Exploring the Molecular Responses of Arabidopsis in Hypobaric Environments: Identifying Possible Targets for Genetic Engineering

Abstract

Environmental stresses to plants have been studied extensively in order to define mechanisms that are used by plants to defend against the stress. The focus of these studies includes drought, cold, heat, salinity, and hypoxia. However, space flights and theoretical missions to the moon and Mars require a low pressure environment to be considered. Plants will be essential in future space mission because they will be included in life support systems designed for astronauts. The molecular mechanisms used by plants to survive in a hypobaric environment are poorly understood, but recent studies have shown that a plant may borrow mechanisms from other stress responses to defend against the low pressures. Research has found that hypoxia, cold, and drought responses seem to be involved in a plant experiencing hypobaria. My studies seek to profile the gene expression patterns of Arabidopsis thaliana during a week long period of time in a low pressure environment by doing a transcriptome analysis. This information will give insight into when the necessary genes are activated or repressed, and the duration of the gene activity will reflect its importance in the stressful situation. It will also provide additional evidence of defense pathways that are shared between the stresses. It has also been shown that low pressures may cause plants to activate a defense response that is unneeded. The cold and drought responses are highly activated in Arabidopsis submitted to low pressures, but the plants showed no signs of wilting, browning, or loss in turgor pressure. This suggests that the plants perceive a lack of humidity and water, but in actuality the plants have enough amounts of water. My second study will attempt to block the defense responses to desiccation stress by creating mutant strains of Arabidopsis with non-functional transcription factors, and seeing the effect on the plants. CBFs and ABIs are known to bind to promoters in order to induce genes that control the production of LEA and COR proteins. By genetically engineering the reduction of the unneeded pathways, the plants will be able to save resources for other important functions.

Introduction

There has been a considerable amount of research done on the effects of environmental stresses such as drought, hypoxia, or heat stress, on plants. And for good reason too, as environmental stresses can reduce the growth rates of plants and decrease crop yields drastically. By studying the changing genome when plants acclimate to a new environment, we can devise biotechnological or genetic techniques to promote plant survival in the stressful conditions (1, 6). For example, by over-expressing LEA proteins, which are responsible for protecting against water deficits, rice gained improved resistance to salinity and drought (6). However, while we know a lot about mechanisms in various extremes, very little is known about the molecular responses of plants to low atmosphere pressures (1). Although this may seem trivial, the significance of hypobaric environments comes into play when the need to grow plants in space, moons, or planets arises. The growth of plants in space is a priority for NASA, since plants will be essential in long-term bioregenerative life support systems for astronauts (1, 3, 7). Greenhouses can be built to provide a somewhat controlled environment, but creating Earth-like pressures is near impossible and highly impractical, thus the ability of plants to tolerate low pressures will be necessary (1,4). Altered atmospheric pressures have been shown to have adverse as well as favorable affects on plant growth, though in all cases extensive adaptations need to happen (4, 7, 9). There is evidence that defense pathways for multiple stresses will simultaneously activate since low pressures will create an environment where: hypoxia can occur since oxygen will be limiting due to a lower partial pressure of oxygen outside of the plant (1, 4, 5, 7); cold and drought stress is perceived because of the lower vapor pressure, and thus lower water potential, outside plant cells (1, 4, 6, 7); heat stress could occur due to lowered conductive and convective cooling via transpiration, which would occur if the plant ceased respiration and closed stomata to defend against desiccation (1, 6). Because of the combined stresses, the final defense mechanism

used by the plant can be very different from when it is only dealing with one stress. It has been shown that the combination of stresses, such as drought and heat, causes a distinct response from when the plant is exposed to either drought or heat alone (2). Genes that are expressed during a single stress may be repressed when exposed to a second stress. Similarly, genes repressed with a single stress, can become active when there are multiple stresses present (2). Hypobaric environments could trick plants into triggering multiple defense pathways, which in some ways could be agonistic or antagonistic to one another. An example of how pathways may be opposing is as following: when plants are under desiccation stress, plant cells will form compatible solutes such as proline or sugars to lower the water potential inside the cell, and also synthesize major intrinsic proteins (MIPs) such as aquaporins to increase water uptake (2, 6). When a plant is facing hypoxic conditions, which often occurs due to flooding, there is a major reduction in the MIPs present (5). If hypobaria does activate hypoxia pathways and drought pathways, then it will be interesting to watch the plant decide to increase or decrease the level of MIPs. There are an innumerable other examples of pathways which may complement each other or work against each other, and so one goal of my research would be to identify the pathways involved in a plant experiencing hypobaria. Additionally, it has been shown that when grown at one-tenth of Earth's atmospheric pressure, plants induce genes to defend against desiccation and hypoxia even though the plants were grown in a humid environment with plenty of water (4). The plants showed no loss in fresh weight or turgor pressure, so it suggests that the plants perceive low pressures as increased water loss. To accommodate for this, the plants start undergoing alternate metabolic and adaptation pathways. However, if these pathways aren't really needed, then the plant is wasting energy that it could use for growing and reproducing. The second goal of my research is to identify what stress responses I can knockout or knockdown, so that the plants can thrive in the environment without any slowdown.

