Expression of 9/13 Hydroperoxide Lyase in Cucumber Leaves

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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 tested the effect of various factors on transcription of the 9/13-HPL gene. We specifically tested factors that have been shown to induce defense responses in other plant systems. Real Time Polymerase Chain Reaction was used 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. In subsequent experiments, the effects of mechanical wounding coupled with treatment with methyl jasmonate, ethylene or norborandiene (an ethylene antagonist), was tested. The RNeasy Plant Minikit from Qiagen was used to isolate mRNA from the plant tissue. SYBR Green was used as the detection system for Real Time PCR.

Background Information

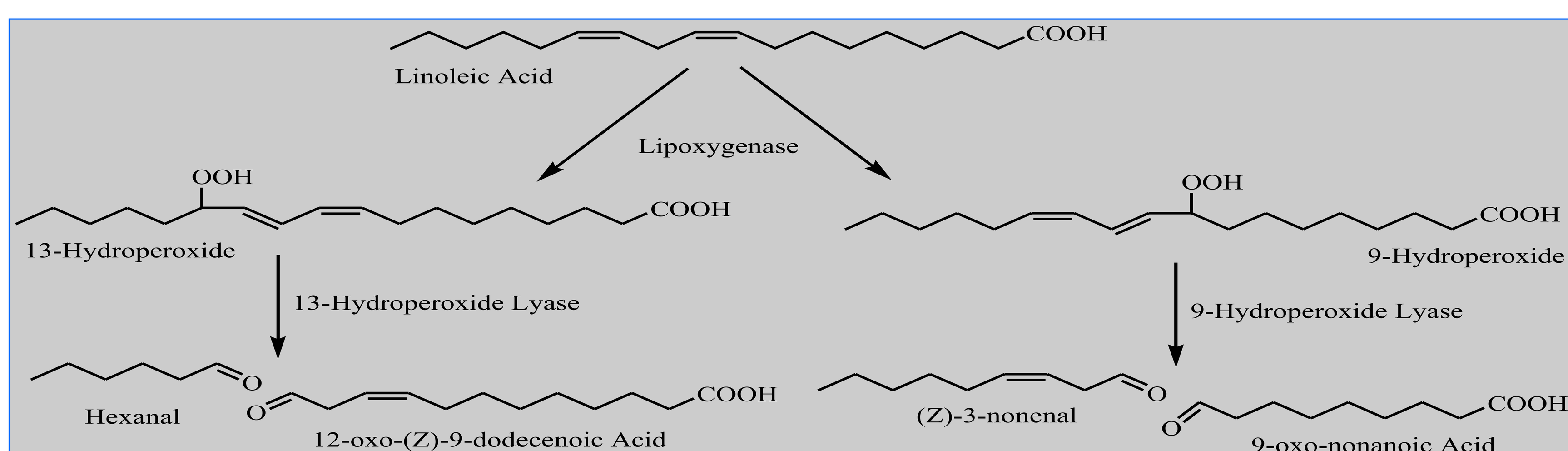
Many plants produce volatile aldehydes such as (Z)-3-hexenal and (E)-2-hexenal in a defense mechanism (Matsui, 2006; Tiget, 2001). The cucumber plant, for example, produces aldehydes in response to mechanical wounding (Matsui, 2006). The aldehydes have been shown to repel insects, lead to the production 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, that 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 among the animal kingdom, fatty acid 9/13-hydroperoxide lyase cannot be detected in every plant, but is found in cucumbers. 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). The figure below shows the HPL facilitated production of volatile aldehydes from fatty acids.

Ethylene and methyl jasmonate are gaseous or volatile regulatory molecules involved in defense responses in some plants, including the induction of plant defense genes (Xu, 1994). This raises the question of whether ethylene or methyl jasmonate affect the gene expression of HPL. The following diagram shows the oxidation of a fatty acid (Linolenic acid) by lipoxygenase into C-9 or C-13 hydroperoxides, which is subsequently cleaved by hydroperoxide lyase to form aldehydes and carboxylic acids.

This project is studying the expression of HPL using Q-PCR. The effect of wounding and phytohormones on the expression of HPL in cucumbers is being examined. This project is significant because it increases our understanding how plants produce natural compounds in defense against natural predators and pathogens. This study could lead to reduced reliance on manufactured pesticides.

