Expression of 9/13 Hydroperoxide Lyase in Cucumber Leaves

By

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Abstract

Fatty Acid 9/13-Hydroperoxide Lyase (9/13-HPL) in the cucumber plant is an enzyme that cleaves either 9- or 13-hydroperoxides of polyunsaturated fatty acids to form volatile C9 or C6 aldehydes respectively. Since these aldehydes may play a role in the plant's defenses against pathogens (K. Matsui, et. al. Phytochemistry 67 (2006) 649-657), the enzyme is stress induced during vulnerable times such as when the plant is injured. In order to better understand how this enzyme is induced, we have developed a Real Time Polymerase Chain Reaction method to quantitate levels of 9/13-HPL mRNA. In initial experiments, the effect of mechanical wounding of cucumber cotyledons on the transcription levels of the 9/13-HPL gene in wounded tissue and unwounded leaves was examined. The RNeasy Plant Minikit from Qiagen was used to isolate mRNA from the plant tissue and SYBR Green was used as the detection system for Real Time PCR.

Introduction

Many plants produce volatile aldehydes such as (Z)-3-hexenal and (E)-2-hexenal as a defense mechanism (Matsui, 2006; Tiget, 2001). The cucumber plant, for example, produces aldehydes among other products, in response to mechanical wounding (Matsui, 2006). These products include hexanal, 12-oxo-(Z)-9-dodecenoic acid, (Z)-3-nonenal, and 9-oxo-nonanoic acid. The C-9 aldehydes specifically are important flavor compounds in cucumbers and melons (Matsui, 2006). These aldehydes also have been shown to repel insects, lead to the production of plant antibiotics such as phytoalexins, and in some cases, act as antibacterial and antifungal agents against plant pathogens (Matsui, 2008). Studies in some plants have shown that absence

of the HPL enzyme in plant leaves can lead to as high as a two-fold increase in aphid fecundity in the leaves (Vancanneyt, 2001).

The enzymes Lipoxygenase and Hydroperoxide Lyase (HPL) play key roles in the production of these aldehydes. Lipoxygenases add molecular oxygen to linoleic acid or linolenic acid to produce hydroperoxides (HPO), while HPL subsequently cleaves the HPO into aldehydes and carboxylic acids (Matsui 1991). There are a variety of substrate specific HPL enzymes, which cleave only specific hydroperoxides. These enzymes specifically cleave either C-9 or C-13 hydroperoxides. Although 13-HPL, which cleaves C-13 HPO is widespread, fatty acid 9/13-hydroperoxide lyase has only been detected in a few species with cucumbers being the best studied.

This enzyme can cleave either C9 or C13 HPOs. The levels of 9/13-HPL are regulated by mechanical wounding or tissue disruption of the plant, as well as the development of the plant, with high levels of the enzyme in female flowers, mature fruit, and hypocotyls (Matsui, 2006). Figure 1 shows the HPL facilitated production of volatile aldehydes from fatty acids.

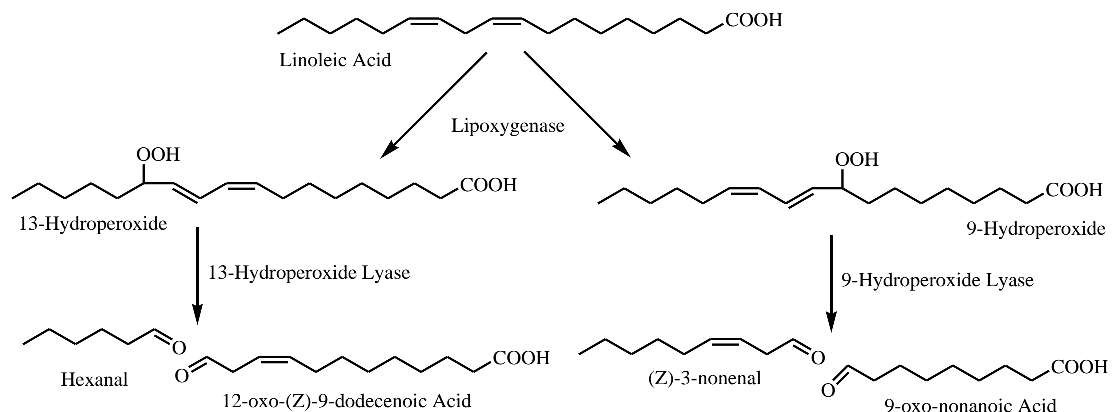

Figure 1: Mechanism of aldehyde production through LOX and HPL catalysis.

