Investigating the Relationship between the Cell Wall Integrity Pathway and Unfolded Protein Response in *Arabidopsis thaliana*

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Abstract

Spaceflight-grown crops are critical to sustaining human presence in space as they are necessary for space diets and astronaut health. This research aims to provide a holistic understanding of the molecular mechanisms underlying plant adaption to spaceflight stressors. Recent multi-omics studies conducted on *Arabidopsis thaliana*, a model plant, have revealed that gene expressions related to cell wall integrity (CWI) and unfolded protein response (UPR) are altered in spaceflight-grown crops. Considering these findings, this research has reanalyzed the *Arabidopsis* gene-expression dataset GLDS-321 from the NASA Open Science Data Repository and identified *bZIP28* and *IRX7* genes as crucial regulators that influence intracellular communication between CWI and UPR. This research proposes a multi-omics experiment with RNA-sequencing analyses and a machine learning vision tool, such as *DeepLabCut*, to quantify the morphological changes in *IRX7* mutants compared to wild-type plants. These data would verify the hypothesis that silencing *IRX7* leads to downregulating UPR and consequent cell wall damage, resulting in weaker crops. Conversely, overexpressing *IRX7* leads to the upregulation of UPR, resulting in thicker cell walls and more robust crops. This research may provide insight into measures necessary to optimize cell wall resilience in space and extreme conditions on Earth.

Keywords: Unfolded Protein Response, Cell Wall Integrity Pathway, IRX7, Secondary Cell Wall Biogenesis, Space Biology, Bioinformatics, Data Analysis, Astrobotany, Astrobiology, Resilient Plants, Genetics, GLDS-321

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Recent studies have shown that spaceflight stressors impact the regulation of cell wall synthesis and growth (Johnson et al., 2017; Kruse et al., 2020; Barker et al., 2023). It is well-understood that the cell wall is essential to maintaining structural integrity, providing flexibility to support cell division, and acting as a barrier against environmental and pathological stresses (Houston et al., 2016). The cell wall has several pathways that foster plant viability, including secondary cell wall biogenesis, which maintains cell wall rigidity, protects against the force of transpiration in plants (Zhong et al., 2019), and regulates the CWI pathway, i.e., a pathway responsible for correcting adverse environmental stimuli that affect cell walls (Zhang et al., 2021). However, how the CWI pathway responds to spaceflight stressors is still understudied.

Recent multi-omics studies conducted on *Arabidopsis thaliana*, a model plant commonly used in space experiments, have revealed that gene expression related to the unfolded protein response (UPR) is altered in spaceflight-grown crops. The UPR is a signaling cascade activated in response to various environmental stresses in the endoplasmic reticulum (ER); the UPR is activated upon the accumulation of unfolded proteins in the cell lumen. For example, Angelos et al.'s (2021) study on how modifications to the UPR may affect gene expression of *Arabidopsis thaliana* in spaceflight. Their study aims to understand the UPR's relevance in space conditions during the SpaceX-CRS12 mission to the International Space Station (ISS) by comparing the UPR transcriptional responses of wild type (WT), i.e., seedlings with no gene alterations, with the deficient UPR transcriptional responses of seedlings with gene alterations, such as mutated seedlings with deleted *bZIP28* gene. The study results indicate

that when comparing differential gene expression on ground control versus spaceflight experiments, seedlings with deficient UPR transcriptional responses had more differentially expressed genes than WT seedlings, leading to an implication that the unique space conditions induce changes in the expression of stress-related genes and activate signaling pathways.

Considering these findings, this research reanalyzed the dataset GLDS-321 available on the NASA Open Science Data Repository (Brandizzi & Angelos, 2021) and found that the *bZIP28* mutant seedlings had the most differentially expressed cell-wall genes in comparison to WT among such seedlings with deficient UPR transcriptional responses as *bZIP28*, bZIP60, *bZIP28* bZIP60, and *atire1*. This finding prompted further investigation of the interconnectedness of the UPR and the CWI pathway.