Reactions Catalyzed by Lipoxygenases and Hydroperoxide Lyases



Description of Q-PCR

Q-PCR (quantitative polymerase chain reaction) is a process that makes copies of DNA molecules in repetitive cycles by doubling the number of DNA molecules through each cycle. The number of copies after any cycle depends on the initial number of copies and the number of cycles that have been completed. Q-PCR monitors the accumulation of DNA copies with each cycle. It is used to quantify the number of initial copies of cDNA, which is equal to number of mRNA. Therefore it can be used to compare gene expression by quantifying mRNAs in samples. We used the SYBR Green method of Q-PCR. This involves using a fluorescent dye (SYBR Green), which fluoresces when it binds to double stranded DNA molecules, As DNA levels increase, so does the fluorescence. Thus, this method allows for both amplification of DNA and the quantification of the initial mRNA being amplified.

Methodology

•Grow *Cucumis sativus (cucumber plant)* seedlings in growth chamber

•Treatment of Plants: Seedlings (~2 weeks in age) were treated as indicated in the table below.

Plants harvested six hours after treatment. The cotyledons and first real leaves were collected, immediately frozen in liquid nitrogen, and stored at -80°C.

•Total RNA from each sample was extracted using the RNeasy Plant Mini Kit from Qiagen.

•RNA concentrations were estimated by the absorbance at 260 nm., assuming an absorbance of 1=40ug/ml.

• RNA purity was estimated from the 260nm/280nm ratio.

•The total RNA was reverse transcribed to create cDNA copies of the mRNA from the sample by mixing 10ul of reverse transcription mastermix (High-Capacity Reverse Transcription Kit, Applied Biosystems) with 10ul of sample. Initially, the crude estimated concentrations of one of the samples were used to determine the volume needed for each of three aliquots needed to construct a standard curve. The amounts of RNA needed for the three aliquots were .02ug, .2ug, and 2ug.

•Samples containing 100, 10, 1, 0.1 and 0.05 ng total cDNA were then amplified by the polymerase chain reaction in a StepOne Plus Real Time PCR System from Applied Biosystems.

Figure 1: Procedural Design of the Project

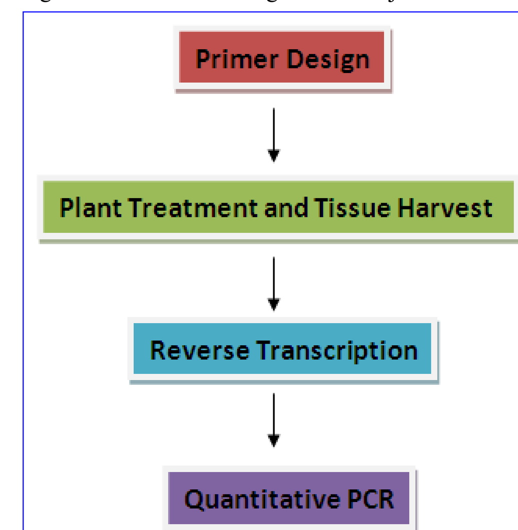


Table 1: Treatment scheme of the Cucumber seedlings

Sample	Injuring	External Treatment
Control Cotyledon	None	None
Control Primary Leaf	None	None
Exp. Sample 1 Cotyledons	Yes	None
Exp. Sample 1 Primary Leaf	No	None
Exp. Sample 2 Cotyledons	Yes	None
Exp. Sample 2 Primary Leaf	No	None
Exp. Sample 3 Cotyledons	Yes	250 ppm Methyl Jasmonate gas
Exp. Sample 3 Primary Leaf	No	250 Methyl Jasmonate gas
Exp. Sample 4 Cotyledons	Yes	1000 ppm Norbornadiene gas
Exp. Sample 4 Primary Leaf	No	1000 ppm Norbornadiene gas