The long term objective of this project is to further investigate the potential role of 9/13 HPL in the cucumber's defense system. Specifically, the project's primary goal is to determine whether plant regulatory compounds such as ethylene and methyl jasmonate, which trigger, defense responses in other plants, affect the expression of 9/13 HPL. The initial goal of this project was to develop and test a Q-PCR method to monitor the expression of 9/13 HPL mRNA

Methods and Materials

Q-PCR Design

The first stage of this project involved designing primers for Q-PCR. It was necessary to design a pair of primers for a housekeeping gene to serve as an internal control as well as for the 9/13 HPL gene. Actin was selected as the housekeeping gene. The Gene Bank file DQ115883 was used as the reference sequence for designing these primers. File AF229811 was used to design primers for the 9/13 HPL gene. Primer Express 3.0 from Applied Biosystems was used to design the primers. The following primers were selected for this project:

HPL Forward	CAAAGTCGAGAAACGCAACATT
HPL Reverse	GCCGGTGAAGGACAAGGA
Actin Forward	GGGTTACGCCCTCCCTCAT
Actin Reverse	TCAGTAAGGTCACGACCAGCAA

Plant treatment

Cucumber plants (National Pickling) were grown in potting soil in a growth chamber with 12 hours of light. Once the plants had well-developed first real leaves (two to three weeks

post germination), they were treated wounded by pinching the cotyledon across its mid-vein three times with forceps. Tissue was harvested 6 hours after treatment and immediately frozen in liquid nitrogen and stored at -80°C.

Table 1 outlines the plant treatments used in this project.

Table 1: Treatment scheme of various sets of *Cucumis sativus* seedlings. At the time of harvest, plants from set 1 had well developed primary leaves.

Sample	Wounded
Control Cotyledon	No
Control Primary Leaf	No
Exp. Sample 1 Cotyledons	Yes
Exp. Sample 1 Primary Leaf	No

Total RNA Extraction

Total RNA extraction of each sample was done using a Qiagen RNeasy plant minikit. Each sample was ground to a fine powder in liquid nitrogen, and 100 mg of each sample was collected in a 1.5 ml microcentrifuge tube, and immediately mixed with 450 µl of the buffer RLT. The tubes was heated at 56°C for 3 min and then cooled. The lysate was transferred to a QIAshredder spin column, placed in a 1.5 ml collection tube and centrifuged for 2 min at 10500 rpm. The flow-through was carefully transferred to a new microcentrifuge tube. Care was taken not to disturb the pellet that had formed in each of the flow-through tubes. 100 µl of 100% ethanol was added to each of the tubes, and the tubes were mixed. The mixture in each tube was transferred into its own RNeasy spin column placed in a 2.0 ml collection tube, and the spin column was spun for 15 s at 10500rpm. The flow through was discarded. 350 µl of buffer RW1 was added to each spin column and the column was centrifuged for 15 s at 10500 rpm (flow

through was discarded). 80 µl of the buffer RDD containing DNase was added onto the membrane of each column, and the column was allowed to sit for 15 min at room temperature. 30 µl of buffer RW1 was added to each spin column and the columns were spun for 15 s at 10500rpm. Then 500 µl of buffer RPE was added to each spin column and the columns were spun at 10500 rpm for 2 min. The columns were spun once more for 1 min to dry any remaining ethanol. New RNase free collection tubes replaced the old ones, 50ul of RNase free water was added to each tube and the columns were spun at 10500rpm to elute the RNA. RNA was stored at -80°.

