The analysis of *Pseudoperonospora humuli and Podosphaera macularis* on *Humulus lupulus* and *Humulus japonicas* and analysis of disease resistant analogues

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INTRODUCTION

Humulus Lupulus, the common hop, belongs in the Cannabaceae family and is used most commonly for the commercial production of beer. Although the genus Humulus has three species: *H. yunnanensis, H. japonicus, and H. lupulus, H. lupulus* is the only species to be grown for its cones (Small, 1978). In the United States, the Pacific Northwest produces 30% of the world supply of hops (Gent 2006). According to the USDA National Agricultural Statistics Service 2015 National Hop Report, 43,633 acres of hops were harvested in that year and the value of production of the United States hop crop was $345.4 million. The 2015 value of production was a 33 percent increase from the 2014 value of $260.6 million, also the total acres harvested increased 15 percent from 2014 to 2015 and 24 percent from 2013 to 2015. Hops have a major role in the brewing of many different styles of beer and also have health benefits such as anti-carcinogenic effects. Female *Humulus* plants produce a hormone known as Lupulin which is a visible yellow liquid inside of the cone. This Lupulin contains three different α and three different β acids that serve as the bittering agent in beer. Hops plants grow profusely, and may grow up to 25cm in one day and may grow up to 7 meters in one growing season (Hawk 2015).

The biggest dangers to commercial hop production are downy mildew (*Pseudoperonospora humilis*) and powdery mildew (*Podosphaera macularis*). While downy and powdery mildew do not kill the plant, they prevent it from producing cones. Powdery mildew is spread through infected rhizomes, windblown spores, and cross hop-yard contamination. Downy mildew is spread through windblown spores, diseased root stock, and cross hop-yard contamination. In 1999 and 2000, powdery mildew is estimated to have cost hop growers over $30 million, or 15% of the total crop revenue (Smith 2005) Fungicides are ineffective against downy and powdery mildew as phenotypic expression is not established until after the infection is present (Hawk 2015). Powdery Mildew appears as clusters of white clumps on hops plants, while Downy mildew appears on hop plants as a combination of light brown or yellow spots (Thomas 2015). Powdery mildew infection can take up to 3-7 days. During the process, spores attach to leaves and spread to burrs and cones, draining the plant of its nutrients and eventually leading to the decomposition of the plant (Bouldin 2015).

One of the most promising ways to prevent the spread of these pathogens in the future is the identification and the manipulation of the possible plant disease resistance genes (R genes) within the hops' genome. R genes encode cell receptors that detect the presence of a specific pathogen and respond by activating a signal transduction pathway. R genes are divided into different classes based on their product structure. The majority of R genes code for the NBS-LRR protein class, with a characteristic nucleotide-binding site domain (NBS) and a leucine-rich domain. The NBS-LRR R genes can be further divided into two classes based on the N-Terminal end of the protein; TIR-NBS-LRR R genes share a homology with the interleukin-1 receptor of mammals (TIR) and the class of non-TIR-NBS-LRR R genes have a coiled coil structure instead of a TIR structure [5]. The hops' genome has been described as having eight genetic sources of gene-for-gene resistance. These R genes were designated as R1-R7 and Rb gene (blistering gene). Neither the function nor the enzymes encoded for these R-genes have been determined (Hemming 2011).

Of the many possible methods to identify the possible R genes within the hop genome, one of the most promising is the use of resistance gene analogues (RGAs) as possible candidates for R genes. Due to the conserved domains and motifs that play specific roles in pathogens' resistance, sequence analysis of cloned R genes from different plant species provides the possibility of designing degenerate primers for PCR amplification in species for which there is no previous knowledge of disease resistance gene-like sequences (Sekhwal 2015). The goal of this experiment is to isolate RGAs from a variety of hop lines using a PCR based strategy and to characterize the resistance-gene-like sequences belonging to the NBS-LRR class. Thirteen total degenerative primer pairs were used in this experiment, all of which have been in identified in one hop culture that showed resistance to powdery mildew. Eight of these primer pairs RGAs products have been sequenced and have the following accession number in the NCBI Nucleotide Database, EF464235, EF464283, EF464240, EF464295, EF464222, EF464228, EF464288, and EF464230. The remainder of the degenerative primers that have

not been sequenced are, RGA1, RGA13, RGA15, RGA17, and *Arabidopsis thaliana* Homolog (Kojzak 2008).

Although much research has been done on hops plants in the Pacific Northwest, there is not a lot of research specifically pertaining to the climate of Virginia. Since climate and soil conditions affect the flavor and aroma of hops, we performed multiple experiments to determine the prime growing conditions for *Humulus lupulus* plants in Virginia. Additionally, due to the time limits of the research, it was necessary to understand how to grow hops in the most expedient way. Knowing that hops plants grow well in soil with maximum drainage, the water holding capacity tests allowed us to tell what type of soil the plants would theoretically grow best in. Additionally, knowing that hops grows best in a pH of 5.0-5.8, a pH test would also give us an idea as to what soil hops would grow best in.

Similarly, there have been multiple studies published that record the properties of downy and powdery mildew in hop yards, but little research is published regarding how to isolate the mildew to study it in a controlled environment. Since downy and powdery mildew spread very rapidly, it is important to be able to grow and observe them in a contained environment. We attempted to grow downy and powdery mildew on agar plates as well as on plants in the greenhouse.

We also extracted DNA from hops leaves in order to perform PCR on them. We included the wild type “Jenkins” and “High Bridge” varieties in our tests because no research had previously been performed on them. By splicing the DNA strands, we were able to run electrophoresis gels and determine if a specific primer caused differentiation. Should there be differentiation, we would have a primer of interest.

We also attempted to isolate Downy and Powdery Mildew, both on agar plates and on plants. By isolating the mildew on plants we would be able to infect multiple plants until we encountered a resistant plant. We would then be able to perform PCR and other tests on the plant to try to isolate the resistance gene.