According to Krysan et al. (2009) and Scrimale et al. (2009), there is evidence of a connection between the UPR and the CWI pathway in *Saccharomyces cerevisiae* (yeast): the communication between the UPR and CWI enables cell wall biosynthesis, providing a buffer against defective proteins. Given that yeast and *Arabidopsis thaliana* have similar cell wall architectures (Voxeur & Hofte, 2016), it is reasonable to draw parallels in the connection between UPR and CWI pathway between yeast and *Arabidopsis thaliana.* As such, this research postulates a relationship between the UPR and the CWI pathway in *Arabidopsis thaliana* (see Figure 1 on page 5). As there has not been published literature on the connection between UPR and CWI pathway in plants, investigating such a connection may provide insight into cell wall synthesis and growth when the organisms are under stress.

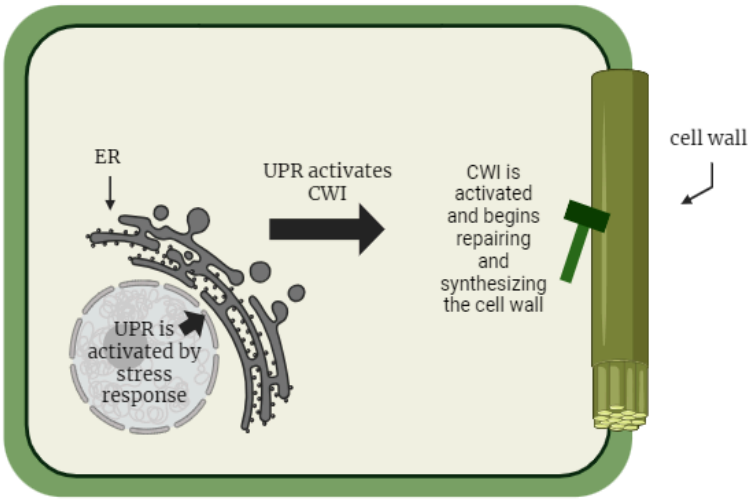
The intercellular communication between the UPR and the CWI pathway in plants has been shown to facilitate cell wall biogenesis. Notably, the *bZIP28* mutant seedlings exhibited differential expression of *IRX7* (Irregular Xylem Gene7), a gene involved in secondary cell wall

biogenesis, as revealed in the Data Analysis section of this research spanning pages 7-10. Therefore, it is imperative to conduct a thorough investigation into the role of the *IRX7* gene in modulating the CWI pathway and, potentially, the UPR to gain deeper insights into the mechanisms underlying the regulation of cell wall synthesis and growth in spaceflight-induced stressors.

Based on the postulation between the intercellular communication between the UPR and CWI pathways and the importance of studying *IRX7*, this research proposes an experimental design with a control group, WT *Arabidopsis thaliana,* and two experimental groups, silenced and overexpressed *IRX7 Arabidopsis thaliana* mutant. This design is further supported by Brown et al. (2005) study on the *IRX7* gene being responsible for secondary cell-wall biogenesis and decreasing cellulose content when mutated. The decreased cellulose content resulted in negatively impacted plant morphology in *Arabidopsis thaliana* compared to WT. As such, the experimental design will also include a method to measure the morphological changes in tandem with the intercellular communication between the UPR and CWI pathway.

Figure 1

A visualization of how the UPR may activate the CWI pathway in plants.



Note. A stress response in the endoplasmic reticulum (ER) activates UPR. The activation of the UPR creates a cascade that evokes the CWI pathway to repair and synthesize the cell wall.

Preliminary Data

Dataset GLDS-321

The dataset GLDS-321 was part of the 2017 SpaceX-CRS12 mission to the ISS. The *Arabidopsis thaliana* samples were tested for the effects of the loss of the UPR regulators. The ground control experiments were conducted at the Kennedy Space Center (KSC), whereas spaceflight experiments on the ISS. Each experiment included five genotypes of *Arabidopsis thaliana*: WT, *atire1, bZIP60, bZIP28, and bZIP28/bZIP60.* Additionally, the five genotypes were placed in either four (*atire1, bZIP28, bZIP60*) or five (*bZIP28*/bZIP60 and WT) Petri dishes, with 70-80 equidistant seedlings and the necessary media supplements.

Arabidopsis thaliana seedling samples were in space for 14 days. Both ISS returned samples and KSC samples were transported to Michigan State University for RNA sequencing. An RNA-sequencing library was then constructed, and *FastQC* analysis was conducted to check the quality of the RNA-sequencing raw data to rule out sequencing data errors such as low-quality reads, low-confidence bases, sequence-specific bias, 3'/5' positional bias, and untrimmed adaptors (Blaber & Samrawit, 2021). The quality-filtered data were then aligned with the *TAIR-10* (*Arabidopsis thaliana*) reference genome to guide the assembly of the transcriptome, and the *DESeq2* was used to interpret the assembled transcriptome to estimate the significance of gene expression between the ground control and spaceflight. The resulting metadata presented in this research is the transcriptomic analysis of the sequenced RNA data.