RNA Concentration and Purity Estimation

Total RNA concentrations were determined for each sample. 2 µl of the original RNA sample was diluted into 98 ul of 25mM Tris buffer pH 7.6. A280 and A260 readings were taken on a SmartSpec Plus spectrophotometer (Bio-Rad) for the diluted sample. To estimate the total RNA concentration of the sample, the reading was multiplied by the dilution factor of the sample and a constant of 40, assuming that 40ug/ml sample has an absorbance of 1.0. To assess the purity of the RNA, a ratio of the A260 to the A280 was taken. An appropriate ratio is 1.9-2.1.

Reverse Transcription

The composition of the reverse transcription master mix is displayed below in Table 2 while Table 3 gives the Reverse Transcription components for the different samples.

Table 2: Constituents of the reverse transcription master mix

Constituent	Volume (µl) for 1 reaction	Volume (µl) for 8 reactions
10X RT Buffer	2.0	16.0
25X dNTPs	0.8	6.4

10X Random Primers	2.0	16
Reverse Transcriptase	1.0	8
RNase Inhibitor	1.0	8
RNase free water	3.2	25.6
Total:	10.0	80.0

Table 3: Reverse transcription protocol for the various RNA samples from cucumber leaves.

Sample	RNA (μl)	RNase free water (μl)	RT master mix (μl)
Control Cotyledon	10.0	0	10.0
Control First Real Leaf .02ug aliquot	3.0 of 1:50 diluted sample	7.0	10.0
Control First Real Leaf .2ug aliquot	0.6 of undiluted sample	9.4	10.0
Control First Real Leaf 2ug aliquot	6.0 of undiluted sample	4.0	10.0
Experimental (injured) Cotyledon	10.0	0	10.0
Experimental First Real Leaf (uninjured)	10.0	0	10.0

The mixtures above were placed in a Perkin Elmer Thermal Cycler. The thermal cycle was as follows: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C, and the remainder of the time at 4°C.

A second RT protocol was run using the same samples, and the same procedure, however, the three standard curve aliquots were prepared from the RNA of the experimental first real leaf sample. This protocol is displayed below in Table 4.

Table 4: Second RT protocol. 1A: control Cotyledon, 1B: control First Real leaf, 1C: Experimental Cotyledon (injured), 1D: Experimental First Real leaf (uninjured).

Sample	RNA (ul)	RNase free water (μl)	RT master mix (μl)
1A	10.0 of undiluted sample	0	10.0

1B	5.7 of undiluted sample	4.3	10.0
1C	10	0	10.0
1D .02ug aliquot	6.2 of undiluted sample	3.8	10.0
1D .2ug aliquot	6.2 of 1:10 dilution	3.8	10.0
1D 2ug aliquot	3.1 of 1:50 dilution	6.9	10.0

Q-PCR

Two master mixes were prepared, one for the actin gene and one for the HPL gene. Forward and reverse primers for the HPL gene were dissolved in 1mL of RNase free water, as were the primers for the actin gene (in a separate 1mL of RNase free water). A 1:100 fold dilution was carried out for both of these primer solutions. These diluted primer solutions were added to other constituents to make a PCR master mix.

Table 5: Q-PCR master mix protocol for both the actin and HPL gene.

<table>
<tr><th colspan="2">Actin</th><th colspan="2">HPL</th></tr>
<tr><td colspan="2">[Initial Forward Primer]= 602 nM
[Initial Reverse Primer]= 572 nM</td><td colspan="2">[Initial Forward Primer]= 473 nM
[Initial Reverse Primer]= 678 nM</td></tr>
<tr><td>Constituent</td><td>Volume (µl)</td><td>Constituent</td><td>Volume (µl)</td></tr>
<tr><td>Syber Green</td><td>315</td><td>Syber Green</td><td>315</td></tr>
<tr><td>Forward Primer</td><td>52.4</td><td>Forward Primer</td><td>66.7</td></tr>
<tr><td>Reverse Primer</td><td>55.1</td><td>Reverse Primer</td><td>46.5</td></tr>
<tr><td>H_2O</td><td>177.4</td><td>H_2O</td><td>171.7</td></tr>
<tr><td colspan="2">[Forward Primer in Master Mix]= 52.58 nM
[Reverse Primer in Master Mix]= 52.54 nM</td><td colspan="2">[Forward Primer in Master Mix]= 52.59 nM
[Reverse Primer in Master Mix]= 52.55 nM</td></tr>
</table>

For each sample, 63 µl of the master mix for either actin and HPL were mixed with 1 µl of the cDNA that was prepared via reverse transcription, and 2.3 µl of H_2O to give a primer of 50 nM. From each of these master mixes, triplicate samples of 20 µl were analyzed by Q-PCR.