We also attempted to determine the best way to root stem clippings taken from clean mother plants, and determine the fungal profile of the hop plant.

The goal of this research was multifaceted. We attempted to determine whether or not these RGAs are present in a wide variety of hop lines in order to determine if these degenerative primers can be used to identify an actual R gene in the hop genome. Additionally, we attempted to find the most feasible method to commercially grow hops in Virginia using propagation and soil studies. We attempted to identify resistance genes to downy and powdery mildew. We looked to establish a method to grow and control downy and powdery mildew in an isolated environment. We worked to isolate root stem clippings from the mother plant. Lastly, we worked to determine the fungal profile of the hop plant.

Principal Approaches

Propagation

To create a repository, we obtained seeds from the National Clonal Germplasm Repository in Corvallis, Oregon. We also obtained plants from various private gardens and breweries in the Farmville, Va area, as well as wild hops from the High Bridge Trail. Multiple propagation methods were tested to determine the most effective method. Fifty two stem clippings, between four and eight inches long, were taken from the plant and included a node and at least one pair of leaves. The clipped ends of the clippings were placed in water, and the plants were set under alternating natural and lamp light for one month. Twenty eight root cuttings, four inches in length and no less than 1mm in diameter, were taken using a razor-knife from a well-developed Mount Hood plant. After the cuttings were taken, they were placed in a seed tray and planted with topsoil. After cutting, the roots were watered and left in the greenhouse. A seed tray was prepared and filled with potting soil. A number of large, healthy Cascade leaves were picked from the plant. A razor knife was used to cut off the leaf at the base of the leaf stalk. The tips of the leaves were also cut off. The midsection of the leaf was then placed into the soil at about one-third its depth, with the latter two-thirds exposed to the open air. Approximately forty cuts were taken. After planting, the cuts were left in a greenhouse and regularly watered. To propagate seeds on agar plates, we created plain agar plates using a solution of Peptone (10 grams/Liter), Dextrose (40 grams/Liter), and Difco Granulated Agar (15 grams/Liter) in 1000ml of distilled water. After agar plates were poured we took seeds from hops from the High Bridge Trail and sorted them on the plates between dark colored seeds and light colored seeds. We placed thirty six seeds on a plate, and had four plates of light seeds and four of dark seeds. The seeds were placed in a growth chamber at 20°C. To grow plants clonally with cell culture medium, we created a medium with MS salts, MS medium, EPure, sucrose, Davis agar, and pyruvic acid that was autoclaved and poured into plates. We cut 11 3cm clippings, 5 from Zeus and 6 from Chinook. Each clipping was then cut in half. To sterilize the plants we washed them in a bowl with a spray bottle of 95% ethanol solution. The leaves set at room temperature for 3 minutes. We then transferred the leaves to a

bowl of Clorox bleach where they set at room temperature for 3 minutes. The leaves were then moved to another bowl where, using a spray bottle, they were sprayed with water three times over. We then removed the clippings from the bowl and placed each clipping into a capped tube. The tubes were filled with the media. The tubes were placed in a growth chamber at 20°C for three weeks before observation.

Callus cell growth with tip whitening

Plates were made using the Sabouraud Method, using five grams peptone, twenty grams glucose, seven and a half grams of granulated agar, and five hundred milliliters of EPure water. The solution was autoclaved and poured into plates. Eight Wilamette and High Bridge leaves were laid on the agar, and set in incubators at 18°C, 20°C, and 25°C, and in the greenhouse which stayed at above 30°C. The plants incubated for 48 hours to isolate growths of interest. We isolated mildew-like growths of interest by using a sterile toothpick and streaking growth spores on a new agar plate. We made a broth using the Sabouraud method using 5 grams peptone, 20 grams glucose, and 500ML EPure water. After autoclaving, the broth was poured into capped tubes. After 48 hours, the isolated plates were observed. Once again, a toothpick was used to remove growth from the plate. The toothpick was placed in the tube with the broth with the cap loosely left on to allow air flow. While we aimed to perform PCR on this broth to determine if our growths were downy or powdery mildew, we did not buy the kit that allowed us to do so. This experiment was re-ran with different agar recipes including the use of hydrochloric acid to adjust pH to a more suitable level and ampicillin to attempt to prevent as much bacterial growth on the plates.

Soil Quality Analysis

To determine water holding capacity, We obtained soil samples from the High Bridge Trail, Metro-Mix potting soil, topsoil used in the hops yards, and soil dug up from around the greenhouse at Hampden-Sydney College in Farmville, Virginia. 3 handfuls of each sample were placed in paper coffee-filters and onto a metal tray that was set inside an oven for 24 at 150°C for 24 hours. A weight was then taken of the samples with a top loading balance. We recorded this as “Dry Weight #1.” The samples were placed resting on top of a 600ml beaker and wet thoroughly with a squirt bottle. We then waited approximately twenty minutes for the samples to drain, or until the samples ceased to drip water. We then took another weight. We then recorded the weight as “Wet Weight #1.” We placed the samples in the oven again for forty eight hours before repeating the process. After acquiring the second set of weights, we calculated the water holding capacity by subtracting Wet Weight #1 from Dry Weight #1 and dividing the resulting number by Dry Weight #1. This was repeated for the second set of weights. The two water holding capacities were then averaged together to get the final average for the test. The final averages for the three tests were then averaged together to obtain a final average for the soil type

Using soil samples from the High Bridge Trail, Metro Mix potting soil, topsoil, and soil around the greenhouse, pH was tested using the LaMotte Garden Guide Soil Test Kit. Using the equipment provided, a small amount of soil was placed in each divet of the tray. Approximately two drops of the indicator was placed in each divet. After two minutes, the tray was tilted to that any excess indicator could drain out. The soil was then compared to the pH card and results were recorded. Three tests were taken for each soil type and the results were averaged.