Although GLDS-321 already used *FastQC* on the raw RNA-sequencing data, this research conducted a separate *FastQC* analysis through the open-source *Galaxy* platform. After

analyzing all five genotypes, the *bZIP28* gene was chosen for further analysis for two reasons. First, there are 8 out of the 27 significant genes related to the cell wall, whereas only 3 out of the 31 genes in WT related to the cell wall (see Figures 2a and 2b). Second, there is little research concerning the relationship between the UPR and the CWI pathway.

Figure 2a
The impact of spaceflight on bZIP28 mutant gene expression in Arabidopsis thaliana.

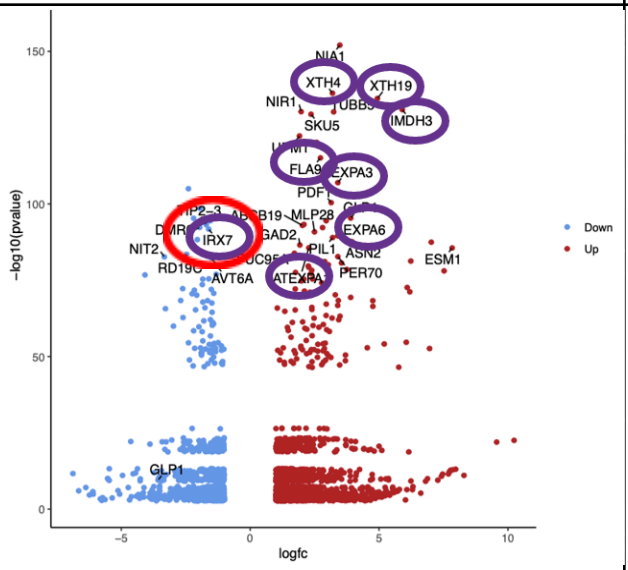
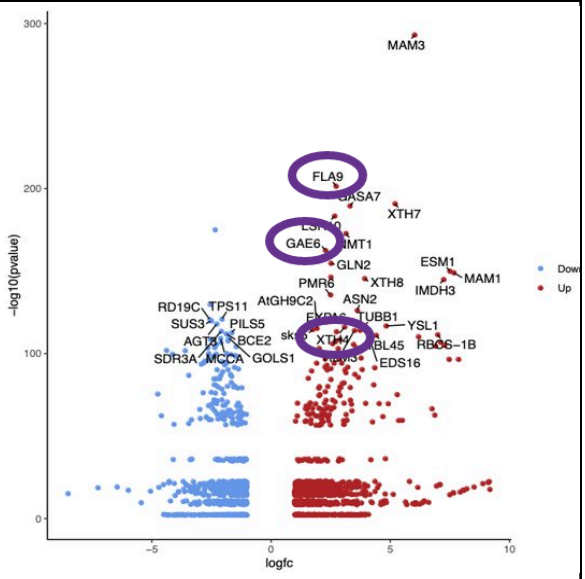


Figure 2b
The impact of spaceflight on WT gene expression in Arabidopsis thaliana.



Note. Volcano Plot showing the relative expression of genes in spaceflight compared to ground control. Figure 2a shows in the *bZIP28* mutant seedlings. 8 of the 27 significant genes were related to cell wall structure (highlighted with purple circles; *IRX7* highlighted with an additional red circle). In WT control, 3 of the 31 significant genes in the *Volcano Plot* were related to cell wall structure (highlighted with purple circles).

