Grow-out #1 plant lines (ISS and Ground controls), these were also initiated on specimens from ISS Grow-out #2 tissues, and the corresponding KSC (Ground control) samples. Following sample preparation as indicated above, these were sent to Los Alamos National Lab (LANL), Pacific Northwest National Lab (PNNL), and UNM (University of New Mexico) consortium members, respectively.

4.1. Consortium Member Shawn Starkenburg (LANL) – Transcriptomics

Grow Out #1

An analysis pipeline was additionally constructed to evaluate possible single nucleotide polymorphism (SNP) level variation in Ground vs. ISS samples to test the impact of microgravity/ISS spaceflight environment on the mutation rate. Fewer mutations were found in stem than leaf samples, regardless of ISS or Ground conditions. Additional validation/analysis is underway to confirm these results.

Grow Out #2

Upon receipt of the total RNA from leaves and stems (September 2020), sequencing libraries (n=94) were constructed for the second set of ISS and Ground control samples. Prior to sequencing all transcriptome samples, a test sample set using 5 random Arabidopsis thaliana transcriptome libraries (5 million reads) were sequenced to verify sample integrity. Sequencing of this test sample set resulted in a predicted pattern of base calls throughout the length of the read when

Task Progress:

mixed with other types of DNA libraries (non-Arabidopsis samples), indicating that viable transcriptomes can be generated from the samples from the Grow-out #2 ISS experiment.

To maximize sequence integrity for additional RNA mutation analysis of the 2nd ISS experiment, a sequencing approach was designed to minimize the impact of sequencing errors and batch effects (run-to-run variability). In short, each sample is to be sequenced a minimum of three times, combining the reads acquired across several sequencing runs until the required sequencing depth is achieved (approximately 25 million reads per sample). Our first batch of samples is being currently sequenced and the plan is to complete sequencing by the end of March 2021.

Covid-19 Impact

Covid-induced shutdowns of Los Alamos National Laboratory (LANL) facilities in the Spring of 2020 prohibited timely processing and transfer of total RNA preps from WSU to LANL. Additionally, domestic travel by LANL personnel to collect samples from WSU was not permitted until late September 2020, despite the fact that the RNA was ready for collection in the Spring.

LANL continues to operate under reduced capacity (approximately 75% efficiency) and reagent & materials sourcing delays for processing of samples continues to hamper research progress. However, it is anticipated that LANL facilities will be fully operational in the summer of 2021.

4.2. Consortium Members Laurence Davin and Norman Lewis (WSU/IBC) – Arabidopsis Metabolomics

Grow-out #2

ISS and KSC (Ground control) stem and leaf samples from Arabidopsis Grow-out #2 were individually processed, extracted, and then subjected to metabolomics analyses. Specifically, each ground leaf and stem sample (~150 to 200 mg) from four biological replicates of six Arabidopsis lines that were grown in APH units on ISS and at KSC (Ground control), respectively, were individually extracted with aqueous methanol. The metabolite extracts of each leaf and stem sample, after solvent extraction, were next individually subjected to UPLC-qTOF-MS for metabolomics analyses. LC-MS analysis and identification of metabolites in each sample were performed as previously described.

In comparison to Grow-out #1, once again the metabolites analyzed in Grow-out #2 were mainly annotated as apocarotenoids, flavonoids, glucosinolates, galactolipids, phenolic glucosides, and phenylpropanoids, respectively. Each metabolite in these classes was identified/annotated and quantified in terms of naringenin equivalents (internal standard).

Next, as one example, statistical analyses were performed to compare the metabolomic profiles of Arabidopsis WT and *adt3/4/5/6* lines, grown on ISS and at KSC (Ground control) in their respective APH units. This involved pair-wise comparisons of the metabolites obtained for ISS and KSC (Ground control) samples, after normalization of data to the internal standard naringenin in XCMS.

Processing the metabolomics data resulted in clear segregation of ISS and KSC (Ground control) Arabidopsis WT and *adt3/4/5/6* lines in terms of either leaf or stem metabolite clusters; indeed, all sample sets were readily distinguished as to both genotype and whether they were ISS or Ground control (KSC) grown (data not shown).

As further examples, heatmap diagrams were also generated from the metabolomics data for WT Ground control (KSC), WT (ISS), *adt3/4/5/6* Ground control (KSC) and *adt3/4/5/6* (ISS) samples. It was evident from these heatmaps that the average relative metabolite levels of leaf and stem samples for WT Ground control (KSC) and *adt3/4/5/6* Ground control (KSC) slightly differed in their levels compared to WT (ISS) and *adt3/4/5/6* (ISS).

ISS and KSC (Ground control) stem samples from Arabidopsis Grow-out #2, processed and extracted exactly as for Grow-out #1, are in progress for lignin content/composition analyses.

4.3. Consortium Members (Kim Hixson and Mary Lipton, PNNL) – Proteomics and Lipidomics

Grow-out #1:

Proteomics: Analysis of Grow-out #1 proteins were further overlaid onto KEGG pathway maps to visualize relevant specific proteome changes. The auxin pathway was also explored as auxin signaling is associated with gravitropism. It was observed that the proteome response was much greater in stem tissue as compared to leaves. In the more extreme mutants (i.e., *adt4/5*, *adt3/4/5/6*), the increases or decreases in specific proteins showed converse abundance profiles. That is, auxin associated proteins highly increased in leaf tissue were conversely lower in abundance on ISS compared to Ground control proteins in the stem tissues.