Root analysis

To conduct the rooting experiments we used many different variables. We first tested a variety of stem clippings attempting to grow them in water inside the growth chamber as well as under the lights in the Biology prep room. Several of the plants rooted, however, we were looking to attempt to get better rooting results so we moved on to trying to root clippings in water with a root hormone added. We used Garden Safe Take Root Rooting Hormone. We followed the directions for the rooting hormone and applied it once as the initial test. We then moved on to testing rooting in soil mediums per the suggestion of Dr. Laban Rutto. We started by testing three different soil types using three different mother plants stem clippings. In this test we used Zeus, Chinook, and Willamette clippings. We then placed two clippings of each type into a soil medium. The soil mediums tested were perlite, regular potting soil, and then potting sand. These clippings were allowed to grow for a week before being removed from the soil and measured for roots and root quality. There was a

noticeably higher amount of roots growing off the stems in the sand and the potting soil than just the perlite on its own. We then eliminated the perlite from further tests. While running this rooting experiment I was also running another rooting experiment in water. We had taken several of our unknown hop varieties labeled as RG(X) (X is the variable of which row the plants were taken from) and placed them randomly into groups testing multiple variables. In this rooting test we tested the environment of different settings and the presence or absence of rooting hormone. The different environments we tested were a dark environment covering the stems immersed in water. This was done to mimic the stems being placed under ground. We also tested the difference between a humid environment and the greenhouse. To create the humid environment we filled a tray with water placed the 4 possible treatment groups into the tray and covered the groups with a medium sized fish tank. This was placed inside the greenhouse. This caused the water from the tray to evaporate and create the increased humidity in the new environment. These plants were taken out of the environment and measured for root growth, health, leaf size, and length of stem.

After these experiments were concluded I waited for our mother plants to grow new stems. We ran into the problem of deer eating some of our plants in the plants that were planted outdoors. This allowed us to take clippings from entire sections of vine rather than just taking the freshly grown shoots. We used these new sections of clippings to start new rooting experiments in both sand and potting soil. The tops of the shoot and clippings that contained two nodes and one node. The clippings all contained leaves. The clippings at the top of the shoot had small still maturing leaves. The one and two node clippings all contained two or more. mature leaves. These were planted into sand or potting soil. Observations were noted ten days later. All of the plants that rooted were taken into the greenhouse for further growth observations.

Isolating Mildew

Plain agar plates were made and brought to a pH of 5.6 with 1-2 drops of Hydrochloric Acid before they were poured. Antibiotic agar plates were also made by making 1/4 liter of the previous solution and adding Sigma-Aldrich Streptomycin (100mg/Liter) when the solution cools to the point that it could be comfortably handled.

Downy mildew growth

Different antibiotic plates were made were also made by using the previous method, but making a 1/2 liter solution and adding Sigma-Aldrich Ampicillin (100mg/Liter). Next, samples were chosen from leaves of hop plants that were suspected of Downy Mildew and Powdery Mildew infection. The samples were placed in separate bags and left to set for 2 days at room temperature. Of these samples, a leaf clearly infected by powdery mildew and a leaf clearly infected by downy mildew were placed on opposite sides of an agar plate without streptomycin. The agar plates were placed in an incubator at 30° Celsius for 2 days. Using a sterile swab, the fungal colonies from the leaves were swabbed. A new agar plate was swabbed with the fungal colonies, and the swab was placed on a CHROMagar plate. The plates were placed in an incubator at 30° for 2 days. This process was performed for both downy and powdery leaves. The plates were observed. Plates showed blue colonies of *Enteroccocus* and pink colonies of E. coli. Agar plates were swabbed using the same fashion as before. The swab was swiped across a streptomycin plate and the swab was left on the plate. The agar plates were sealed in plastic wrap and left at room temperature for 2 days and observed.

Blue Enteroccocus growth

Analyzing DNA

DNA extraction was performed on a total of six lines of hops, Chinook Zeus, Cascade, Willamette, a wild type from the High Bridge trail, and an unknown line (nicknamed Jenkins), by following the modified CTAB method as described in Plant Molecular Biology Reporter [8]. 1.0g of leaf tissue was collected from each line, liquid nitrogen was poured on the leaf tissue and then the tissue was ground into a powder with a mortar and pestle. The powdered leaf tissue was then placed into Eppendorf tubes, with each tube containing approximately a spatula tip of tissue. 750ul of 2X CTAB buffer (50ml of 1.0 tris-HCL, pH 8, 140ml of 5 M NaCl, 50 ml of 0.25 M EDTA pH 8, 10g of cetytrimethylammonium bromide (CTAB)), distilled water to 500ml) and 3.0ul of 2-mercaptoethanol was added to each tube. The tubes were then vortexed and incubated in a water bath at 55-60°C for 4 hours while mixing by inverting every 15 minutes. 700ul of SEVAG (chloroform: isoamyl alcohol 24:1) was added to each tube and then they were centrifuged at 9240g for 15 minutes. At this time the aqueous phase was transferred to a new Eppendorf tube with 0.33 the volume of ice-cold isopropanol. The tubes were then stored at 80°C for one hour. This process was repeated an additional time. The tubes were then spun at 12,000g for ten minutes at room temperature, the supernatant was discarded without disturbing the DNA pellet, and the pellets were air dried overnight. The following day the pellet was suspended in 200ul of EB buffer, vortexed, and incubated for 30 minutes at 37°C. 20ul of 2.5M NaOAc and 500ul ice-cold 95% ethanol was added to each tube, and stored at -20°C for one hour. The tubes were then centrifuged at 12,000g for 5 minutes and the supernatant was discarded. The pellet was washed with 1ml of 70% of ethanol, spun at 9240g for four minutes, and the ethanol was poured off. The pellet was allowed to air dry overnight, and the following day was suspended in 100ul of EB buffer, and stored at -20°C.