Data analysis

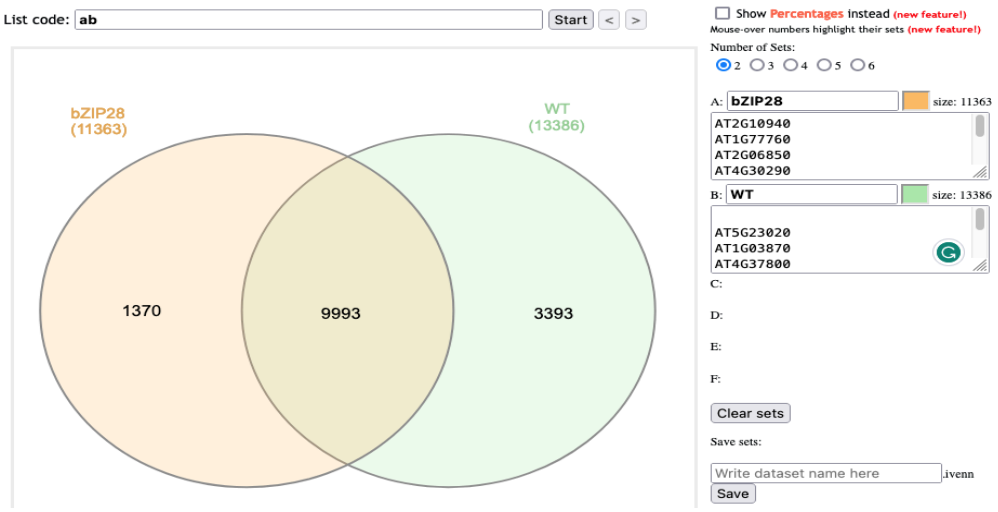
This research reanalyzed the GLDS-321's raw RNA-sequencing data using *FastQC* available on the open-source *Galaxy* platform to ensure the data was of high quality and low

bias. It also used *Trim Galore!* to cut untrimmed adaptors, *RNA STAR* to align sequences, and *featurecounts* to quantify RNA sequencing reads. The *DESeq2* tool from *Galaxy* was used to determine the most significant and differentially expressed genes in the *bZIP28* mutant seedlings and WT (*p-value* < 0.05; *LogFC* < 1.0), and a *Volcano Plot* was created (see Figures 2a & 2b on page 7). In Figures 2a and 2b, the adjusted *p-value* accounted for false discovery rates. The *LogFC* indicates the magnitude of the up-or-down-regulation of genes from the ground control versus spaceflight, ensuring control over differential gene expressions.

The values depicted in the *Volcano Plot* indicate the significance of the genes in the WT and *bZIP28* mutant seedlings. Genes from WT and the *bZIP28* mutant seedlings were sorted by *InteractiVenn Diagrams* to find genes unique to WT and the *bZIP28* mutant seedlings (see Figure 3). There are 1370 genes from the *bZIP28* mutant seedlings that are *not* expressed in WT. Of the 11,363 genes from the *bZIP28* mutant seedlings, 13,386 genes from WT were analyzed. Among the 1370 genes from the *bZIP28* mutant seedlings, the *IRX7* gene stands out because it is the only downregulated cell wall gene, as shown in Figure 2a. It is worth noticing that compared to the *bZIP28* mutant seedlings, there are no downregulated cell wall genes in WT, as shown in Figure 2b.

Figure 3

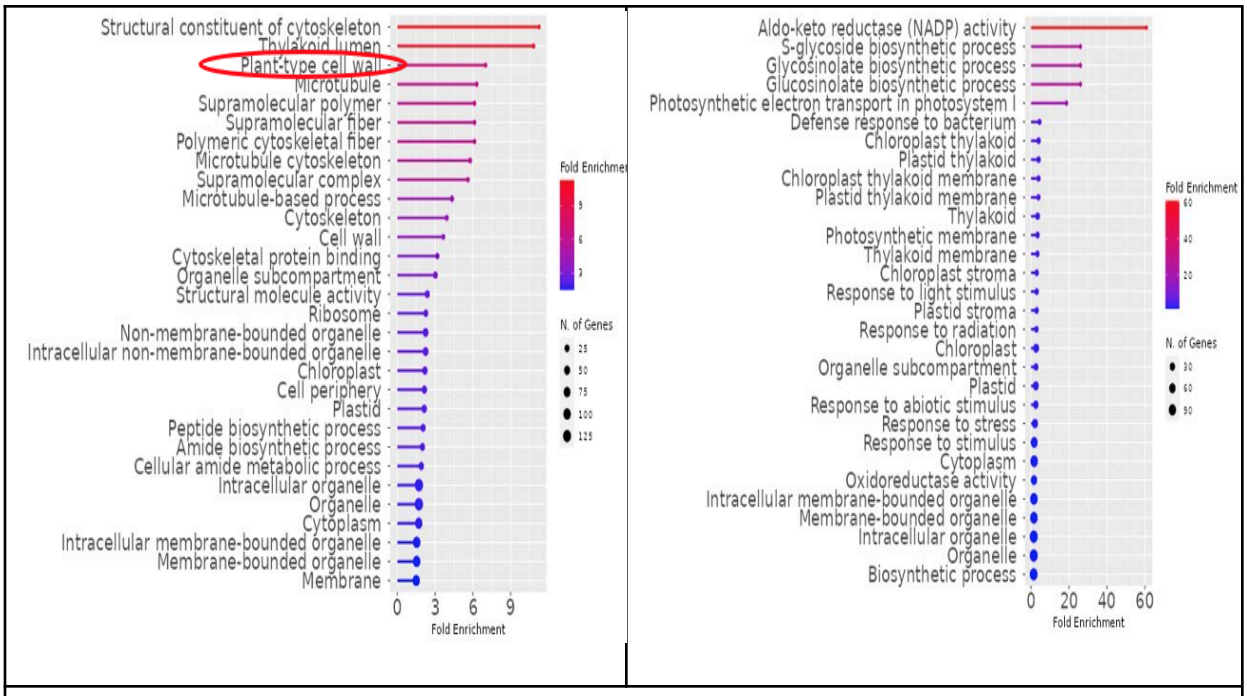
Shared gene expression changes in bZIP28 and WT Arabidopsis thaliana.



