The effects of high gravitational stress on the expression of stress response genes in *Arabidopsis thaliana*

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Abstract

As humans venture farther into space, we are increasingly interested in how plants respond to the unique conditions of spaceflight. This experiment focuses on how gene expression in the model plant *Arabidopsis thaliana* is affected by high-gravitational stress that is comparable to the amount experienced on a sounding rocket. The genes chosen, have been studied previously for their roles in mechanical stress and pathogen response. Our results showed an increase in expression of the genes TCH2, TCH3, and MPK3, no change in the expression of CBP60g, and a decrease in the expression of RCI3 and ICS1. These results are largely consistent with spaceflight data and warrant further investigation into the effects of rocket launch on plant growth.

1. Background

Signaling mechanisms in plants are highly complex and responsive to a variety of stimuli. This experiment focuses on three groups of genes involved in plant stress – Calcium signaling, peroxidases, and pathogen response genes. The genes are involved in response to a variety of both abiotic and biotic stressors.

Table 1. A brief description of each gene in this study.

Gene Name	Abbreviation	AT locus	Category	Brief Description
Touch 2	TCH2	AT5G37770	Ca2+ signaling	Involved in flooding response and mechanical stress.
Touch 3	TCH3	AT2G41100	Ca2+ signaling	Involved in mechanical, cold, and light stress.
Cam-binding protein 60-like G	CBP60g	AT5G26920	Ca2+ signaling, Pathogen Response	Triggered by a variety of abotic and biotic stressors. Recruited to the promoter of ISC1.
Mitogen-activated protein kinase 3	MPK3	AT3G45640	Pathogen Response	Increases in response to touch, cold, and fungus attack.
Isochorismate synthase 1	ICS1	AT1G74710	Pathogen Response	Involved in Salicylic Acid signaling.
Rare cold inducible 3	RCI3	AT1G05260	Peroxidase	Peroxidase activity gene that is induced by cold and desiccation.
Peroxidase 22	PRX22	AT2G38380	Peroxidase	A peroxidase family protein that is involved in salinity stress.

Using data from the STRING database (Snel B. et al., 2000), we generated a map of the connections in molecular pathways of these genes. Note that CBP60g binds to the promoter of ICS1.

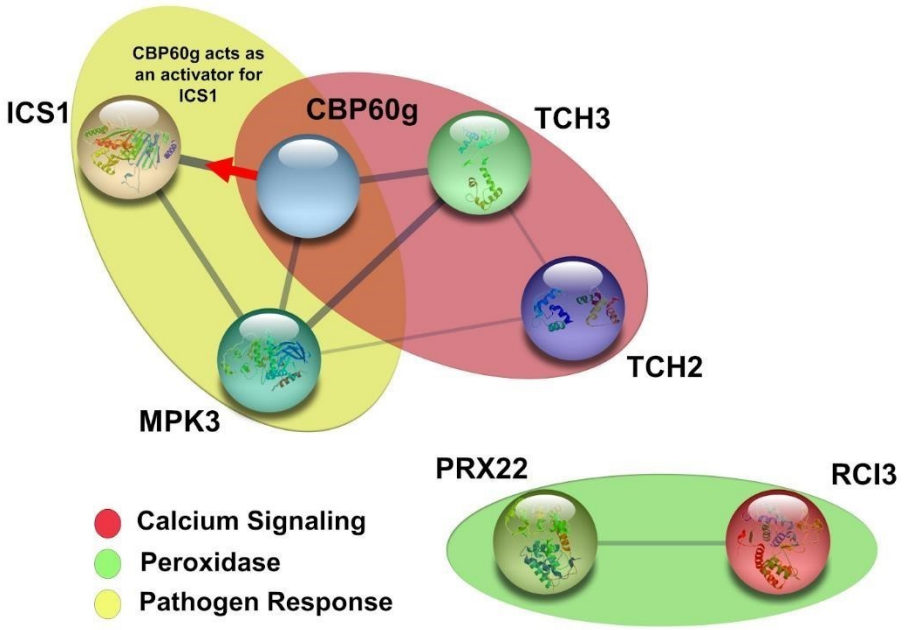

Fig. 1. A map of relatedness between the genes studied. A thicker line indicates a closer connection.

Many of the genes have been shown to be highly correlated in previous research. RNA coexpression stores from the STRING database are shown in Table 2, where a higher co-expression score indicates that the genes have been affected in similar ways in previous experiments.

Table 2. RNA co-expression scores based on previous research. Data from the STRING database (Snel B. et al. 2000).