The thermal cycle was as follows: 10 minutes at 95°C for the initial melting of the double stranded DNA, then 40 cycles of 15 seconds at 95°C, and 1 minute at 60°C, followed by a melt curve (60°C to 95°C over the course of 1 hour).

Results

Initial RNA Extraction

RNA was extracted from the leaves of two plants (cotyledons and first real leaves). A crude estimation of the RNA content is provided below.

Table 6: RNA concentrations of the initial samples

Sample	A_{260} reading	A_{280} reading	[RNA] estimation (ug/ml)	A_{260}/ A_{280} ratio
Control Cotyledon	.021	.002	42	10.5
Control real leaf	.176	.080	352	2.2
Wounded cotyledon	.086	.038	172	2.26
Real leaf from wounded plant	.162	.081	324	2.00

First Q-PCR run

The first Q-PCR run yielded amplification of both the actin and HPL gene. The C_T of the amplification curve of the HPL gene was slightly lower than that of actin's gene. The negative controls showed no amplification. Figure 2 shows typical amplification curves.

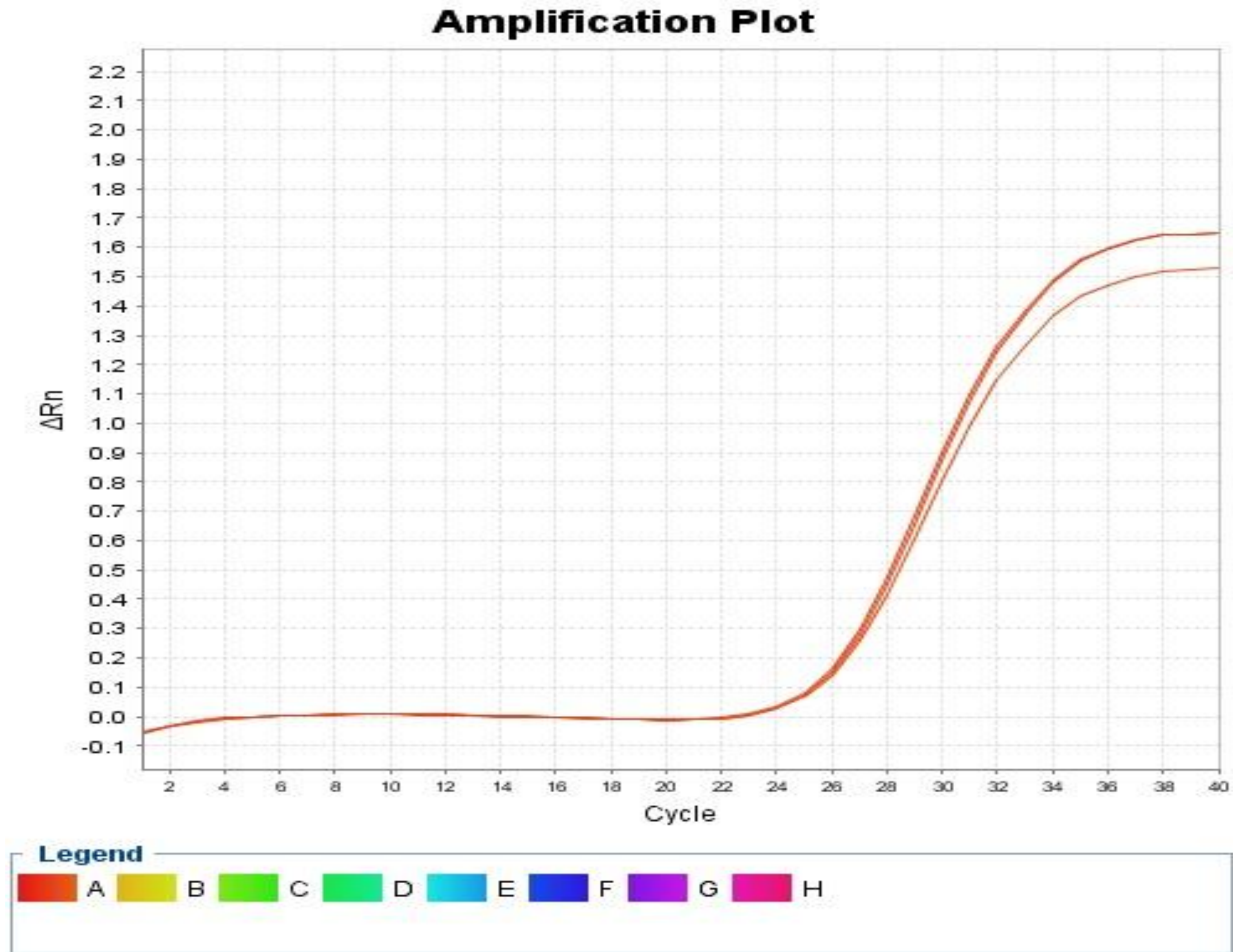

Figure 2: Q-PCR amplification plot of both the Actin (red) and HPL (orange) gene of Cucumber from the initial Q-PCR run.

Figure 3 shows efficiency curves for these samples. The efficiency of the standard curve prepared with the control cotyledon sample yielded slopes of -3.15 for the HPL gene and -3.14 for the Actin gene. These slopes indicated efficiencies of 113% for both genes.

