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### Abstract

Autophagy is a stress reaction process by which eukaryotic cells internalize and degrade cytosol in a vacuole or lysosome and recycle the contents to where they are needed in the cell. The affect on autophagy genes Atg6 and Atg13 were examined in response to sucrose deprivation and increased salt concentration in the plant *Arabidopsis thaliana*. It was concluded that although these genes are always being expressed, they do not increase in expression in response to these stress conditions.

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### Subject Categories

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**The Effect of Sucrose Starvation and Salt Stress on Atg6 and Atg13 in *Arabidopsis***

By

Lindsey Gish

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Eastern Michigan University

Honors Program

in Partial Fulfillment of the Requirements for Graduation

with Honors in Biology.

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Honors Advisor (Print Name and have signed)

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Department Head (Print Name and have signed)

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Honors Director (Print Name and have signed)

## **Abstract**

Autophagy is a stress reaction process by which eukaryotic cells internalize and degrade cytosol in a vacuole or lysosome and recycle the contents to where they are needed in the cell. The affect on autophagy genes Atg6 and Atg13 were examined in response to sucrose deprivation and increased salt concentration in the plant *Arabidopsis thaliana*. It was concluded that although these genes are always being expressed, they do not increase in expression in response to these stress conditions.

## Introduction

We can see from the recent tomato shortage resulting from the hurricanes in the Atlantic that any fluctuation in weather can cause a cascade of ramifications nationwide and even internationally. It is important that we try to understand how plants harness and distribute nutrients so that we can avoid food shortages in the future. Just as genetic manipulation of crops has provided a greater yield, an understanding of how plants maintain nutrient concentrations and production of stress-resistant crops can lead to more economic stability when the weather takes a turn for the worse.

Eukaryotic cells use a process known as autophagy to maintain the proper nutrient concentrations under stress conditions. Autophagy, which literally means "to eat oneself," is the process by which eukaryotic cells transport parts of their cellular components into a vacuole where they are degraded into basic nutrients and redistributed to where they are needed most. Through this the cell is able to maintain a nutrient balance and can ensure that the organism will operate efficiently in times of stress.

While autophagy is an induced process in yeast and mammals when under periods of stress (Reggiori and Klionsky 2002), plants appear to undergo autophagy constantly, perhaps increasing the rate of under stress. My goal throughout this research has been to identify and characterize the levels of expression of autophagy genes in *Arabidopsis* when the plants are under salt stress and when lacking sucrose. The stress conditions used in this experiment are an increase in salt concentration and a lack of sucrose in the media.

Autophagy has been studied most extensively in yeast and mammalian cells and is not nearly as well understood in plant cells. In mammalian cells, the organelle that is the site of degradation is the lysosome while in yeast it is the vacuole. Since the site of degradation in the

plant cell is the vacuole, as it is in yeast, we have been using the yeast autophagy model for comparison to autophagy in plant cells. In yeast, an autophagosome envelopes cytosol and then transports it to the membrane of the vacuole. The membrane of the autophagosome then fuses with the membrane of the vacuole and deposits its contents into the vacuole. The vacuole contains enzymes such as hydrolases and protease that break down macromolecules. The vacuole then degrades the contents and redistributes the nutrients to where they are needed (Figure 1) (Wang and Klionsky 2003).

To do this research, I needed a plant that is ideal for keeping in laboratory conditions and that has been completely sequenced. I used the plant *Arabidopsis thaliana* otherwise known as Thale Cress or Mouse Ear. In nature it is a common weed, growing wherever it finds room and the right conditions. *Arabidopsis* is an ideal model plant to use because of its relatively short lifecycle. It germinates, grows, sets seed (in the thousands), and dies within a matter of weeks. It is also easy to grow and keep in the lab. *Arabidopsis* naturally grows in the winter time, however its optimum growing conditions are easily synthesized in the lab and therefore we can keep it growing all year long.