Note. Using *InteractiVenn*, shared genes were compared between *bZIP28* and WT *Arabidopsis thaliana*.

These 1,370 genes in *bZIP28* mutant seedlings and 3,393 genes in WT were plugged into *ShinyGo* to identify significant pathways. The results showed a recurring pattern such that the gene expression of the *bZIP28* mutant seedlings was altered in cell wall pathway (see Figure 4a), whereas there are no pathways related to the cell wall in WT (see Figure 4b). This implies that even though the *Lollipop Plots* detect small changes in gene expression, WT shows no change in expressions of cell wall genes.

Figure 4a *The impact of spaceflight on pathways in bZIP28* mutant seedlings	**Figure 4b** *The impact of spaceflight on pathways in WT*



Note. Lollipop Plots highlight the most enriched pathways. One of the most recurring pathways labeled as significant in *bZIP28* was cell-wall-related pathways (highlighted with a red circle). This implies that the deletion of *bZIP28* impacts the cell wall pathway. In WT, there are no pathways related to the cell wall in fold enrichment, which highlights the impact of the deletion of *bZIP28* on the cell wall pathways.

Because the *IRX7* gene is down-regulated by a log fold change (*LogFC*) of -1.608 and has a statistically significant *p-value* (as shown in Figure 2a), it is reasonable to claim the cell wall genes are only affected when a gene responsible for UPR transcriptional response, i.e., *bZIP28* is silenced. That is, the deletion of the *bZIP28* gene could catalyze changes in the cell wall pathway. As the *IRX7* gene is a key regulator in the CWI pathway for correcting adverse environmental stimuli that affects cell wall (Zhang, et al., 2021) and *bZIP28* gene is the key regulator in the UPR, it is reasonable to claim that the *IRX7* gene may also play a role for the intracellular communication between the UPR and CWI pathway.

In a word, based on these data analysis results, it is reasonable to assert that the study of intracellular communication between the UPR and CWI is crucial to gain a holistic understanding of how the cell wall responds to the environmental stress and that the investigation of the IRX7 gene is necessary for the study of the intracellular communication between the UPR and CWI.

Hypothesis and Aims

This research hypothesizes that the *IRX7* gene, which oversees secondary cell wall biogenesis, affects the communication between the UPR and the CWI pathway, leading to cell wall integrity and rigidity changes. This hypothesis is generated based on the results from the *Preliminary Data* section: the *bZIP28* gene (a key transcription factor in the UPR) and *IRX7* gene (Irregular Xylem Gene7) are identified as crucial regulators that influence intracellular communication between CWI and UPR. As the *IRX7* gene regulates cellulose, lignin, and xylem production and essential components in the cell wall structure (Brown et al., 2005) and prevents cell wall defects (Ramírez et al*.,* 2019), the modification to the *IRX7* gene will be expected to impact the cell wall architecture and unfolded protein accumulation when *Arabidopsis thaliana* seedlings are under environmental stress.