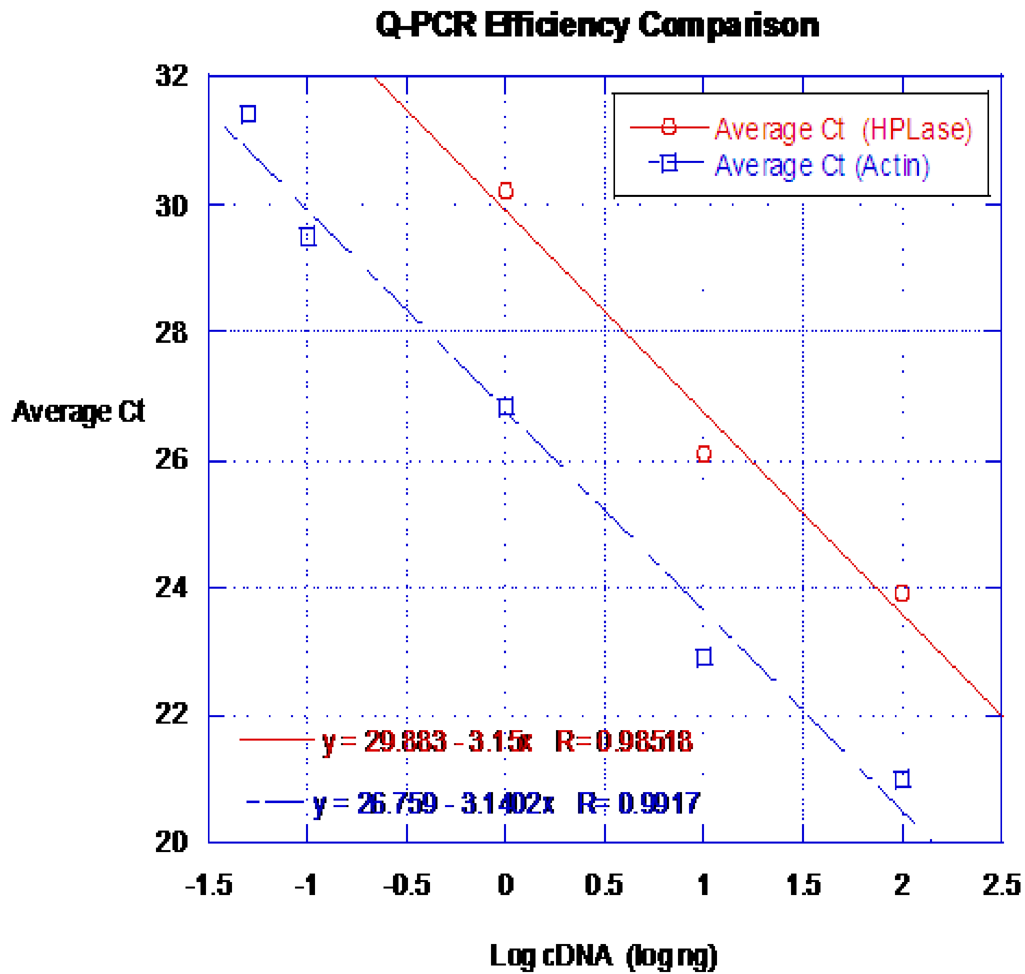

Figure 3: Q-PCR Efficiency curve for both the HPL and Actin genes of Cucumber from the initial Q-PCR run.

A melt curve was obtained for all samples after Q-PCR amplification. Figure 4 shows the melt curves obtained with these samples. The negative controls did not show any T_m peaks, indicating the absence of DNA. Most of the sample peaks showed one large T_m peak and a smaller peak before the large T_m peak. One of the samples exhibited a small shoulder peak appearing after the large T_m peak. Ideally, a single peak should be observed.