Essentially, *Arabidopsis* is the fruit fly of the plant world in the field of genetics because of its short life-span, simply identifiable physical characteristics, the ease with which its genetics can be manipulated, and its relatively simple genetics. The *Arabidopsis* genome consists of 5 chromosomes and is diploid, meaning it has 2 copies of each chromosomes, like humans. This

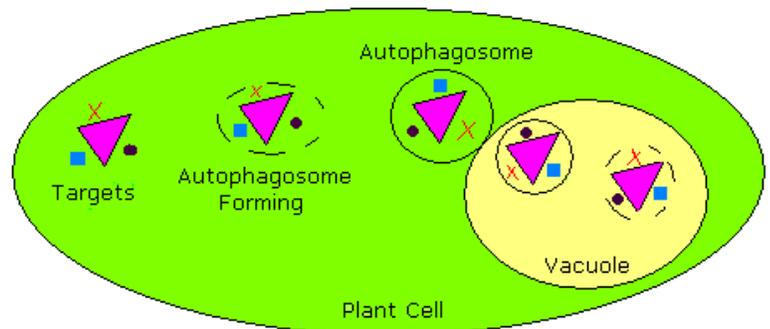


Figure 1. In autophagy, cytosol and contents are engulfed by an autophagosome and transported to the vacuole for degradation and reallocation

makes it much easier to work with than other plants which can be polyploid, having many copies of each chromosome.

In order to analyze gene transcription through amplification of RNA transcribed by genes, we must know the location of the genes being studied. For this, it is essential that the genes of interest be sequenced. Genome sequencing projects such as the Human Genome Project are what make this type of research possible. The sequencing of the *Arabidopsis* genome was completed in 2000 and is now available online ([www.arabidopsis.org](http://www.arabidopsis.org)). Although the sequences of these genomes is known, it will take years of research to fully understand the function of the genes. Once we do, plants will be able to be manipulated in order to grow under the harsh conditions; perhaps in the nutrient-poor soils of many impoverished nations and even in soils contaminated with substances plants normally cannot handle.

I studied autophagy genes in *Arabidopsis Thaliana* known as Atg6 and Atg13 which are homologous to autophagy genes found in yeast. In yeast, Atg6 is involved in the formation of the autophagosome and the targeting of the autophagosome to the vacuole. Atg13 combines with another autophagy protein, Atg1, to induce autophagy (Klionsky and Reggiori 2002). In yeast, these genes are involved in the targeting pathway which selects proteins for vacuolar degradation (Hanaoka et al, 2002). In autophagy, a section of cytosol is engulfed by an autophagosome and then is transported to the vacuole for degradation and reallocation (Figure 1).

To analyze the expression of these genes I determined the amount of mRNA that these genes were transcribing. Only when RNA is transcribed from template DNA can a protein be made from the RNA code. The amount of RNA present is proportional to the amount that gene is being expressed and therefore can sometimes correspond to how much protein that gene is

making. After amplification by RT-PCR, the bands that had original samples with a greater amount of RNA in them should appear as brighter bands than those that had less. This is how I was able to examine the increased, or lack of increased, expression of the genes in question. My goal in this project is to determine whether or not Atg6 and Atg13 are turned on and/or increase in expression under stress conditions. I expect the transcription of both genes to increase under both stress conditions I impose which will be increased salt concentrations and sucrose starvation.

## Methods



Figure 2. Arabidopsis Plant growing on solid MS media

The culture system was a vital part of this research. The plants and subsequent callus (undifferentiated) cells were grown in sterile boxes on solid media. Through the use of the solid media I was able to control the nutrients the plants were getting. Through manipulation of the liquid media, I was able to add and subtract sucrose and NaCl (table salt) and analyze the effect on my genes of interest.

Wildtype *Arabidopsis* plants were grown on MS media from Phytotechnology Laboratories. Wildtype callus cells were then grown on callus-induction media (Table 1). Making callus cells consists of taking healthy leaves and cutting them to inducing a



Figure 3. Arabidopsis callus cells growing on solid callus-induction media

wounding response and placing these wounded leaves onto the callus-induction media. After the



Figure 4. Arabidopsis callus cells in liquid media

callus cells proliferated, they were transferred to liquid media. Half of the callus was transferred to full liquid media, and half to liquid media with no sucrose.

After three days the callus grown in the full media was put into new media: half into full

media and half into full media + 100 mM NaCl. These

samples were again split and half of the callus grown in the media with no sucrose was put into new media, again with no sucrose, and the other half into new media, again with no sucrose + 100 mM NaCl (Figure 5). For the sake of simplicity, the treatments were referred to by letter:

- A, full media
- B, no sucrose media,
- C, full media + 100mM NaCl
- D, no sucrose + 100mM NaCl.