Experiment 1

The plant of choice for my experiments will involve Arabidopsis thaliana, since the most is known about its genome, and almost every previous research study done on plant stresses has used Arabidopsis. To compare my results with them, I will also use Arabidopsis. To start off, I want to grow seedlings in a Low-Pressure Growth Chamber (LPGC) as done by Paul et al. and Musgrave et al. (4, 9). Unlike the previous two experiments, I will set the pressure even lower to 2 kPa, which is slightly above the average atmospheric pressure of 1 kPa on Mars (3, 7). The lowest pressure possible for plant survival is estimated to be around 1 kPa; this value takes into account the minimal partial pressures of water vapor, oxygen, nitrogen, and carbon dioxide needed by plants (8). I want to avoid going to the extreme low end to steer clear of unexpected complications. Since hypoxia is inevitable in low pressures, I will fix the air flowing through the LPGC to have an oxygen concentration of 9 mmol/L. At this concentration, I expect plant growth and respiration to be normal or even enhanced as described by Musgrave et al. (9). If necessary, a higher concentration of oxygen could be used if the seedlings continued to struggle with hypoxia. The other Martian conditions such as sunlight availability, atmospheric composition, and lower gravity are presumably not obstacles to plant growth as long as a greenhouse is providing an environment with optimal levels of CO2, O2, and UV protection (8). For my control, I will grow seedlings at the normal Earth pressure of 101 kPa. Past data has already shown that low atmospheric pressures do not negatively affect plant growth from a visual standpoint (1, 4, 9). The plants seem to tolerate the low pressures very well, and show no signs of wilting or loss of growth when germinated at low pressures; instead the plants are actually experiencing enhanced growth in many scenarios. To determine how the seedlings are altering their biochemical pathways to acclimate to a low pressure environment, I will use a microarray to do a transcriptome analysis on the hypobaric plants and control plants. I will utilize the same

GeneChip for the Arabidopsis genome as used by Paul et al. in their experiment (4). Instead of just using tissue from the shoots of the plants however, I will also collect leaf tissue, and root tissue as well. This way I can see if there is differential expression between tissues. Detecting mRNA for a gene does not necessarily mean that it is being translated, but it will give insight on how the plant is preparing to defend against a stress. By comparing the microarrays from the control and hypobaric plants, it will be possible to tell which genes are differently expressed in the hypobaric plant. I will label mRNA from the control plant red, and mRNA from the hypobaric plants green. On the microarray, areas that are only green are genes uniquely expressed by hypobaric plants, yellow areas are genes expressed by both plants, and red areas are genes that are inferably repressed in hypobaric plants. I will be doing a time-course for gene expression microarrays by taking the RNA samples at different time intervals (30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 16 hours, and 24 hours) and then taking a final RNA sample a week after germination. The purpose of the time-course is to make sure we aren't overlooking genes that are being activated or repressed early in the stages of adaptation or late in the stages. Additionally, the Paul et al. experiment used 10 kPa whereas I'm using 2 kPa, so the effects of the lower pressure on the genome have yet to be seen. I expect to see a similar result as described in the Paul et al. experiment (4), but an added time-course will show if these genes they listed are activated from the start or at different times. With this information, we can identify which genes are crucial for the first step towards adaptation to a low pressure environment. Also, genes that become activated from the start and stay active throughout the whole process are more likely to be essential for dealing with stresses. For example, alcohol dehydrogenase (ADH) is an enzyme that is essential for glycolysis to proceed, and since glycolysis is upregulated during times of stresses, I would expect much higher levels of ADH in the hypobaric plants the whole time (1, 2, 4, 5, 6). Identifying genes that are repressed is equally important, because certain genes