Each PCR reaction was run at a total volume of 20ul, using a BIO RAD T100tm Thermal Cycler. Each mixture consisted of 10ul of Mean Green Master Mix 2x, 3ul of sterilized water, 1ul of DNA template (diluted 1:10), 3ul of Upper Primer (diluted 1:100) and 3ul of lower primer (diluted 1:100). All six samples were tested with thirteen total primer pairs. For analysis of resistance genes, a PCR cocktail was made by mixing 3 µl Mean Green Mastermix, 3 µl each primer from the primer pairs, 2 µl sterile/deionized water, and 2 µl template DNA into a .5ml PCR tube. The PCR tubes were placed into a thermal cycler. For our work with downy and powdery mildew, PCR was run based on the following steps: 95°C for 3 minutes, 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, repeat steps 2 through 4 thirty four times.,72°C for 5 minutes, 12°C for infinite hold. For our work with resistant analogues, PCR was run on the following steps : a 1minute hot start at 95°C, followed by 40 cycles consisting of denaturation (1 minute at 95°C), annealing (1 minute at 55°C), and extension (1minutes and 30 seconds at 72°C) and a final extension at 72°C for 10 minutes. After PCR is complete, 1µl of EZ-Vision was added to each of the tubes.

After extraction, a DNA reader was used to examine the purity of the extracted DNA. First, the program was turned on and the blank tray was extracted. The tray was wiped with a Kimwipe. A 1µl drop of sterile water was placed on every cell. The tray was placed in the reader, and the cells were selected that were to be read. After the program ran the trays with the water, the tray was removed and cleaned with a Kimwipe. A 1µl drop of DNA was placed on as many cells as necessary. The tray was placed in the reader and the cells with DNA were selected to be read. The ng/µl and 260/280 numbers for each cell were recorded.

Approximately thirty minutes before the PCR process is complete, a 1% agarose gel was made by weighing 1 gram agarose on a top loading balance. The agarose was poured into a 250 ml Erlenmeyer flask and adding 100ml TAE buffer. The solution was microwaved to a rolling boil and then poured into a prepared tank. 3µl of dye and 3µl of EasyVis were added to each of the PCR products. 10µl of ladder was inserted into the first well. For both our work with analogs and mildew, a 100bp ladder was used. was drawn from each PCR tube and placed in the individual wells. The tank is connected to the power supply and turned on to 103 Volts for 30 minute. The gel was then removed from the tank and then placed into a UV trans-illuminator for observation.

Root analysis

We grew cascade plants using the stem clipping propagation method. Multiple rooting hormones were tested. We placed two clippings of Zeus, Chinook, and Wilamette clippings in a soil medium. The soil mediums tested were perilite, potting soil, and potting sand. These clippings were allowed to grow for a week before being removed from the soil and measured for roots and root quality. Other rooting experiments were run in water by taking an unknown hop variety and tested the presence or absence of rooting hormone. We also tested the effect of light on rooting hormone by covering the clipping containers with aluminum foil entirely, halfway, and not at all. We also tested the effects of humidity on rooting hormone. To create the humid environment we filled a tray with water and placed the treatment groups into the tray.

Propagation

	Number Trials	Number Propagated
Stem Clippings	52	25
Root Cuttings	28	3
Leaf Cuttings	40	0
Seed Plates	288	0
Cell Culture	11	0

Propagation results

Soil Analysis

Water Holding Capacity	*Average*
Behind Greenhouse	0.5407748636
High Bridge Average	0.5820854426
Organic Potting Soil	1.832520753
Ed's Organic Potting Soil	0.9888789803

Water holding capacity results. "Behind Greenhouse" trial is red clay

SOIL pH	PH 1	PH 2	PH 3	Average
High Bridge	8	7	7	7.333333333
Top Soil	6.5	7	6.4	6.633333333
Potting Soil	6	6.8	7	6.6
Green House	6	7	6.4	6.466666667

Soil pH results

DNA Extraction

DNA Sample	Ng/ul	260/280
Chinook Sample-1	301.625	2.012
Chinook Sample-2	27.952	2.048
Zeus S-1	43.196	1.921
Jenkins 18 S-1	265.009	2.056
Jenkins 16 S-1	45.688	2.013
Jenkins 15 S-1	107.562	1.989
Jenkins 13 S-1	153.813	2.015
Jenkins 12 S-1	46.387	2.072
Jenkins 11 S-1	163.916	2.013
Jenkins 10 S-1	83.343	1.964
Jenkins 9 S-1	82.502	2.072
Jenkins 8 S-1	366.333	2.071
Jenkins 7 S-1	252.343	2.05
Jenkins 6 S-1	438.362	2.06
Jenkins 5 S-1	79.766	2.067
Jenkins 4 S-1	83.957	2.089
Jenkins 3 S-1	49.156	2.081
Jenkins 2 S-1	168.288	2.107
Jenkins 1 S-1	226.209	2.094
High Bridge S-1	4.517	1.783
High Bridge S-2	29.697	2.087
Willamette S-1	183.424	2.106
Cascade S-1	283.504	2.035
Mount Hood S-1	294.041	2.071
RG S-1	0.341	7.333
RG S-2	98.429	2.13

DNA purity results. 2.0 is generally accepted as pure.