Once the callus went into this new media, a sample was taken and this was time 0. Samples were taken at 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours. The RNA from these callus samples was then extracted

using Qiagen's RNeasy kit. RT-PCR was run using Promega Accessquick RT-PCR system

(Table 2). The RT-PCR program began with a 45 minute incubation at 48 °C. Then it was held

at 94 °C for 2 minutes for the initial elongation, then 22 cycles of 94 °C for 30 seconds, 60 °C for

Table 1. RT-PCR components and concentration used in analyzing gene expression

<b>RT-PCR (25 <math>\mu</math>L Final Volume)</b>	
<b>Component</b>	<b>Final Concentrations</b>
2X AccessQuick Master Mix	1X
Atg6 Primer	1 $\mu$ M
$\beta$ -Tubulin Primers	100 nM
RNA Template	200 ng
Nuclease-Free Water	to bring to final volume
AMV Reverse Transcriptase (5units/ $\mu$ L)	0.08 units/ $\mu$ L

1 minute, and 68 °C for 2 minutes. There was then a final elongation at 68 °C for 7 minutes and the reactions were held at 4 °C. The expression of Atg6 was analyzed with respect to the expression of the internal control reaction for  $\beta$ -Tubulin.  $\beta$ -Tubulin is a structural protein within the cell that is always needed and so the expression of the gene should not change due to stress conditions (Bhattacharya and Cabral

2004).

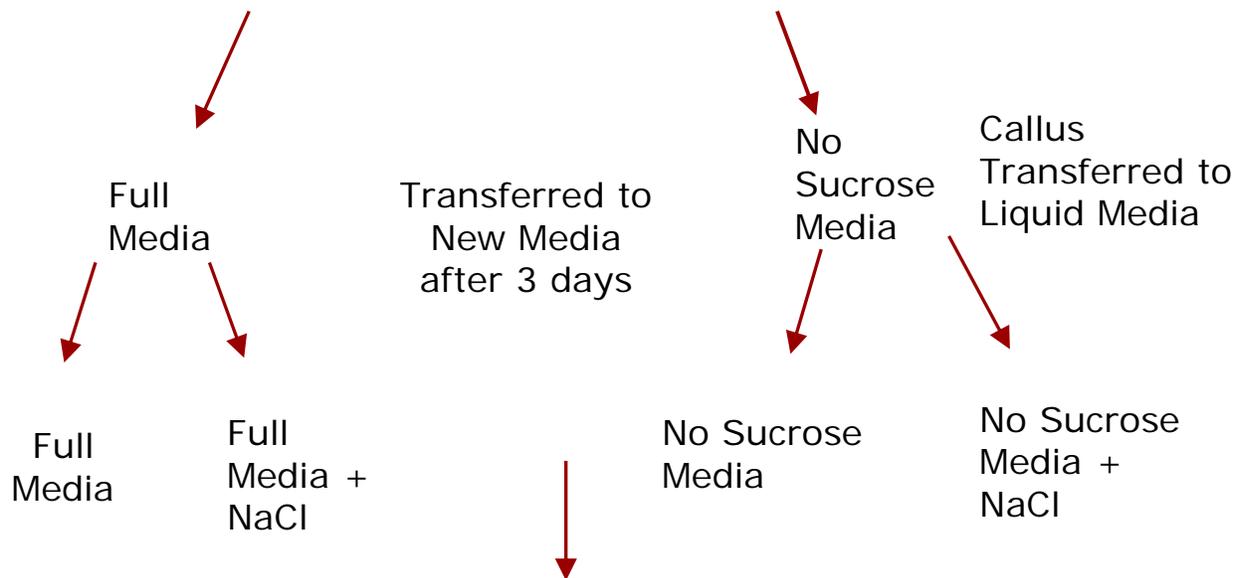
Table 2. RT-PCR primers and sequences used in analyzing gene expression

<b>Name</b>	<b>Sequence</b>
$\beta$ -Tubulin Right	5'- TTC AGC ATC TGC TCG TCA AC -3'
$\beta$ -Tubulin Left	5'- TTC TTC ACA TCC AGG GTG GT -3'
Atg6 Right	5'- CAC TTC AAG AGA CAG ATT GTG AGA A -3'
Atg6 Left	5' GGA AAG AGG AGA TTC CAG ATA AAA G -3'
Atg13 Right	5'- TTG GGA CTT CTT CGG GTA TG -3'
Atg13 Left	5'- CCT TGC TCT CGT CAT CTT CC -3'

Arabidopsis grown on solid media with all sugar and essential nutrients



Callus (undifferentiated) cells grown on solid, callus-induction media



Samples taken from each at intervals over 5 hours



RNA extracted using Qiagen's RNeasy kit.