Two assess the hypothesis, two aims are configured. They are:

1) To investigate the effect of over-expressing and silencing *IRX7* on the CWI pathway and the UPR, which would provide evidence that genes involved in the UPR regulation are affected.

2) To determine how cell wall integrity affects the morphology of *Arabidopsis thaliana*, presuming that cell wall defects and change in overall plant morphology will change.

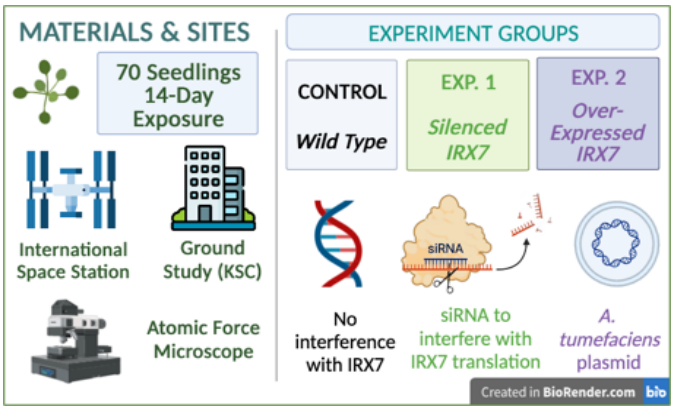
Experimental Design and Expected Results

The research experiment investigates the effects of silencing and overexpressing *IRX7* on plant cell wall resilience. To achieve this, the study proposes a multi-omics experiment that combines RNA-sequencing analyses with a Machine-Learning tool, such as DeepLabCut vision technique to quantify plant growth and morphological changes. The experiment aims to quantify the morphological changes in *IRX7* mutant plants compared to wild-type plants and verify the hypothesis that silencing *IRX7* leads to downregulating UPR, resulting in weaker crops due to cell wall damage. Conversely, overexpressing *IRX7* leads to the upregulation of UPR, resulting in thicker cell walls and more robust crops.

The methods and materials are built on the baseline procedures of dataset GLDS-321 to enable comparison between the biological data of this experiment and dataset GLDS-321. There will be a control group and two experimental groups (see Figure 5 on page 12). In the control group, *Arabidopsis thaliana* WT will be used. In the respective experimental groups, the study will silence and overexpress *IRX7*. All groups will be tested in Biological Research in Canisters (BRICs) hardware on the ISS or the ISS environment simulator at KSC. Like the methods in dataset GLDS-321, each group will have five Petri dishes, each with 70 seedlings (for a total of 350 per experimental condition). See Table 1 for the overview of the proposed experiment.

Figure 5

Visualization of Proposed Experimental Design



Notes. Materials and Sites will be the same as dataset GLDS-321: Experiments will be done via Ground Control at KSC and the Experimental Site on the ISS for 14 days. There are two experimental groups: Exp.1 with silenced *IRX7* and Exp. 2 with over-expressed *IRX7*.

Table 1

Dependent and predicted independent variables for RNA sequencing, cellulose concentration, atomic microscopy, and DeepLabCut experiments.

Group (Method)	**Independent Variable (*Arabidopsis thaliana*)**	**Predicted Dependent Variable (RNA Seq)**	**Predicted Dependent Variable (Cellulose Concentration)**	**Predicted Dependent Variable (Atomic Microscopy)**	**Predicted Dependent Variable (*DeepLabCut*)**
Control Group	WT	Baseline expression of CWI and UPR genes	Baseline in cellulose concentration	Baseline in the cell wall, and ER	Baseline plant morphology
Exp. Group 1	Silenced *IRX7*	Decreased expression of CWI and UPR genes	Decreased cellulose concentration	Decrease in the cell wall, and ER	Negative change in plant morphology metrics
Exp. Group 2	Over-expressed *IRX7*	Increased expression of CWI and UPR genes	Increased cellulose concentration	Increase in the cell wall, and ER	Change in plant morphology