Target Sequence	Primers Pairs	Nucleotide primer sequences	PCR product length (bp)
EF464235	WT2-B1 2/1e WT2-B1 2/1e	5'-AGACGACTCTAGCACATGCCC-'3 5'-ACTCAGCATAAAGCTTTTCCC-'3	132
EF464283	WT2-D9 WT2-D9 WTb	5'-TCGTCATCAATTTGATCATGGGTGC-'3 5'-AGCAACTTTTTATGACTTAGCATTC-'3	165
EF464240	WT1-D7 2/1spl WT1-D7 2/1spl	5'-TTACCTGAAGTTGATTTAAGACGTTG-'3 5'-ATGATGACAGAGTGAAGGTCTTTG-'3	150
EF464295	WT2-B1 WTc HL-WT2-B1	5'-ACGACTCTAGCACATGTCTTATTTAC-'3 5'-TGTCTTCCTTCAATAACTCAGCA-'3	138
EF464222	HL-RGA2 RGA2-2/1f	5'-AGACGACCCTTGCTTATGTCATGT-'3 5'-TATGGAACCCAAATCTTCAAAGTC-'3	172
EF464228	RGA8 2/1d RGA8 2/1d	5'-GATCTTTGAGGGGGTCACTTC-'3 5'-TTCCATGTGCTCCAGACTCG-'3	179
EF464288	RGA8 WTa RGA8 WTa	5'-AGAGATCTTTGAGGGGGTCAC-'3 5'-AAGACTCGTTCCAAACATCATCAA-'3	131
EF464230	RGA8 2/1g HL-RGA8	5'-CGACAAATGTGATACTGATAACTTG-'3 5'-AATTTTACTTCCATGTGCTCCAG-'3	164
RGA1	RGA 1 RGA 1	5'-GCAAGTTTATGAGGATCCGAAAGTG-'3 5'-TGGTGGACACTTCTGGTGCAACTGG-'3 -	155
RGA13	WT3B-A7 WT3B-A7	5'-TTACTTAGAGATAGTCGTGTTTGG-'3 5'-TTGAGCATAATTCACCAACTC-'3	234
RGA15	WT1-B8 WT1-B8	5'-GGAGAATATGGGTTTCCGTATCTC-'3 5'-GCCGGACGTCATACGGGACGATCG-'3	171
RGA17	WT2-A12 WT2-A12	5'-AATGATGACAGAGTGAAGGTG-'3 5'-TTACCTGAAGTTGATTTAAGACG-'3	152
***Arabidopsis* thaliana Homolog**	WT3B-B12 WT3B-B12	5'-GTCTTCGTCTGCTCTCATGCAGAC-'3 5'-TGGGGTATTGTGCCCCCGATG-'3	

List of primer pairs used

Resistance PCR/Gels

PCR Results	Rough Size in BP	Characteristics Gel 1	Rough Size in BP 2	Characteristics Gel 2	Rough Size in BP 3	Characteristics Gel 3
Willamette 1	150	Single	100	Single	100	Single
Willamette 2	200	Single	100	Single	250	Single
Willamette 3	250	Single	Nothing	Nothing	150	Single
Willamette 4	300	Double	Nothing	Nothing	100	Single
Willamette 5	Nothing	Nothing	100	Single	80	Single

Willamette 6	Nothing	Nothing	Nothing	Nothing	200	Single
Willamette 7	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing
Willamette 8	150	Single	100	Doublet	100	Single
Willamette 9	400	Single	100	Single	150	Single
Willamette 10	150	Double	100	Single	100	Single
Willamette 11	150	Single	Nothing	Nothing	50	Single
Willamette 12	250	Single	80	Single	80	Single
Willamette 13	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing
High Bridge 1	100	Single	110	Single	125	Single
High Bridge 2	200	Single	125	Single	60,150	Double
High Bridge 3	150	Single	110	Single	60	Single
High Bridge 4	Nothing	Nothing	100	Single	125	Single
High Bridge 5	60,600	Double	200	Single	50,150	Double
High Bridge 6	Nothing	Nothing	200	Single	150	Single
High Bridge 7	80	Single	100	Single	125	Single
High Bridge 8	80	Single	90	Single	200	Single
High Bridge 9	175	Single	200	Single	100	Single
High Bridge 10	125	Single	125	Single	150	Single
High Bridge 11	60	Single	60	Single	150,600,800	Triple
High Bridge 12	70	Single	70	Single	200	Single
High Bridge 13	Nothing	Nothing	Nothing	Nothing	Nothing	Nothing
Jenkins 1-1	80	Single				
Jenkins 1-2	200	Single				
Jenkins 1-3	125	Single				
Jenkins 1-4	175	Single				
Jenkins 1-5	60	Single				
Jenkins 1-6	200	Single				
Jenkins 1-7	100	Single				
Jenkins 1-8	200	Single				
Jenkins 1-9	200	Single				
Jenkins 1-	100	Single				

10						
Jenkins 1-11	100	Single				
Jenkins 1-12	175	Single				
Jenkins 1-13	Nothing	Nothing				
Jenkins 2-1	125	Single				
Jenkins 2-2	200	Single				
Jenkins 2-3	175	Single				
Jenkins 2-4	100	Single				
Jenkins 2-5	80,100	Double				
Jenkins 2-6	150	Single				
Jenkins 2-7	100	Single				
Jenkins 2-8	100	Single				
Jenkins 2-9	200	Single				
Jenkins 2-10	80	Single				
Jenkins 2-11	80, 900	Double				
Jenkins 2-12	100	Single				
Jenkins 2-13	Nothing	Nothing				
Cascade 1-1	Bad Ladder	Single	Bad Ladder	Single		
Cascade 1-2	Bad Ladder	Single	Bad Ladder	Double		
Cascade 1-3	Bad Ladder	Nothing	Bad Ladder	Double		
Cascade 1-4	Bad Ladder	Double	Bad Ladder	Double		
Cascade 1-5	Bad Ladder	Single	Bad Ladder	Double		
Cascade 1-6	Bad Ladder	Single	Bad Ladder	Single		
Cascade 1-7	Bad Ladder	Single	Bad Ladder	Double		
Cascade 1-8	Bad Ladder	Single	Bad Ladder	Single		
Cascade 1-9	Bad Ladder	Single	Bad Ladder	Double		
Cascade 1-10	Bad Ladder	Double	Bad Ladder	Double		
Cascade 1-11	Bad Ladder	Quadruple	Bad Ladder	Quadruple		
Cascade 1-12	Bad Ladder	Single	Bad Ladder	Double		
Cascade 1-13	Bad Ladder	Nothing	Bad Ladder	Nothing		
Zeus 1-1	Bad Ladder	Nothing	Bad Ladder	Single		
Zeus 1-2	Bad Ladder	Single	Bad Ladder	Double		