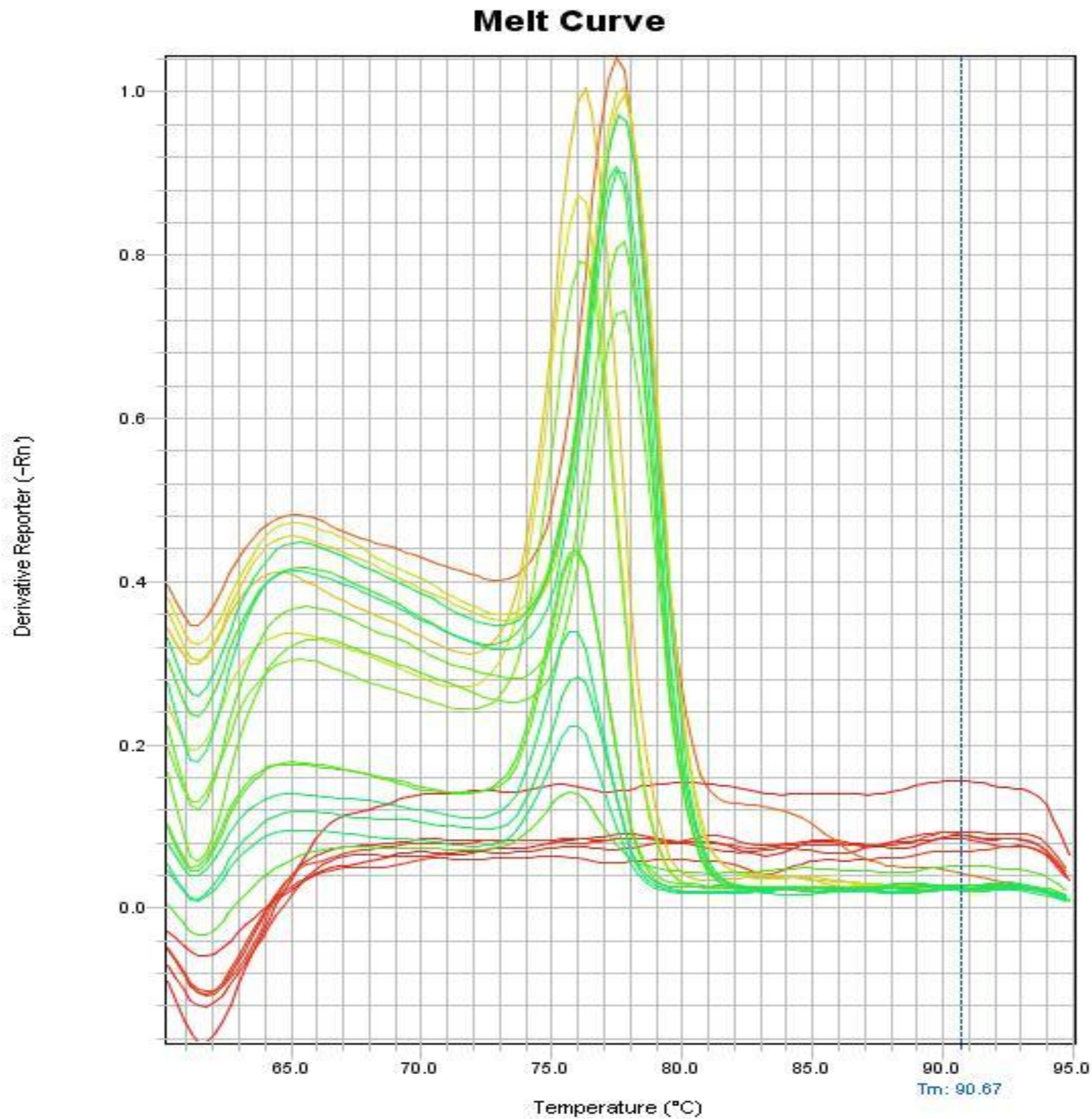

Figure 4: Melt curves of the various samples including the Actin and HPL genes from cucumber, and the negative controls from the first Q-PCR run.

Second Q-PCR run

The control cotyledon was used as the reference sample for comparison of HPL levels of the other various samples. Compared to the control cotyledon, the level of HPL mRNA increased in the injured cotyledon. The level of HPL in the first real leaf of injured plant increased in

comparison to that in the first real leaf of the uninjured plant; however, its HPL levels were lower than that of