Expression of Atg6 analyzed using RT-PCR and gel electrophoresis.

Figure 5. Flow-chart of methods and media used.

## Results

The level of gene expression for all treatments for genes Atg6 and Atg13 were similar(Figures 6 and 7). To the naked eye there did not appear to be any difference in gene expression for either gene in any treatment. Neither gene showed any increase in transcription for any of the treatments and the band intensity of the experimental primers, Atg6 and Atg 13, did not increase relative to the intensity of the control primers  $\beta$ -Tubulin.

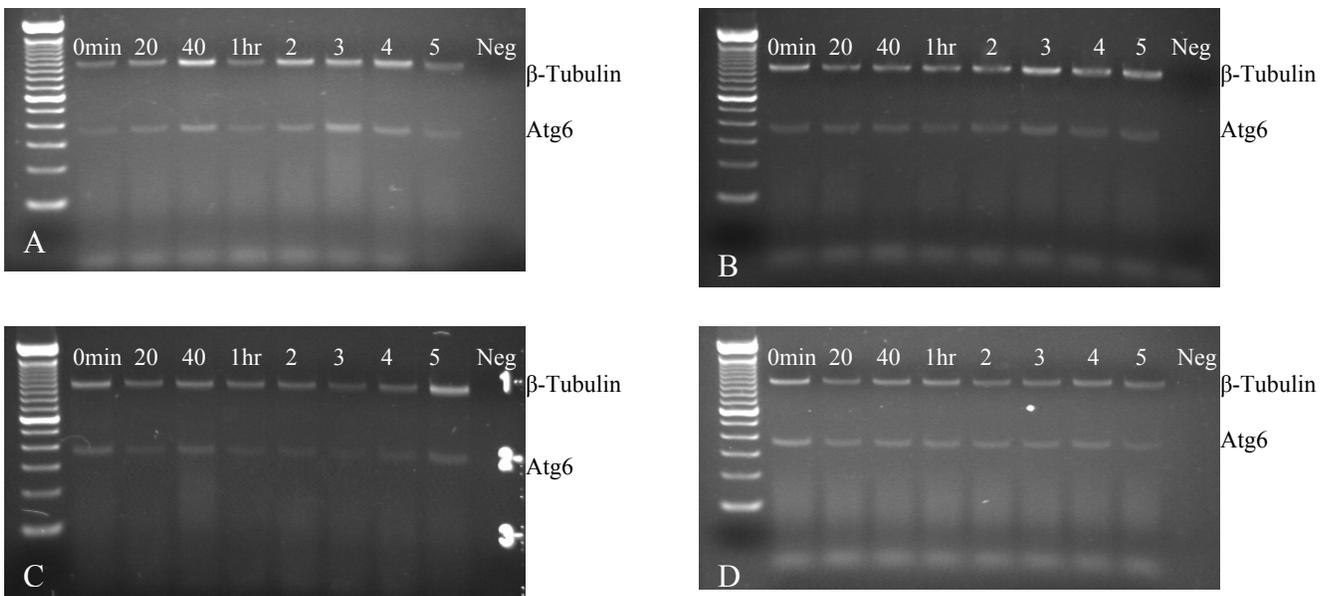


Figure 6. Atg6 expression gels. Treatment A- full media, treatment B- no sucrose media, treatment C full media with 100 mM NaCl, treatment D no sucrose media with 100 mM NaCl.