Zeus 1-3	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-4	Bad Ladder	Single	Bad Ladder	Double		
Zeus 1-5	Bad Ladder	Double	Bad Ladder	Double		
Zeus 1-6	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-7	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-8	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-9	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-10	Bad Ladder	Single	Bad Ladder	Double		
Zeus 1-11	Bad Ladder	Quadruple	Bad Ladder	Quadruple		
Zeus 1-12	Bad Ladder	Single	Bad Ladder	Single		
Zeus 1-13	Bad Ladder	Nothing	Bad Ladder	Nothing		

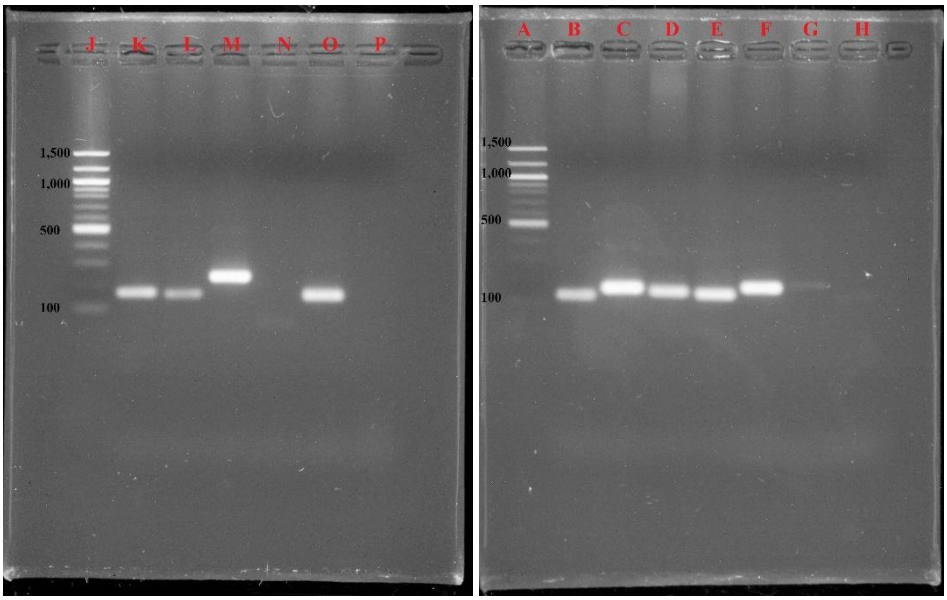
Electrophoresis results for finding resistance genes. "Bad Ladder" means results were unable to be obtained

First Run of Gel Electrophoresis for Analogues

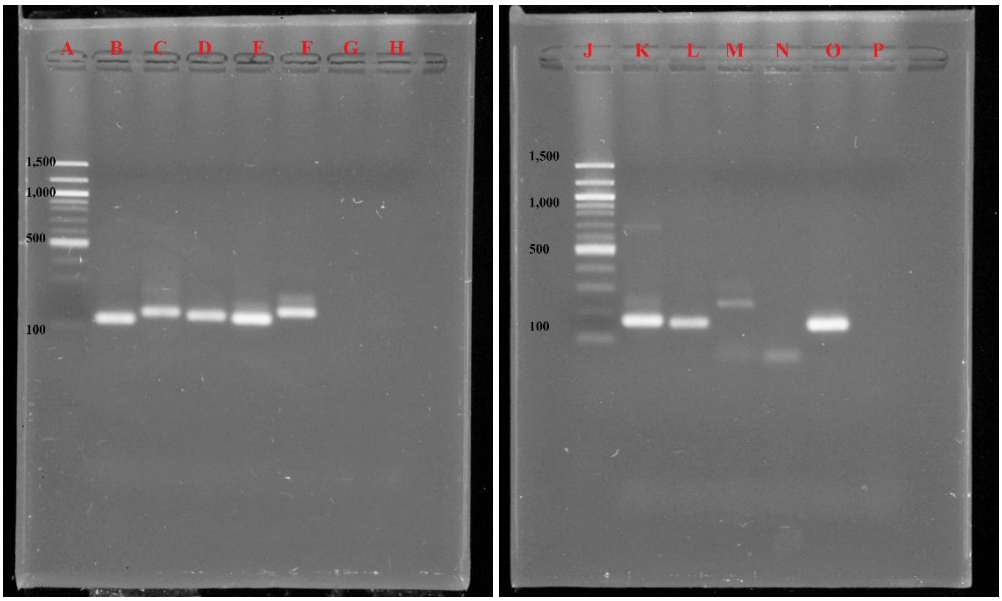
All six lines of hops' DNA samples were run individually on gels and annotated the exact same way. Table 2, displays the lane ID, target sequence, primers used in the particular PCR reaction, and the expected length of the product. Each gel is used to determine whether or not the RGA is present in the particular line of hop's genome, by determining if a band is present or not. Since degenerative primers were used some primers had results with more than one band; however the desired RGA bands show up in-between 100 and 300 base pairs. The main objectives of the first run of gel electrophoresis were to determine if the conditions of the PCR run were correct and determine how many RGA bands were clearly present in the hops genome.