the cotyledons of both plants. Table 7 compares the RQ or relative quantities of cDNA in these samples while Figure 5 presents the comparison as a graph.

Table 7: Comparison of HPL mRNA levels in various tissue samples from cucumber leaves via use of RQ values

Sample	RQ	Error
Control Cotyledon	1	0.033
Control First Real Leaf	0.524	0.031
Experimental Cotyledon (injured)	1.271	0.093
Experimental First Real Leaf (uninjured)	0.856	0.056

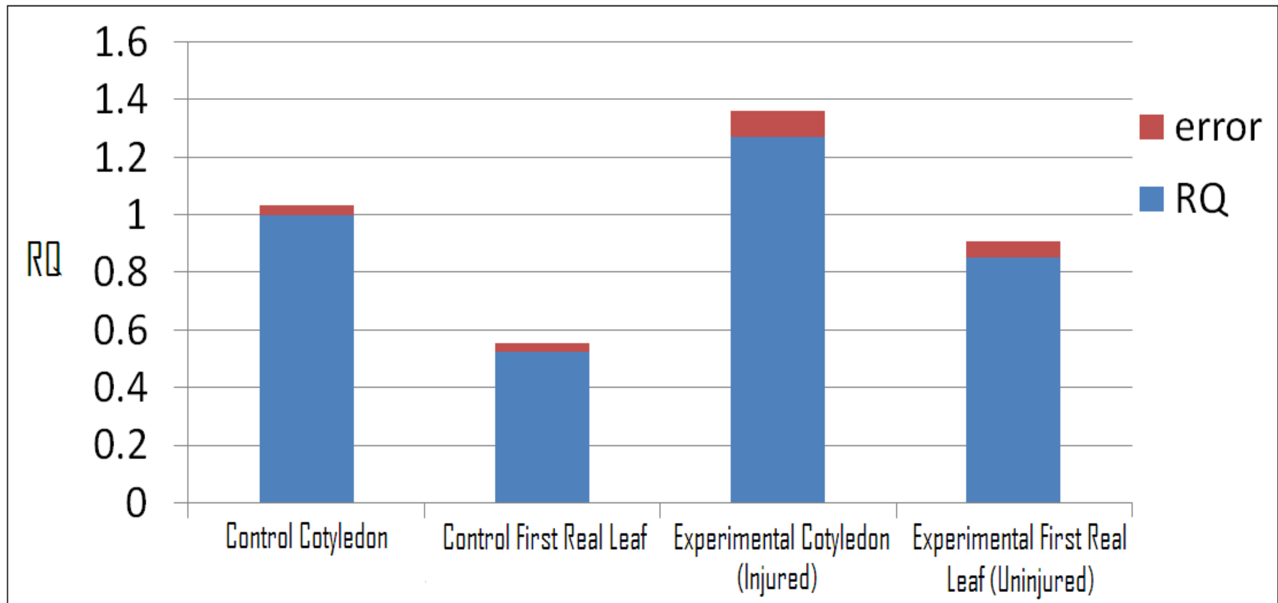

Figure 5: Comparison of HPL gene RQ values of various control and experimental samples from the second Q-PCR run.