Primer Alignment in the HPL cDNA Sequence

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   1 ccattctctt ccaacgtgaa gataagaaac cttttgttta ctttttgtac gatcacaggt
  61 cacagcaatg gcttcttcct cccctgaact tcctctcaaa cccattcccg gtggctatgg
 121 cttccccttc ctcggtccca tcaaagaccg ttacgattac ttctatttcc aaggtagaga
 181 cgaattcttc cgttcccgaa ttaccaaata caactccacc gtcttccacg ccaacatgcc
 241 accgggcccc ttcatctcct ccgattccag agtcgttgtc ctcctcgatg ccctcagttt
                                  caaagtcga gaaacgcaac at
 301 tcccatcctc ttcgacacca ccaaagtcga gaaacgcaac attctcgacg gaacttacat
          aggaac aggaagtaac cg
 361 gccctccttg tccttcaccg gcggcattcg cacctgtgct tatttggacc catcggaaac
 421 agagcacact gttctcaaac gcctctttct ctcctttctc gcttctcacc atgacaggtt
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1021 agggggactg actttctccg ccttggagaa aatgagtctg ctgaagtcgg tcgtgtacga
1081 agctctgagg atcgaaccgc cggtgccgtt ccagtacggg aaagcgaagg aggatatcgt
1141 gattcagagc cacgattctt gtttcaagat caaaaaaggg gagacgattt ttggttatca
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1261 gttcgtggga gaagaagggg agaagctttt gaagtatgtt tactggtcaa atgagcggga
1321 gacggtggag ccgacggcgg agaacaagca gtgtccgggg aagaatctgg tggtgatgat
1381 gggcaggatt attgtggtgg aattcttcct ccgttatgat acgttcactg tggacgtcgc
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1561 tgattggttg atgcagggaa gctgtggtgg acttgagcac acatgacaca tcattgattt
1621 gttggggtta taaagtaatt tcctcgtgat ccgcgtcgtt tttaatttga gatctcattg
1681 tgtgttgtaa cccgcgggtg atcttatttt tatagtttct tttttctcaa ctatgctcca
1741 atttttaaat taaataaata ccaaatatca acttcataga c