Lane	Target Sequence	Forward Primer	Reverse Primer	Product Length (Base Pairs)
A	100 BP ladder			
B	EF464235	WT2-B1 2/1e	WT2-B1 2/1e	132
C	EF464283	WT2-D9	WT2-D9 WTb	165
D	EF464240	WT1-D7 2/1spl	WT1-D7 2/1spl	150
E	EF464295	WT2-B1 WTc	HL-WT2-B1	138
F	EF464222	HL-RGA2	RGA2-2/1f	172
G	EF464228	RGA8 2/1d	RGA8 2/1d	179
H	EF464288	RGA8 WTa	RGA8 WTa	131
J	100 BP ladder			
K	EF464230	RGA8 2/1g	HL-RGA8	164
L	RGA1	RGA1	RGA1	155
M	RGA13	WT3B-A7	WT3B-A7	234
N	RGA15	WT1-B8	WT1-B8	171
O	RGA17	WT2-A12	WT2-A12	152
P	*Arabidopsis thaliana* Homolog	WT3B-B12	WT3B-B12	

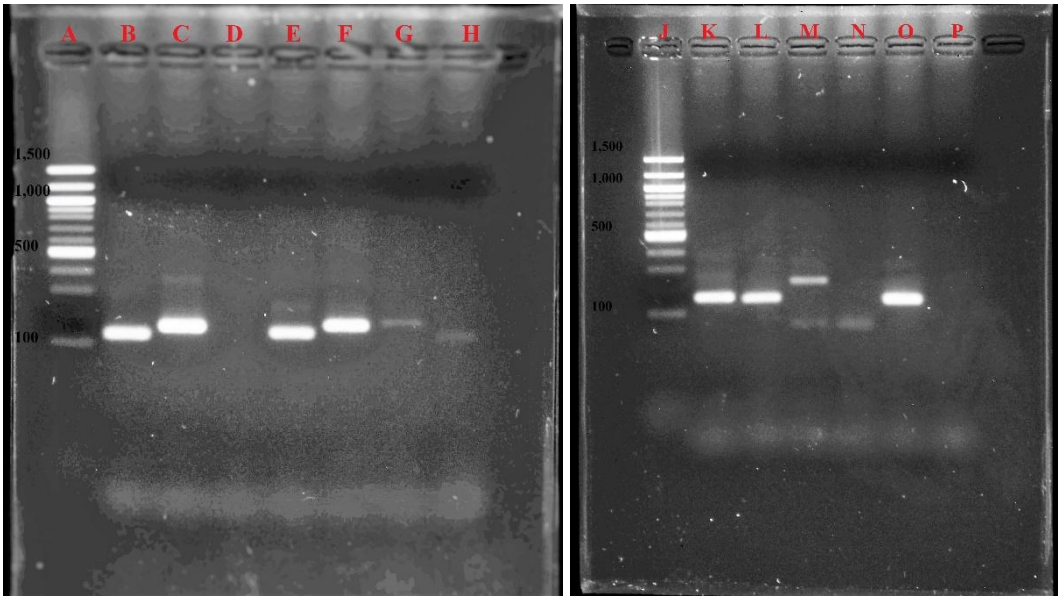
Letters which correlate to the same target DNA in all six lines of hops in the first run of electrophoresis for analogue analysis.



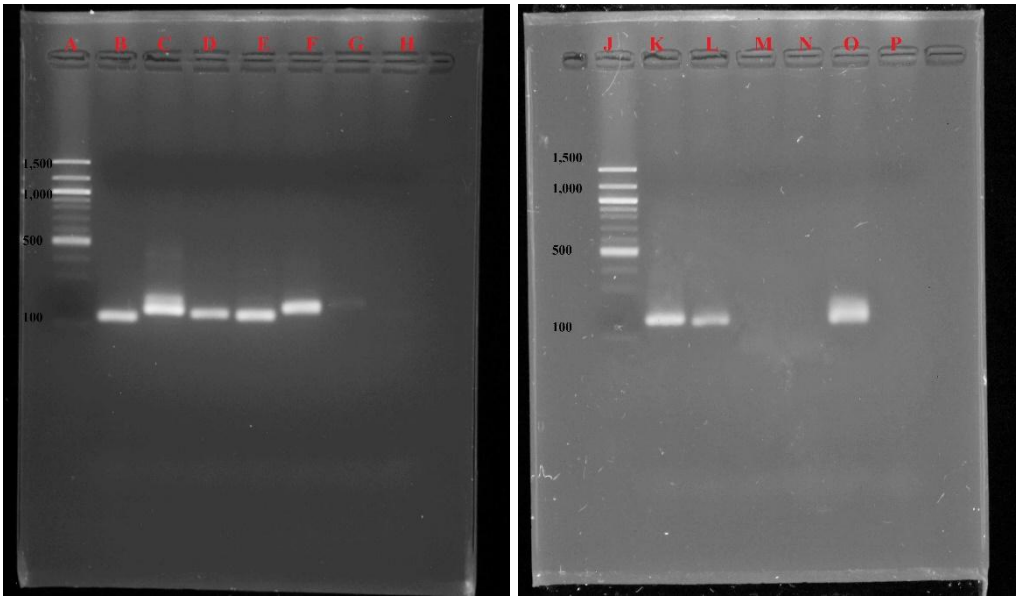
This figure consists of the two gels ran with DNA samples from the Chinook line of hops. These gels confirm that the following RGAs are present in the Chinook genome: EF464235, EF464283, EF464240, EF464295, EF464222, EF464230, RGA1, RGA13, and RGA17. The following RGAs were not identified in these gels: EF464228, EF464288, RGA15, and Arabidopsis thaliana homolog.