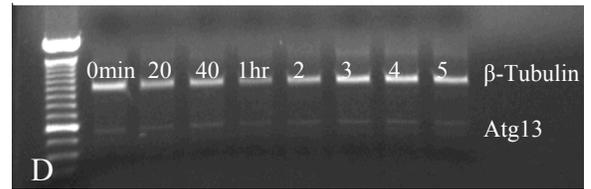
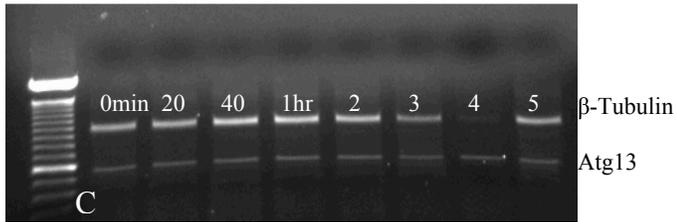
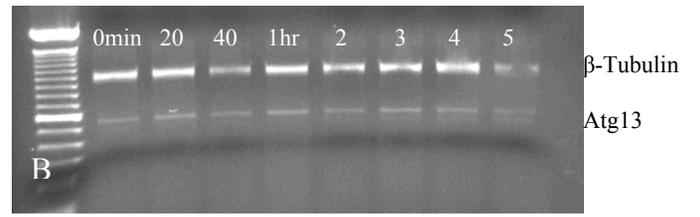
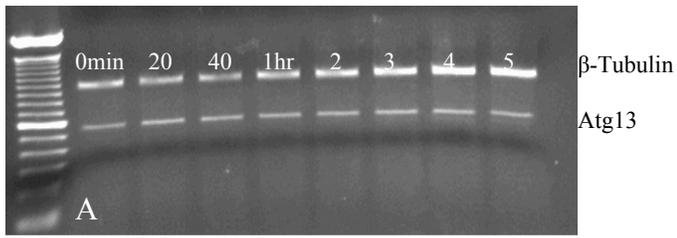


Figure 7. Atg13 expression gels. Treatment A- full media, treatment B- no sucrose media, treatment C full media with 100 mM NaCl, treatment D no sucrose media with 100 mM NaCl.

## Discussion

In order to examine the expression of autophagy genes Atg6 and Atg13 in *Arabidopsis thaliana*, timed experiments were carried out with callus cells in a full media, media lacking sucrose, and each of these with 100 mM NaCl for a total of four different media. Callus cell samples were extracted at time 0, 20 minutes, 40 minutes, 1 hour, 2 hours, 3 hours, 4 hours and 5 hours. RNA was extracted from each of the samples, amplified through RT-PCR and visualized through gel electrophoresis.

It did not appear as if the intensity of bands for either gene changed, neither increasing nor decreasing, in response to any media. According to these results, I conclude that the expression of Atg6 and Atg13 does not change in response to sucrose starvation or salt stress. However, that there were bands for every treatment at every time interval supports the hypothesis that these autophagy genes are always expressed, even when plants are not under stress conditions.

In order to confirm these results the experiment should be repeated. The expression of the genes in the callus before it enters into the liquid media should also be analyzed as well as the callus living in liquid media before the experiment begins. In the future, analysis of the expression of these genes over a longer period of time, several days, should also be carried out to confirm that the expression does not increase under these conditions. This may be a possibility and it may take several days for the gene to be expressed at increased levels. It may also be possible in the future to analyze protein expression levels of these two genes. This would be done to confirm the results because the level at which mRNA is transcribed does not always correspond with the level of protein expressed.

## **Troubleshooting**

I encountered problems at many points along the way throughout my research. The most frustrating and overwhelming problem was contamination. The media used are packed with everything that the plants need to survive, however, they also contain everything needed for bacteria and fungi to thrive as well. I had contamination in every kind of media at one point, which set me back about 2 months overall. It took stringent sterilization of all components, including my hands, to rid my experiment of the contamination.

Other troubles came about when I extracted RNA with the Quiagen RNeasy kit. It took a while to refine the technique and timing of this protocol. In this protocol, the callus cells and all of the equipment being used, including a mortar and pestle, must be kept frozen with liquid nitrogen. After the RNA extraction problems, the RT-PCR program troubles began. I had to pick a number of cycles that would amplify to the point of visualization both the experimental and control bands without saturating either to the point of being too intense. After several weeks of adjustment, I finally discovered an RT-PCR program that worked.

## **Future Directions**

When I began this research, my intentions were to analyze the expression of autophagy genes in mutant plants. As it turns out, full mutant Atg6 plants are not viable, and do not usually grow or set seed. As far as heterozygous Atg6 and Atg13 plants, I was not able to isolate any heterozygous of either in time to obtain data for my thesis. Therefore my first direction in the future will be isolate heterozygous Atg6, Atg13 and mutant Atg13 plants and analyze the expression of those genes under sucrose starvation and salt stress conditions. I also would like to look at the physical characteristics of both kinds of mutant plants if they are able to be isolated and germinate. In addition to looking at Atg6 and Atg13, I will examine other autophagy genes within *Arabidopsis*.

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