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The Effect of Hydroponics on *Glycine max* Root Systems through the Analysis of the Gene SUBI2

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Abstract

The gene expression of SUBI2, a gene that regulates the protein Ubiquitin, was analyzed to understand the effects of hydroponics on the root systems of *Glycine max* (var. Williams 82). Following a 21 day growth period of 6 *Glycine max* plants, the root systems of each plant were taken to isolate their RNA using the NucleoSpin® RNA Plant by Machery-Nagel. The RNA samples were then used for reverse transcription, to translate to cDNA, which was used for a 25 cycle Polymerase Chain Reaction (PCR). The resulting strands were placed on an agarose gel electrophoresis to analyze the contrasting brightness of their bands. The results of the gel electrophoresis show that SUBI2 was expressed more in hydroponics rather than in traditional forms of agriculture. Using the expression analysis of SUBI2, future applications may include using CRISPR Cas9 to reliably amplify the gene expression to increase yield and growth rate in hydroponic systems.

Keywords: Hydroponics; *Glycine max*; Ubiquitin; SUBI2; Root Systems, RT-PCR

Introduction

Soybeans (*Glycine max*) are one of the most widely grown plants in the world. It is one of the United States' larger agricultural exports, accounting for 21.5 billion dollars in 2018 [1,2]. Due to its large variety of uses, it is an extremely valuable and important crop to many industries. Besides its traditional uses for human consumption, it is even more widely used for cattle feed and cooking oil. Additionally, soybeans are a nitrogen-fixing crop (legumes), which makes it a staple for crop rotation. Soybeans are in extremely high demand due to the increasing world population, which is estimated to reach 9.7 billion by 2050. As the human population increases, the need for more livestock will also increase. However, many countries are unable to produce the amounts of soybeans needed for livestock and consumption [3]. A plausible solution is to use a different method of growing called hydroponics.

Hydroponics is a rapidly spreading growing method that is revolutionizing the agricultural community. Through the mere use of light and a water-based nutrient solution, a single gardener can produce 11 times more yield than they would be able to use soil [4]. Using these findings it is possible to infer that hydroponics changes the plants' physiology due to the introduction of possible abiotic stresses such that of water abundance. Furthermore, the root systems of plants are affected the most by the introduction of this new medium. There has been little research regarding the effects of hydroponic conditions on the plant's gene expression, especially genes related to root adaptation and growth. By better understanding the genetic changes of plants, researchers can genetically modify new breeds to better suit hydroponic growing methods; therefore causing an increase in yield and growth rate. A gene called SUBI2 (Glyma13g17830) is a possible target gene that lies in a Quantitative Trait Loci (QTL) related to plant height [5].

SUBI2 has been linked to regulating the functions of a small protein called Ubiquitin [6]. Ubiquitin's main function is proteasomal degradation (called ubiquitination), to maintain homeostasis. However, its single role has many uses, such as cellular growth, abiotic stress reaction and embryogenesis [7]. Additionally, a study conducted by Leitner et al. [8] concluded that Ubiquitin is of "significant importance" in root systems, especially "for auxin distribution in root meristems" and "environmentally controlled adaptations of root growth." It has a vital role in the formation of roots and the adaptation of the plant as a whole. The root meristem is the location of root elongation and growth. Thus, Ubiquitin's prominence in the root meristem can be inferred to have a profound effect on the root's adaptation to its natural environment, especially the growing medium [9]. The growth of a plant is impossible without a strong foundation in the root system to intake nutrients and water. Its wide variety of functions all relies on environmental factors to trigger this protein.

Furthermore, the location of the protein determines its function, such as if the protein was located in the nucleus it would help "constitute a concise signaling pathway" [10]. When a plant is placed under more environmental stresses, Ubiquitin will be more prominent in the cellular processes of root cells and therefore the expression of its regulatory gene(s) will be higher [11]. Ubiquitin is present in all plants, and by understanding the expression levels of SUBI2 under a

hydroponic growth medium, the results of this experiment can be applied to other studies for other plant breeds in hydroponics [12].

Materials and Methods

Experimental design

To properly estimate the gene expression, the experiment devised has three trials, each trial consisting of a plant grown in hydroponics and a plant grown in soil. The plants are grown in soil (via traditional forms of agriculture) are considered to be the experiment's control to help analyze the difference in the gene expression hydroponics causes for the gene SUBI2. Each plant was grown for 21 days to allow for the gene to be activated at the same stage of growth.