	RCI3	PRX22	TCH2	TCH3	CBP60g	MPK3	ICS1
RCI3		0.424					
PRX22	0.424						
TCH2				0.177		0.180	
TCH3			0.177		0.438	0.471	
CBP60g				0.438		0.418	0.168
MPK3			0.180	0.471	0.418		0.054
ICS1					0.168	0.054	

PRX22 and RCI3, the two peroxidases, are strongly correlated in expression levels. TCH3, CBP60g, and MPK3 are strongly correlated with each other. TCH2 is moderately linked with TCH3 and MPK3; ICS1 is moderately linked to CBP60g and weakly to MPK3.

2. Methods

Wild-type *Arabidopsis thaliana* seedlings were grown with 6 seeds each in 24 small pots using Sungro Fafard® Germinating Mix, which provided all necessary nutrients. They were grown under 24hr light for 11 days and watered every three days. The pots were divided evenly in an alternating fashion into treatment and control groups. The treatment group was loaded into the centrifuge, where they received 12 ±1 Gs of force, which is comparable to the maximum force of 12Gs experienced on sounding rockets (European Space Agency, n.d) They were spun for a duration of 30 seconds, then all plants were flash frozen in liquid nitrogen.

RNA was extracted from the roots and shoots of the plants using the protocol described by Choi et. Al (2018), and RNA concentration, OD260/280 ratios and OD260/230 ratios were obtained using a nanodrop machine. The four samples from each group with the highest concentration and purity underwent further analysis.

Reverse Transcription-QPCR was used, with Ubiquitin 10 as a housekeeping gene. Three QPCR plates were used, which contained 96 wells each. Each well contained 24 ng of RNA, and each row contained a primer for the target gene. Each reaction well was run with four technical replications per gene per biological replicate.

The plates were ran using a 7500 Real-Time PCR System. The thermocycler went through a warming step then forty PCR cycle steps. At each step, the fluorescence level was measured, and the cycle number was recorded when fluorescence reached the threshold level, giving the C_T (Cycle threshold) value.

Fold change values of each gene were calculated using the $2^{-\Delta\Delta C_T}$ method. The Cycle C_T value was obtained for each of the 96 wells on the three plates. Using Python and Jupyter, the ΔC_T values for each target gene by sample were calculated by subtracting the control gene Ubiquitin 10 C_T values from the target gene C_T. The control ΔC_T values for each gene were then subtracted from the treatment ΔC_T values to give the $\Delta\Delta C_T$ value. Taking $2^{-\Delta\Delta C_T}$ gave the fold change value.

3. Results

The results showed an increase in the expression of TCH3, MPK3, and TCH2. There was no significant change in the expression of CBP60g, and there was an extremely significant decrease in RCI3, and a fairly significant decrease in ICS1. PRX22 had overall low expression levels and was excluded from analysis due to low signal-to-noise ratios. A heatmap of expression (ΔC_T) is in Fig. 2. Red indicates a lower ΔC_T value, indicating higher expression values, and blue higher ΔC_T value (lower expression).

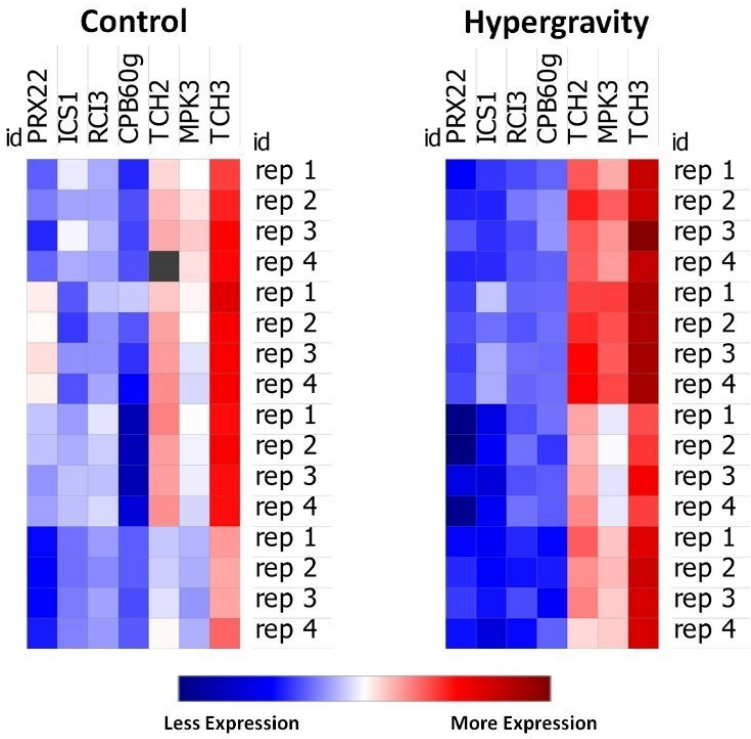

Fig. 2. A heatmap of expression levels in the two treatment groups. Created using Morpheus.