can be harmful to the plant during a time of stress. For example, when dealing with drought stress, plants activate pathways to create higher levels of proline in the cell in order to lower the water potential among other reasons (6). But when faced with drought stress and heat stress, the pathways creating proline is repressed, and pathways creating sucrose and glutamine are induced instead since proline is thought to interfere with the heat stress response (2). After a week in the hypobaric environment, the seedling will have acclimated to the new low pressure environment, so the transcriptome produced at that time period will most likely reflect the final mechanisms the Arabidopsis seedlings are using. At the 24 hour time period and week time periods, I will use RT-PCR in order to quantify the degree of transcription for the hypobaria-induced clusters of genes as well as other genes of interest such as genes involved in glycolysis, C4 pathway, photorespiration, and aerobic respiration. The RT-PCR method I use will be the same one used by Paul et al. (4). I expect higher levels of mRNA for genes belonging to a hypoxic stress response, such as alcohol dehydrogenase and other genes in the fermentation pathway, as well as higher levels of genes corresponding to a drought stress response, such as LEA proteins and COR proteins, in the hypobaric plants than in the controls. I'm also interested in examining whether the levels of certain transcription factors (CBFs and ABIs) are raised. These transcription factors are essential in a drought stress response, and will be important in my second experiment (1, 6, 10). Seeing if Heat Shock Proteins (HSPs) rise in levels will be important as well for future experiments. I hypothesize that by the week time-period, the levels of mRNA in the hypobaria-induced cluster will have risen, or possibly dropped, to their optimal level (I will compare the expression between the week time-period and the 24 hour period). It is possible for mRNA levels for defense genes to rise in number, and then decline as time progresses as seen in the hypoxic stress response (5). Microarrays and RT-PCR will give us a much more comprehensive picture of what is happening in the hypobaric stress response. To end the first

experiment, I will use Expression Analysis Systematic Explorer to investigate the results of my microarray experiment as done by Liu et al. (5). With this program, I will be able to identify biological themes within the gene list, identify the molecular functions and localization of gene products present, and I will have a more in depth perspective into the possible biochemical/metabolical pathways that are occurring as part of the stress response for hypobaria. The information provided from the first experiment will greatly increase understanding of the responses necessary to defend against low pressures, and will also help immensely in the design of genetically engineered plants for space missions.

Experiment 2

There have been countless observations that when plants are pre-acclimated to stressful environments, such as heat shock, hypoxia, or drought, the plants acquire a sort of tolerance to that stress (1, 6). A major strategy in creating plants that are pre-acclimated to a stress would be to engineer them to constitutively express genes that are responsible for the adaptive response (1). This might include introducing genes which take part in the adaptive response, or introducing genes coding for transcription factors to activate multiple genes that are part of the response. While this strategy will be useful for dealing with the hypoxia stress response and heat stress response, it could be ineffective with the cold and drought stress responses that the plants are exhibiting during hypobaria. When grown at 10kPa, Arabidopsis plants showed no signs of wilting, browning, or other symptoms of desiccation stress, even though the defense pathways for cold and drought stresses were being activated (4). If the desiccation response isn't necessary for adaptation to low pressure, and really is a side effect of the plant perceiving low pressures as lower humidity, then the induction of the pathways is causing a drain on metabolism. My second experiment would be to investigate how important the desiccation stress response actually is. I will do this by blocking the response pathways responsible for reacting to the stresses, and then growing the mutant plants in the LPGC at 2

kPa. Two classes of proteins have been identified as the major players in cold and drought stress. The LEA (late embryogenesis abundant) genes are important in the response against desiccation, and the COR (cold responsive) genes are vital in the response against freezing temperatures (1, 6). Since cold and drought stress are related stresses, as both cause water deficits, it is not uncommon to see both stresses sharing defenses. One way to knockdown the expression of the LEA and COR proteins would be to stop the activation of their promoters. There are two main pathways to activate these promoters: one that is dependent on the hormone ABA, and one independent of it. I will try to inhibit both pathways individually, and then at the same time. Since blocking ABA independent pathways is easier, it will be my first target. Numerous cold-induced genes and water-deficit genes are controlled by transcription factors, called CBFs (C-repeat binding factor) or DREBPs (DRE-binding protein), that bind to a DRE cis-element on the promoter (1, 6). I will create, or purchase, mutant Arabidopsis plants with dysfunctional CBFs/DREBPs. The preferred method of mutation would be gene knockouts so that the plants do not transcribe the mRNA, and thus no chance of translating the transcription factors. Silencer RNA would be a second option, but since it is more of a knockdown instead of a knockout, proteins could still sporadically be expressed. Also, I would have to apply the siRNA to all the tissues multiple times during the experiment. Without the proper transcription factors, almost none of the ABA-independent stress response genes could be activated. For example, CBF3 causes the activation of a few COR proteins and the activation of pathways to form compatible solutes such as proline and sugars to protect against water loss (1, 6). CBF1 has been shown to increase the expression of all of the COR proteins, showing its great importance (1, 6). By mutating the CBF/DREBP family of transcription factors, the stress response will be severely diminished and resources will be free to use for other cell functions. Similarly, ABA-induced genes have cis-elements on the promoter to bind different transcription factors. In Arabidopsis, the ABRE (ABA-