According to Le et al. [13], the gene SUBI2 is highly expressed in the soybeans' VE growth stage. There are various methods of measuring gene expression, such that of RT-qPCR, Northern Blotting, and Southern Blotting. A reverse transcription-polymerase chain reaction (RT-PCR) was used to gain information about the expression levels of the gene SUBI2. Then the expression levels were analyzed by being placed on an agarose gel electrophoresis.

Using RT-PCR

An RT-PCR was used due to the utilization of the plant's RNA, which is required for any gene expression analysis. In order to gain accurate details about the gene expression of SUBI2, the RNA inside of the plants must be isolated for reverse transcriptase. RNA is a type of single-stranded nucleotide which replicates DNA in a cell's cytoplasm, therefore it also contains the expression of genes. Additionally, RT-PCR was chosen because it is also an affordable way to estimate gene expression as it mainly utilizes a generic thermal cycler; unlike an RT-qPCR which requires a special thermal cycler specially designed for gene expression analysis.

Growing constants and methods

In this comparative experiment, constants were kept to ensure that the only major variable changed was the growing medium. Generally, abiotic stresses cause genes to express differently, therefore the growing environment's temperature and daylight cycles were kept the same. These constants are especially important for the soybean; because soybeans are dependent on their environment due to their morphological growth habits, which are termed "maturity groups" (University of Wisconsin, 2015). In order to simulate a real-life environment, the daylight cycles and temperatures the soybeans were grown in were similar to the environment that the plants would have been grown in **(Appendix A).**

The nutrient contents of the hydroponic solution were also the same as the soil's (Scotts' Moisture Control Potting Mix) nutrient saturation. Based on Scotts' Miracle-Gro, the nutrient saturation of the Moisture Control Potting Mix is 0.21-0.11-0.16 (NPK format). To help keep nutrients a constant the nutrient saturation of the hydroponics mix was also 0.21-0.11-0.16. The macronutrients were only kept the same because they are considered to be the nutrients that affect plant growth the most. Micronutrients were not included as constants and therefore could have had a small impact on the results of the experiment. In addition to the nutrient saturation, the light received by each of the plants was kept constant. The light received by each plant was measured daily and averaged approximately 9000 lux for all plants **(Appendix B).**

Gene expression using RT-PCR

RT-PCR is an extremely precise tool in genetics. Thus, the need for an error-free procedure is required in order to have a successful experiment. The procedures in this experiment followed the MIQE guidelines for qPCR experiments as a way to help ensure that the most accurate information is gained from the RT-PCR.

Primer design

Over the course of the soybeans' evolution, its genome was replicated numerous times (polyploidy); therefore for each gene found there would be another gene with the same function. Many plants are polyploid, as having multiple copies of their chromosomes often leads to higher survival rates. The soybean's diploid predecessor was subjected to an aneuploid loss, polyploidization, and diploidization. Additionally, its genome may have had 2 duplications or hybridizations. Its long evolutionary history results in the soybean being a "partially diploidized tetraploid" [14]. Therefore in this experiment, it was of utmost importance to design a primer sequence specific to SUBI2 **(Figure 1)**. A primer is utilized in RT-PCR to help locate the gene that is needed for replication. It is possible to think of the primer as a key molded specifically to a single gene or segment of DNA. Therefore, when designing a primer, the sequences must only fit the gene of interest and not to a similar gene in a different repeated chromosome which is termed a paralog. The forward and reverse primers are base-pair specific and were updated from a former study conducted by Le et al. **(Figure 1)**. The primer sequences made, spanned an exon to exon junction (intron) to only replicate the coding portion of the gene sequence [5]. In order to ensure that the primer was only specific to SUBI2, NCBI's BLAST was used to ensure that the primer was specific to the one gene of interest in the soybean and not to any other eukaryotes. BLAST uses an algorithm to compare the base pairs of a primer to the DNA of other species. The BLAST comparison used ensures that the primer will only bond to the gene (SUBI2) inside of a specific organism (soybean).

SUBI2:

ATTCATAGCTATTCGCAGTTCCCAAATTCATTTTAATCCTAATTCTG
CACTTCTTGATCATTCGATTTTTCTCCTCAAGATGCAGATATTCGTA
AAAACTTTGACCGGCAAGACCATCACCCTTGAGGTGGAATCCTCC
GACACCATTGACAACGTAAAAGCTAAGATTCAAGACAAGGAAGGG
ATCCCACCTGACCAGCAGAGACTTATCTTTGCGGGTAAACAACTTG
AGGATGGTCGCACCCTTGCCGATTACAACATCCAAAAGGAATCAA
CCCTCCATCTCGTGCTTCGCCTCAGGGGTGGCATGCAAATCTTTGT
CAAGACTTTGACCGGGAAGACCATCACCCTTGAGGTGGAATCCTC
GGACACCATTGATAATGTTAAGGCCAAGATCCAGGACAAAGAGGG
CATCCCCCCAGATCAGCAGAGGCTGATTTTTGCCGGTAAACAACTT
GAGGATGGAAGAACCCTTGCTGATTACAACATTCAGAAGGAATCC
ACCCTTCACTTGGTGCTTCGTTTGAGAGGTGGTATGCAGATTTTCG
TTAAAACTTTGACCGGAAAGACCATAACCTTGGAGGTGGAGAGTT
CAGATACCATAGACAATGTGAAAGCTAAGATTCAGGACAAGGAAG
GTATCCCACCAGACCAACAGAGGCTTATTTTTGCTGGGAAGCAACT
GGAAGATGGTCGTACCTTGGCAGATTACAATATTCAGAAGGAATC
CACCTTGCATCTCGTCCTTCGCCTTCGCGGTGGTTTTTAAGCGTGTT
TTTTTTCAAGTGTTCTTGTTTTAAGCGTGTCTTTTTTCAAGTGTTCTT
GTATGTCATGTCTGTTATGTTCAATTTCTCCCTTGAACTTGTTTGCT
GTTCTGGTTTAGGTGCTGCTTTTTATGTTTTAATATGATGTATTGAG
CTCGTTTCGCCAAAAGAATTAAATATCAATATCGTATCATAGTTAA
ATTCATATTTCGT

Figure 1: Primer sequence specific to SUBI2.

RNA extraction procedures

The RNA of the soybeans was extracted for reverse transcription into cDNA for gene expression analysis. 50 milligrams from each of the plant's roots were cut, washed and then ground under dry ice to help ensure that the cells were flash-frozen and the RNA retained was pure. Plant roots contain starches and other RNA inhibitors, therefore the roots must be flash frozen. Following, the cells were lysed using 350 µL Buffer RA1 (Machery Nagel) and 3.5 µL ß-mercaptoethanol, so that it could be filtered to reduce viscosity and clear the lysate. Next, the lysate was mixed with 70% ethanol to allow for DNA binding. The resulting solution was then passed through another filter in addition to 350 µL of a Membrane Desalting Buffer. The RNA samples obtained were then purified to remove and contaminate DNase. Lastly, the silica membrane was washed and dried resulting in a pure RNA to be used for reverse transcription. All of the procedures used for RNA extraction followed the User manual for NucleoSpin® RNA Plant by Machery-Nagel.

Reverse transcription and PCR procedure

The resulting RNA from the plant was mixed with 10 µl of 2X Reaction Mix, 19 µl of 1X nuclease-free H2O, and 1 µl of OneScript® Plus RTase (200 U/µl). Then the components were centrifuged and incubated for 50 minutes at 50°C. The incubation reaction was stopped by heating at 85°C for 5 mins. The resulting cDNA was then used in PCR. All of the procedures used for reverse transcription followed the suggested user manuals by ABM Good [15].

The cDNA achieved by reverse transcription was then used in PCR for a 25 cycle replication. All PCR reactions have a maximum saturation point after 30-35 cycles **(Figure 2)**; to better see differences between the gene expressions in hydroponics compared to soil, a 25 cycle PCR was run. 100 ng of cDNA, 1 µl of the forward primer, 1 µl of the reverse primer, 25 µl 2X PCR Taq Plus MasterMix, and 20 µl Nuclease-free HO were mixed and centrifuged. Then they were placed in the thermocycler for 25 cycles. The resulting products were then placed on an agarose gel for gene expression analysis.

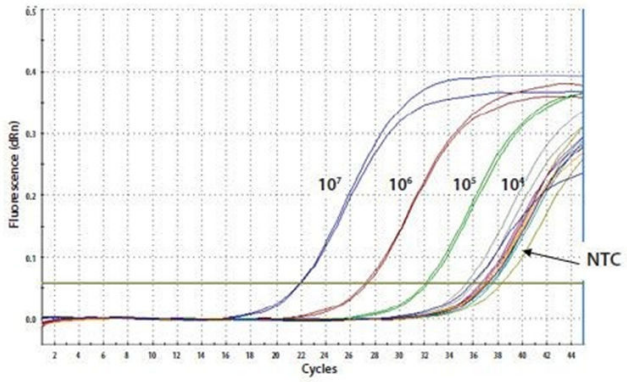

Figure 2: A qPCR curve.

Gel electrophoresis

The Mini One Gel Electrophoresis System was used for gel electrophoresis. 5 µl obtained from each sample was placed in each of the columns including one negative reaction. Lastly, the gel electrophoresis was run for 20 minutes, as recommended by Mini one, and a photo was taken of the resulting bands.