We also examined the log₂ ratio z-scores [ISS grown/Ground control] onto the immediate upstream (shikimate/chorismate) and downstream (phenylpropanoid) pathways relative to arogenate dehydratase. In almost all enzymes detected in both tissues, and in different ADT knock-out combinations, we observed these enzymes to be increased in abundance. It seems that the ISS spaceflight environment has a consistent and significant effect of increasing abundance of these enzymes. A notable exception was observed in the first enzyme of this pathway in the leaves of *adt5*, namely 3-deoxy-7-phosphoheptulonate synthase. Interestingly, there was a significant decrease in abundance observed in the ISS grown leaves compared to those grown as Ground control.

In these analyses, there was a mix of enzymes that were increased as well as decreased in abundance. The greatest increases or decreases in abundance were identified in the double knock-out (KO) ADT mutant *adt4/5* both in leaves and stems. The KO mutant with more ADTs knocked out, *adt3/4/5/6* showed more moderate abundance changes. In both stems and leaves and in all examined ADT KOs, trans-cinnamate 4-hydroxylase was increased in the ISS grown plants as compared to the Ground control plants. This variety of increases and decreases in a single pathway with plants that contain various ADT isoenzymes provisionally demonstrates how the phenylpropanoid pathway enzymes are collectively controlled by additional mechanisms under ISS spaceflight environment conditions beyond just the amount of phenylalanine available to the pathway.

Lipidomics: Analyses on pertinent mass spectrometer platforms and pipelines have all been completed for WT, ADT mutant, and CCM-engineered Arabidopsis from Grow-out #1. The data is currently being assessed and interpreted.

Grow-out #2

Proteomics: Precipitated proteins were extracted in an identical way by WSU IBC personnel as for the Grow-out #1

experiments and provided to Pacific Northwest National Laboratory (PNNL). At PNNL, precipitated proteins were then individually solubilized in a denaturing solution and digested into peptides using trypsin, with each sample desalted and quantified. As in the Grow-out #1 experiment, the isobaric tagging protocol (iTRAQ) method was used to label peptides and to determine relative quantitation of each peptide in each sample. We are currently waiting for all of the proteomics data from the Grow-out #2.

Lipidomics: Lipid samples for Grow-out #2 have also been completed and the two grow-out datasets are currently being integrated and analyzed for assessment of the significant lipid changes in leaves and stems due to plants being subjected to ISS spaceflight environment growth conditions.

Covid-19 Impact

WSU/IBC provided samples of all stem and leaf tissues from Grow-out #2 from ISS and the corresponding Ground controls. Covid-19 stay-at-home orders and instrumental resource diversion at the Environmental Molecular Sciences Laboratory (EMSL) at the Pacific Northwest National Laboratory (PNNL), significantly delayed progress on the second flight proteomics analysis, until late Fall 2020.

5. Consortium Member David Hanson (UNM) – 13C Analyses

13C analyses were carried out on plant leaf samples from Grow-out #1 (Ground control and ISS) and will be completed on plant leaf samples from Grow-out #2 (Ground control and ISS). Samples have been freeze-dried and will be packed (2 mg) for analysis via isotope ratio mass spectroscopy through the Center for Stable Isotopes Facility at UNM. 13C/12C analyzes will provide insight into the photosynthetic function and water use efficiency (WUE) over the lifetime of the plants during their growths in the APH chambers. This is important in understanding stomatal usage and closure that may help detect symptoms of stress from flight.

Covid-19 Impact

Delay on these analyses has occurred because of back order issues on the supplies needed due to COVID-19.

Bibliography Type:	Description: (Last Updated: 04/30/2024)
Articles in Peer-reviewed Journals	Overbey EG, Saravia-Butler AM, Zhang Z, Rath KS, Fogle H, da Silveira WA, Barker RJ, Bass JJ, Beheshti A, Berrios DC, Blaber EA, Cekanaviciute E, Costa HA, Davin LB, Fisch KM, Gebre SG, Geniza M, Gilbert R, Gilroy S, Hardiman G, Herranz R, Kidane YH, Kruse CPS, Lee MD, Liefeld T, Lewis NG, McDonald JT, Meller R, Mishra T, Perera IY, Ray S, Reinsch SS, Rosenthal SB, Strong M, Szeewczyk NJ, Tahimic CGT, Taylor DM, Vandenbrink JP, Villacampa A, Weging S, Wolverton C, Wyatt SE, Zea L, Costes SV, Galazka JM. "NASA GeneLab RNA-seq consensus pipeline: Standardized processing of short-read RNA-seq data." <i>iScience</i> . 2021 Apr 23;24(4):102361. https://doi.org/10.1016/j.isci.2021.102361 ; PMID: 33870146; PMCID: PMC8044432 , Apr-2021
Articles in Peer-reviewed Journals	Hixson KK, Marques JV, Wendler JP, McDermott JE, Weitz KK, Clauss TR, Monroe ME, Moore RJ, Brown J, Lipton MS, Bell CJ, Pasa Tolic L, Davin LB, Lewis NG. "New insights into lignification via network and multi-omics analyses of arogonate dehydratase knock-out mutants in <i>Arabidopsis thaliana</i> ." <i>Frontiers in Plant Science</i> . 2021;12:664250. Published: 25 May 2021. https://doi.org/10.3389/fpls.2021.664250 , May-2021