Discussion

Initial Q-PCR run

The results of the first Q-PCR run show that amplification of the HPL and Actin genes took place (Figure 2), indicating the presence of the HPL gene in the samples. The cDNA in each sample is doubled through each cycle. The C_T value is the number of cycles it takes for the amplified DNA to reach a threshold value. The more initial cDNA present in the sample, the lower the C_T value will be. The HPL gene was compared to that of Actin, a common protein expressed at constant levels in a plant to correct for differences in efficiency of RNA extraction between samples.

The log linear standard curve (Figure 3) indicates that this Q-PCR method can be used to quantify the cDNA obtained from different samples. Care must be taken to improve the efficiency of the slope, as the efficiencies were slightly higher than ideal. Efficiencies should be between 95 and 110 percent; however, our efficiencies for both genes were approximately 113 percent. This is not far off of the accepted range, but should be improved with more careful technique. For the HPL gene, the linearity of the standard curve was only over three points because at the lowest cDNA concentrations, the C_T was too high, leading to high error. The curves also showed a linear decrease of C_T with the increase of starting cDNA, which is expected.

The melt curves exhibited single large peaks for most the samples. The large peaks give the T_m values of each of the products, the temperature at which half the DNA molecules melt. Each product formed during amplification has a specific melting point. The presence of a single large peak indicates that the target gene was being selectively amplified with minimal

amplification of extraneous genes. Most of the samples have a small peak with a low Tm. Further analysis will be done to identify the cause of these peaks. One sample had a shoulder peak appearing after the main peak. The fact that there are no shoulder curves in most of the samples indicated that only the target DNA was being amplified. This indicated low contamination of most of the samples. However, more care must be taken because a shoulder was observed in one of the samples, indicating contamination or secondary amplification products.

The melt curves with a single predominant peak along with the apparent amplification and log linear increase of C_T with decreasing amounts of cDNA indicate that this Q-PCR method can be used to analyze HPL expression in cucumber leaves.

Second Q-PCR run

In the second Q-PCR run, the HPL levels of the various control and experimental samples from the first set of samples were compared (Figure 5). The control cotyledon was used as a reference. The 27% increase in HPL mRNA in the injured cotyledon in comparison to the control cotyledon agrees with our hypothesis that mechanical injuring triggers an increase in expression of the HPL gene in the injured leaf. The level of HPL mRNA in the first real leaf samples was lower than in the cotyledon samples suggesting tissue differences in HPL expression. However, there was a 63% increase in the HPL levels in the first real leaf of the experimental plant compared to that of the control plant. This suggests wounding may cause a systemic increase in HPL expression.

Mechanical wounding seems to increase HPL expression in the leaves of cucumber plants; however, more samples must be analyzed via Q-PCR to ensure validity. Since this Q-

PCR method can be used to compare HPL expression in different samples, the effect of methyl jasmonate and norbornadiene can also be assessed.

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Author's Biography

Samee Ranginwala joined Minnesota State University, Mankato in the Fall of 2006. He is pursuing a BS in Biochemistry and Human Biology. Samee began his research in the spring of 2009 under the guidance of Dr. James Rife and Dr. Terry Salerno the Department of Chemistry and Geology. His focus was mainly on the biochemical basis of plant defence mechanisms. Samee presented his research at the 11th Annual Undergraduate Research Conference at Minnesota State University. Upon graduating, Samee plans to pursue medicine.

Mentor's Biography

Jim Rife earned a B. S. in Chemistry from the University of Illinois at Champaign-Urbana in 1972. After serving in the Army, he began graduate studies at the University of Wisconsin at Madison in 1974 where he earned a Ph.D. in Biochemistry in 1978. He did post-doctoral research in the Department of Chemistry at the University of Wisconsin from 1978 until 1980. He taught Chemistry at Mundelein College in Chicago from 1980 until 1986. He joined the

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