Results and Discussion

Analysis of the gel electrophoresis

Based on the gel electrophoresis **(Figure 3)**, it is possible to conclude that the gene expression of SUBI2 was more prominent in a hydroponic growing medium rather than in soil. Although the data obtained from the gel electrophoresis is qualitative, the bands correlating to the gene expression of SUBI2 in hydroponics appear to shine brighter, than the bands of the plants grown in soil. Based on the results, it is possible to conclude that SUBI2 is heavily influenced by growing medium and environmental stresses, which was confirmed by [14].

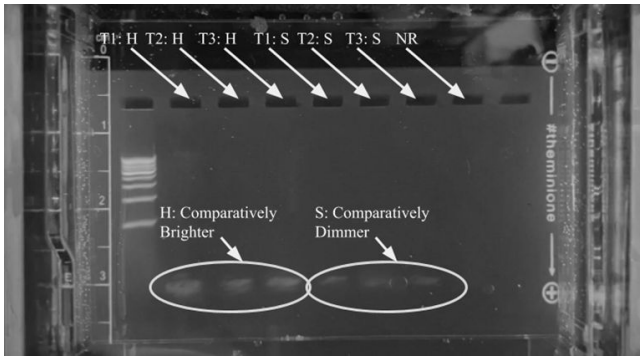

Figure 3: Gel electrophoresis results: The "H" represents hydroponically grown plants while the "S" represents plants grown in soil.

Ubiquitin is known to degrade proteins causing the protein to turn over in the protein cycle

This process is known to affect many different and important cellular components such as transcription factors and cytoskeletal proteins. Additionally, protein turnover in the roots can be used to explain why hydroponic plants are able to grow faster. Due to the introduction of this environmental stress, the plant tries to intake as many nutrients as possible. However, it also has to adapt to this artificial environment that was created. Therefore we can infer that the ubiquitination pathway resulted in antibiotic tolerance of the hydroponic growing medium [16]. The choice of root growth media has an extensive effect on gene expression and protein half-life. Therefore SUBI2 should be considered as a factor in comparing expression and protein assays of root growth from different experimental protocols **(Figure 4).**

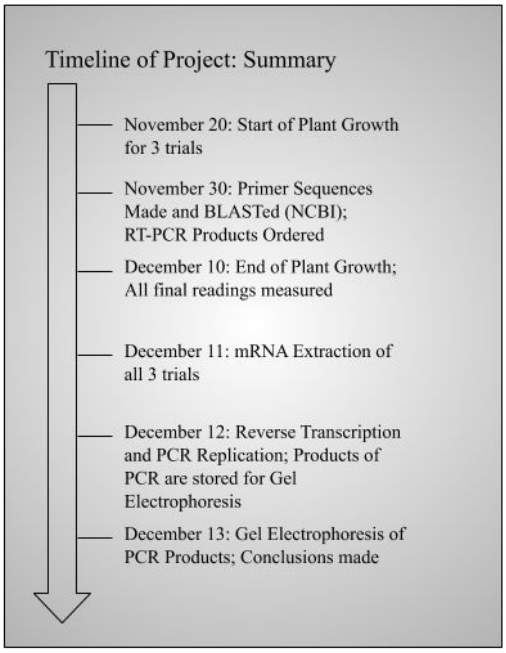

Figure 4: Project summary timeline.

Conclusion and Further Scope

The results of the reverse transcription PCR proves that the growth of a soybean causes some genes to be expressed more than in soil due to the introduction of new abiotic factors. Ubiquitin is an extremely important protein with many functions, and the introduction of a new growing medium causes it to work more than it would normally be in the soil. The changes in the environment caused by growing using hydroponics are the main reason why many plants grow better in hydroponics. Genetically modifying plants to better suit hydroponics can increase yield and decrease the number of resources wasted. CRISPR Cas9 is an alternative method than can be used to reliably increase the expression levels of a designated gene. When breeding new forms of soybeans, it is possible to genetically modify other parts of the genome while using CRISPR Cas9 to increase the expression levels of SUBI2. However, the side-effects of the genetically editing SUBI2 are still very much unknown and should be proceeded with caution. Hydroponics is an inexpensive and reliable way to grow plants; due to its versatility where it can be used in many harsh environments. By increasing the efficiency of hydroponics we can grow more using fewer resources to feed an ever-growing population sufficiently. Additionally, the use of hydroponics also reduces the strain of the nearby ecosystem due to nutrient runoff often occurring from traditional forms of agricultural farming. Therefore, it is important that more research is done on the physiological effects the hydroponic growing medium has on plants, because of the large impact that it will have on society and our planet.

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