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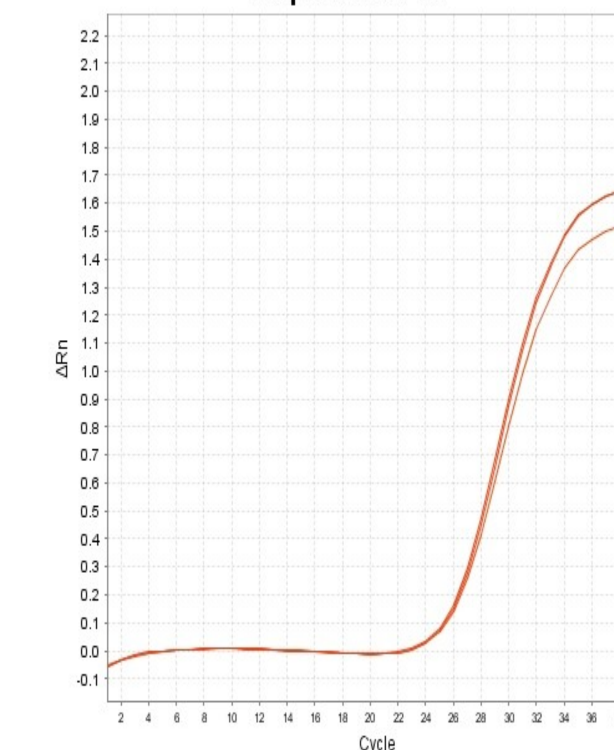
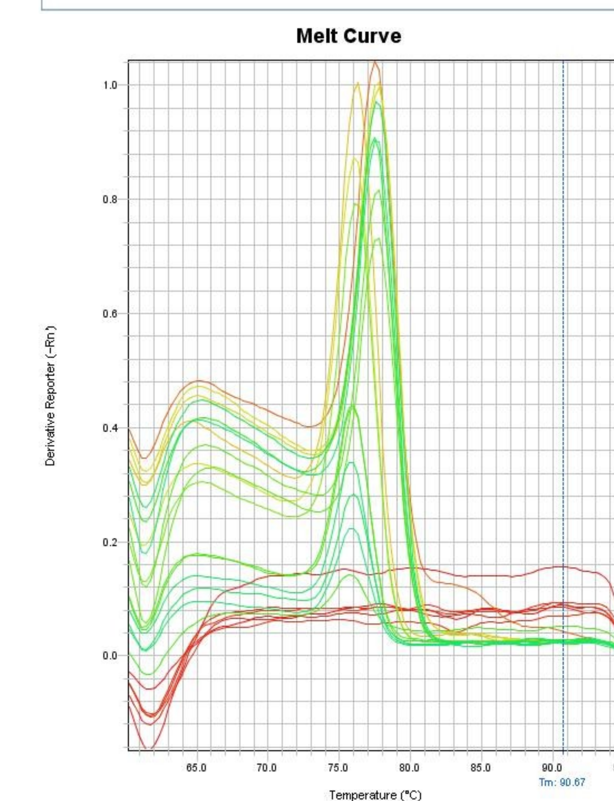
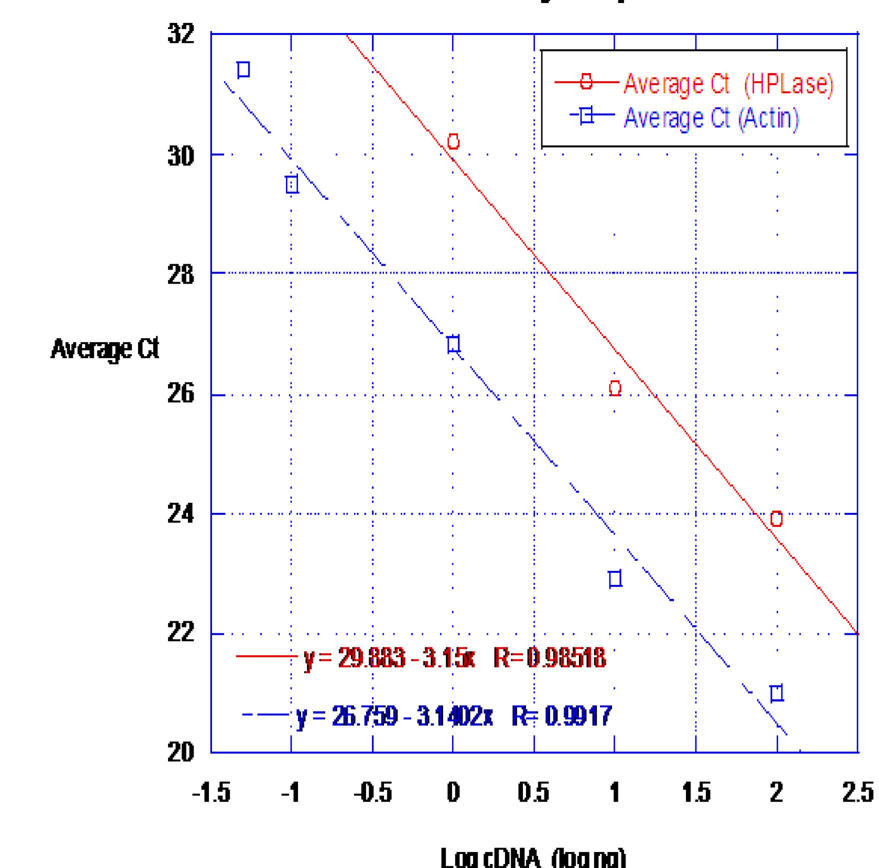
Results







Analysis of Results

The Q-PCR Efficiency Graphs - Ct versus the log cDNA.

Ct = number of cycles for the amplification curve to reach a defined threshold.

Ct increases as the initial copies of cDNA decreases.

Results indicate both the housekeeping gene, Actin, and the HPLgene are successfully being amplified.

Both genes show a log-linear decrease in Ct with increasing initial amounts of cDNA. HPL is only linear over a 3 log range because has significantly fewer initial cDNA copies.

Ideally the lines should have a slope = -3.3. The slopes of -3.15 for HPL and –3.14 for Actin indicate amplification efficiencies of 113% for both genes

It is important that a single target be amplified in Q-PCR If SYBR is used. The melt curves should show a single peak. Shoulders in some HPL and Actin, indicate a contaminating product. Since these extraneous peaks are not in all samples, one of the samples must have a contaminating DNA.

Conclusions

The fact there was amplification for both HPL and Actin indicates that the primers were successfully designed. The linear efficiency curves indicate this system can be used to measures changes in the expression of the HPL gene. Tissue samples collected from plants that have been wounded and treated with methyl jasmonate and norbornadiene can now be analyzed. Care must be taken to avoid contamination with extraneous DNA. Melt curve must be closely monitored to ensure validity of the experiments.

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