responsive element) and CE (coupling element) sites bind ABI transcription factors, which are activated after an ABA signal-transduction event (6). I will create, or purchase, mutant Arabidopsis plants with dysfunctional ABI1 and ABI3 transcription factors since this combination totally disrupts desiccation stress responses (10). The method of mutation will be the same as above. Even though the ABI transcription factors aren't necessary for all of the water-deficit response genes, their loss of function will definitely reduce the quantity of LEA proteins and other desiccation induced proteins in the cell. For example, there are 5 groups of LEA proteins, and the ABI3 transcription factor is responsible for activating at least Group 4 LEA proteins, but Group 2 LEA proteins do not require ABI3 (6). Once again, the reduction of superfluous proteins will free up resources for other cell processes. The last part of the experiment will involve creating mutant Arabidopsis plants with dysfunctional CBFs, ABI1, and ABI3. This strain will lack any sort of protection against the cold or desiccation, so the water source and temperature will be carefully monitored (10). If the plants grow at least somewhat normally after blocking the cold and drought pathways, then it will be a good idea to further pursue methods to permanently block the unneeded responses of cold and desiccation protection in the plants with a high possibility of being sent to Mars. If the desiccation response is necessary, then it is very possible to enhance desiccation tolerance via genetic engineering or other biotechnological methods to increase production at low pressures.

There are a lot more was to help plants cope with low pressures. For example, if the desiccation response and ABA is causing stomata to close, then it hinders the plant's ability to exchange gases like CO2, and photorespiration could occur. By blocking this response, perhaps the plants will have a more normal respiration rate. Also, investigation into the role of Heat Shock Proteins in hypobaric plants is another possible route. This project will serve as a model for future projects to engineer plants to thrive in hypobaric environments.

References

1. Ferl, RJ; Schuerger, AC; Paul, A; Gurley, WB; Corey, K; Bucklin, R. 2002. Plant Adaptation to Low Atmospheric Pressures: Potential Molecular Responses. *Life Support Biosph Sci*. 2002; 8(2):93-101.

2. Rizhsky, L; Liang, H; Shuman, J; Shulaev, V, Davletova, S; Mittler, R. 2004. When Defense Pathways Collide. The Reponse of Arabidopsis to a Combination of Drought and Heat Stress. *Plant Physiology*. April 2004, Vol. 134, pp. 1683-1696

3. Graham, JM. 2004. The Biological Terraforming of Mars: Planetary Ecosynthesis as Ecological Succession on a Global Scale. *Astrobiology*. Volume 4, Number 2, 2004.

4. Paul, A; Schuerger, AC; Popp, MP; Richards, JT; Manak, MS; Ferl, RJ. 2004. Hypobaric Biology: Arabidopsis Gene Expression at Low Atmospheric Pressure. *Plant Physiology*. January 2004, Vol. 134, pp. 215-223

5. Liu, F; VanToai, T; Moy, LP; Bock, G; Linford, L; Quackenbush, J. 2005. Global Transcription Profiling Reveals Comprehensive Insights into Hypoxic Response in Arabidopsis. *Plant Physiology*. March 2005, Vol. 137, pp. 1115-1129.

6. Buchanan, et al., *Biochemistry and Molecular Biology of Plants.* 2000. American Society of Plant Biologists.

7. Lehto, KM; Lehto, HJ; Kanervo, EA. 2005. Suitability of different photosynthetic organisms for an extraterrestrial biological life support system. *Research in Microbiology*. 157 (2006) 69-76.

8. McKay, CP; Marinova, MM. 2001. The Physics, Biology, and Environmental Ethics of Making Mars Habitable. *Astrobiology*. Volume 1, Number 1, 2001

9. Musgrave, ME; Gerth, WA; Scheld, W; Strain, BR. 1988. Growth and Mitochondrial Respiration of Mungbeans Germinated at Low Pressure. *Plant Physiology*. Vol. 86, pp19-22

10. Ooms JJ; Bartels, D; Koornneef M; Karssen, C. 1993. Acquisition of Desiccation Tolerance and Longevity in Seeds of Arabidopsis thaliana. *Plant Physiology*. Vol. 102, pp1185-1191