The values were graphed A T-test was performed, and P values were obtained for each of the genes and can be seen in Table 3. A graph of fold change values is shown in Fig. 3. A value greater than one indicates gene activation, and a level less than one indicates gene suppression.

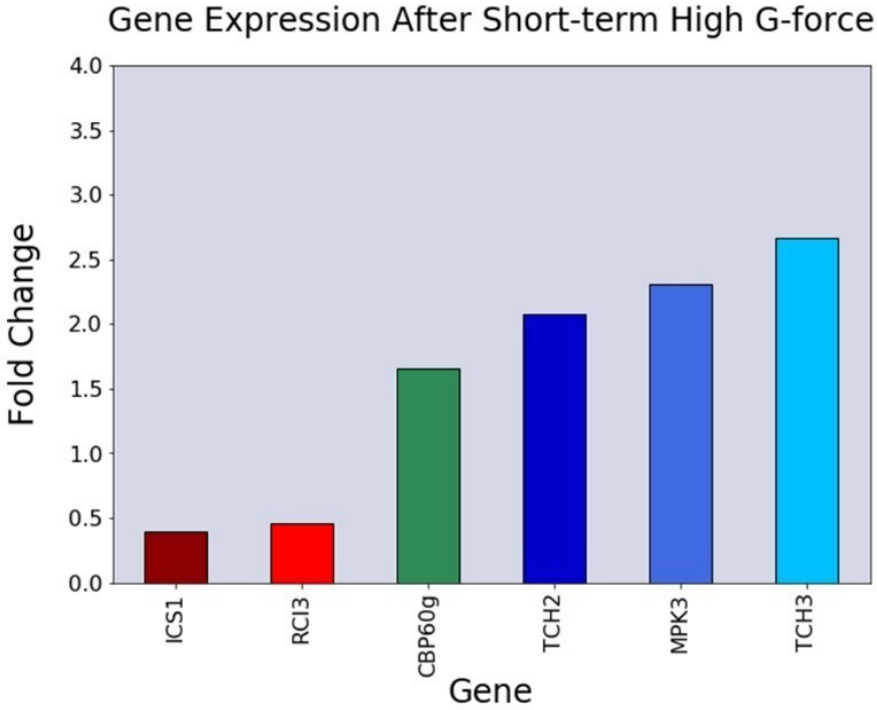

Fig. 3. A bar chart showing the fold change values for each gene, where 1.0 is baseline expression.

Table 3. A table of fold change values and P values for each gene studied.

Gene	TCH3	MPK3	TCH2	CBP60g	RCI3	ICS1
Fold Change	2.587	2.255	2.158	0.950	0.506	0.409
Significance	0.0792	0.0706	0.0805	0.3442	0.0027	0.0654

The results indicate that the genes involved in mechanical stress (TCH2, TCH3, MPK3) are significantly increased when compared to plants that underwent similar handling, but not centrifugation.

RCI3 sharply decreased, which may indicate that peroxidase activity, which is known to be reduced during spaceflight (Kwon T. et al., 2015), may also be affected by the mechanical stress of the rocket takeoff. Both are of major concern for plants launched on commercial spaceflight launches as mechanical stress, and a reduced capacity to withstand oxidative stress can severely affect the growth and yield of crop plants.

4. Further Research

Agravitropic mutants such as *arg1* and *toc132* lack a gravitropic response. They would be used to determine whether gravitropic sensing, specifically through statolith granules, are responsible for the change in gene expression during rocket launch.

Lanthanum chloride can be used to chemically suppress gravitropic and other stress responses (Friedman et al., 1998) and potentially numb the plants to gravitational stress during rocket launch. This would allow us to test whether the gravitational stress can be mitigated by chemical agents.

The duration of force experienced may show different effects, particularly in genes later in the stress response pathway such as CBP60g. As this experiment shows only the immediate responses, taking samples at specific intervals after launch will allow us to see into the long-term mechanisms of gravitational stresses. In addition, the centrifuge is currently being upgraded to support exact thrust curve simulations of commercial rockets such as Blue Origin's *New Shepard* and the SpaceX *Falcon 9.*

Another method for quantifying the effect of gravitational stress is using G-CAMP, a "high affinity Ca^{2+} probe composed of a single GFP" (Nakai et al., 2001). This technology allows us to see calcium signal propagation in living cells, giving a dynamic view into calcium signaling responses.

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