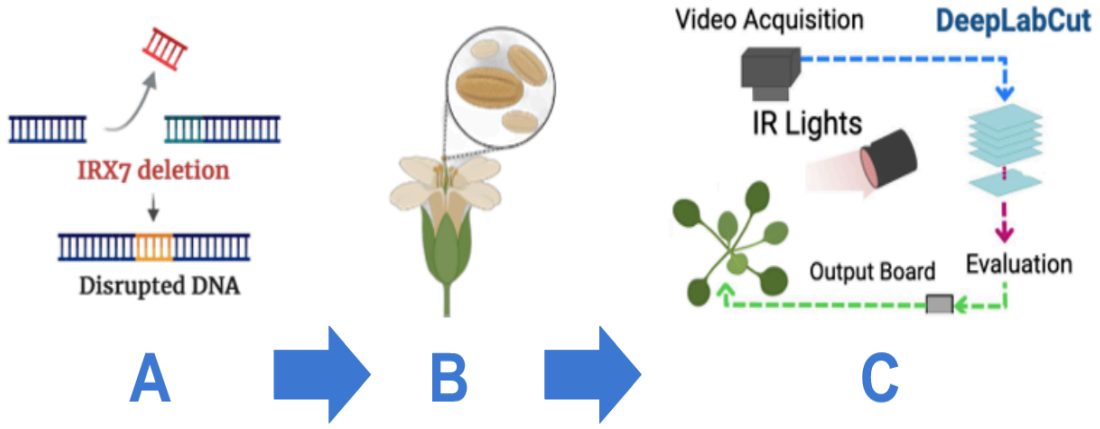
Experimental Group 1

IRX7 will be silenced using siRNA (small, interfering RNA). The siRNA will be developed to interfere with the translation of IRX7, inserted into a plasmid, and then transferred

into *Agrobacterium tumefaciens* bacteria (Xu and Qingshun, 2008). These bacteria will be placed on the *Arabidopsis thaliana* flowers to insert the plasmid, express the siRNA, and silence IRX7 in the resulting seedlings (Hwang *et al.*, 2017). (See Figure 6). It is hypothesized that silencing IRX7 will impair cell wall integrity and, thus, downregulate the UPR.

Figure 6

DeepLabCut offers real-time quantification of plant morphology by capturing plant growth stages through a BRICs camera.



Note. A) *IRX7* gene is knocked out the DNA of *Arabidopsis thaliana.* B) The subsequent DNA is then inserted into an *Agrobacterium-tumefaciens* bacterium plasmid. The bacterium is placed onto the *Arabidopsis thaliana* flower. The germinated seed will have a knocked-out *IRX7* gene. C) *DeepLabCut* will be used to trace and record the morphological changes. This enables the verification of crucial data: 1) Silencing *IRX7* results in the downregulation of the UPR, causing cell wall damage and weakened seedlings. 2) Overexpressing *IRX7* leads to the upregulation of the UPR, strengthening both cell walls and seedlings.

Limitations of Experimental Group 1

Incomplete silencing of *IRX7* might occur, as achieving consistent and complete gene suppression can be difficult. Potential off-target effects, where gene silencing could

inadvertently affect other genes due to sequence similarities, could further lead to unintended consequences. To address these problems, multiple siRNAs will be created. Polymerase chain reaction will be used to screen for siRNAs that successfully silence *IRX7*, and then RNAseq will be used to test for effects in other unrelated pathways. This will identify an optimal siRNA that is effective at silencing *IRX7* and has minimal off-target effects.

Experimental Group 2

IRX7 will be overexpressed using the same *Agrobacterium tumefaciens*-mediated transformation strategy described in the *Experimental Group 1*, with a plasmid containing *IRX7* (Hwang *et al.*, 2017). The predicted result is that increased *IRX7* expression will enhance cell wall resilience against environmental stresses by upregulating the UPR and CWI pathway.

Limitations of Experimental Group 2

Because the effects of *IRX7* overexpression have not yet been studied on the cell wall, its impact on plant morphology remains uncertain. While the study of dataset GLDS-321 doesn't show an upregulation of *IRX7*, there is a possibility of altered cell wall rigidity.

Methods for Experimental Group 1 and 2

Cellulose content measurement is an objective indicator of *IRX7* overexpression or silencing effects. To test how *IRX7* mutations affect the cell wall, the cellulose content of the plant will be measured. Cellulose, forming the plant cell wall's backbone, will be quantified using the Updegraff assay method. The Updegraff assay method eliminates other components that make up the cell wall's structure, allowing only cellulose to be present (Dampanaboina *et al.*, 2021). Silenced *IRX7* plants are expected to exhibit reduced cellulose concentrations, indicative of inhibition of the CWI pathway. Conversely, overexpressed *IRX7* plants are predicted to demonstrate elevated cellulose concentrations.

Additional analysis

Additionally, the study aims to quantify changes in plant morphology at both the macroscopic and microscopic levels. To measure the microscopic mechanical properties of the cell wall, atomic force microscopy will be used. Atomic force microscopy is an imaging technique that utilizes a cantilever to repeatedly tap the cell wall to estimate the rigidity and cell wall shape accurately (Main *et al.*, 2021).

DeepLabCut

To measure the macroscopic morphology of the plants, *DeepLabCut* machine vision techniques will be leveraged. While *DeepLabCut* is primarily used for tracking animal movements, this study will extend this approach to quantify plant growth. A BRICs camera will image the plant throughout the different stages of growth. Using *DeepLabCut*, a machine vision system will be trained to quantify plant morphology, including root branching and length measurements, leaf shape, size, numbers, and plant height. Differences across these categories are expected in ground control and spaceflight conditions, including microgravity-induced truncated roots and plant heights. By releasing the videos produced by BRICs and the *DeepLabCut* quantification for public use, this study can help enhance machine vision technology and contribute to the NASA Open Science Data Repository's mission to provide accessible data to the public.

Limitations of DeepLabCut

DeepLabCut was originally designed to track animals, so applying the approach to *Arabidopsis thaliana* plants may be difficult. Machine learning researchers will be contacted to develop a new plant-specific morphology measurement system if issues arise.

Expected Results

Silencing *IRX7* is predicted to reduce cell wall rigidity, impacting nutrient transport due to impaired xylem and transport functionality (Zhong *et al.*, 2019). In contrast, plants with overexpressed *IRX7* should have more rigid cell walls. The stresses of spaceflight are predicted to induce a reduction in cell wall rigidity and deformations in cell wall shape. The deformities in cell wall rigidity are hypothesized to be more pronounced in the silenced *IRX7* group, while a strengthening in cell wall structure and rigidity are expected in the overexpressed *IRX7* group.

Significance & Conclusions

As space missions extend in duration and the impact of uncertain climate change events on Earth continues, the optimization of cell wall resilience has become a significant concern for enhancing crop quality and production and reinforcing astronauts' physical and psychological health during long-term space missions. A holistic understanding of the molecular mechanisms underlying the adaptation of plants to spaceflight conditions is thus vital. This research proposes an effective study of the intracellular communication between the Unfolded Protein Response (UPR) and Cell Wall Integrity (CWI) may provide valuable insight into such a holistic understanding. Additionally, the use of *DeepLabCut* in plant morphology may bring new innovative Artificial Intelligence technology to aid in the omics research and techniques in plants’ adaptation to various environmental stressors.

One of NASA's main objectives is cultivating edible plants for long-term space missions, owing to their nutritional, psychological, and cost benefits. On the physical health front, plants can offer micronutrients such as vitamins C and B1 and dietary antioxidants that would degrade in pre-packaged foods. Pre-packaged foods have been shown to degrade in critical nutrients and will not have a long shelf life relative to long-term space missions (Cooper et al., 2017). On the psychological side, growing plants in space can help alleviate

homesickness among astronauts, as they remind them of Earth (Zhang et al., 2022). For instance, NASA Astronaut Megan McArthur expressed her love for growing Chile Peppers on the International Space Station (ISS), as these peppers brought a spiciness not often tasted in her freeze-dried diet (M. Hitt, personal communication, August 1, 2023). This phenomenon occurs because, in spaceflight, fluid shifts to the head, thus dampening the sense of taste through the ears, nose, and throat.

Studies suggest that plants successfully developed in space are more resilient to stress. Non-resilient plants have a greater chance of programmed cell wall death, thus diminishing the cost-effectiveness of growing plants in space (Angelos et al., 2021). On Earth, plants must tolerate stressors like heat, cold, mold resistance, pathogen resistance, and overwatering (Ezquer et al., 2020). Even in Artemis missions, astronauts will need plants resilient to pathogens, which are more virulent in space.

In the broader research community, the findings from this research will provide valuable insight for NASA scientists, astronauts, and bioengineers into maintaining cell-wall health in plants for spaceflight. The relationship between the UPR and CWI pathway will highlight cell-wall maintenance, and the objective data collected from this study may support the development of strategies/genetic engineering to optimize plant health for astronauts in future Artemis missions and beyond.

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