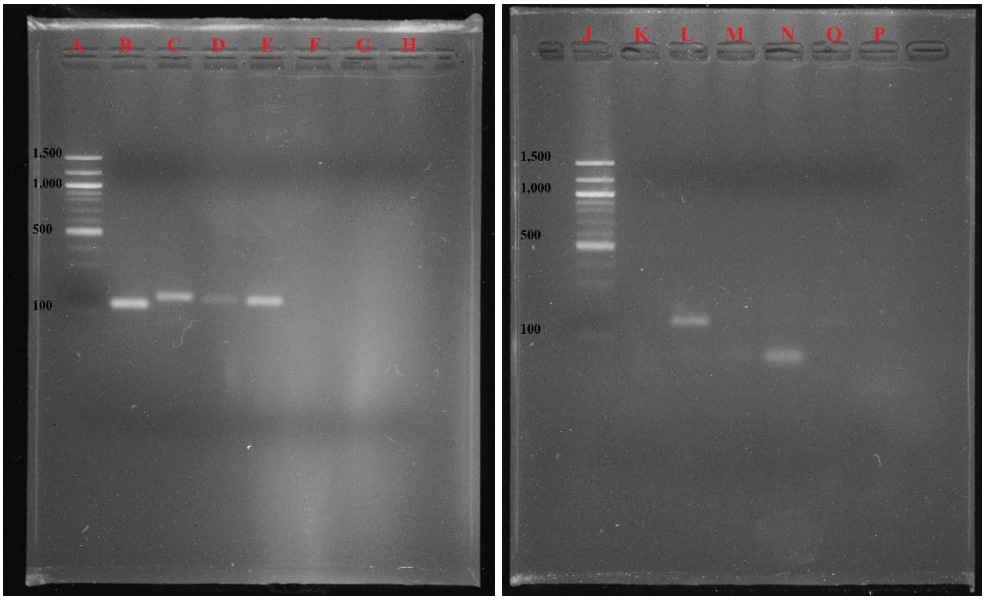
This figure consists of the two gels ran with DNA samples from the Zeus line of hops. These gels confirm that the following RGAs are present in the Zeus genome: EF464235, EF464283, EF464240, EF464295, EF464222, EF464230, RGA1, and RGA17. The following… …RGAs were not identified in these gels: EF464228, EF464288, and Arabidopsis thaliana Homolog. The following RGAs were identified; however, these bands were very faint: RGA13 and RGA15.



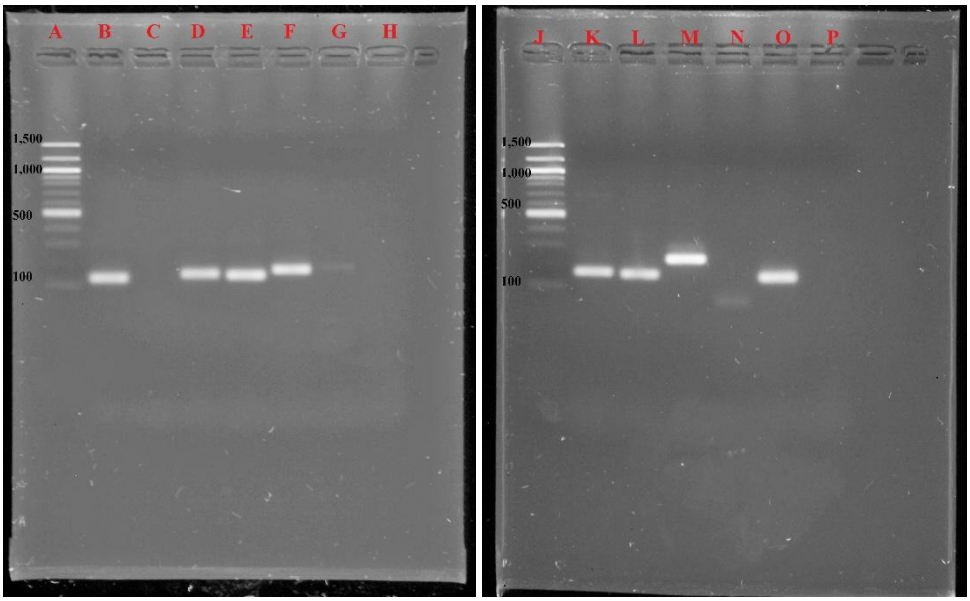
This figure consists of the two gels ran with DNA samples from the Cascade line of hops. These gels confirm that the following RGAs are present in the Cascade genome: EF464235, EF464283, EF464295, EF464222, EF464230, RGA1, RGA13, and RGA17. The following RGAs were not identified in these gels: EF464240 and Arabidopsis thaliana homolog. The following RGAs were identified; however, these bands were very faint: EF464228, EF464288, and RGA15.



This figure consists of the two gels ran with DNA samples from the Willamette line of hops. These gels confirm that the following RGAs are present in the Willamette genome:… …EF464235, EF464283, EF464240, EF464295, EF464222, EF464230, RGA1, and RGA17. The following RGAs were not identified in these gels: EF464288, RGA13, RGA15, and Arabidopsis thaliana homolog. The following RGAs were identified; however, these bands were very faint: EF464228.



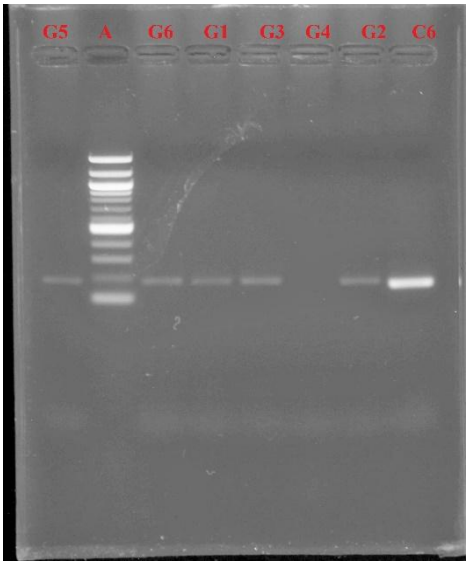
This figure consists of the two gels ran with DNA samples from the wild type of hops found on the High Bridge Trail. These gels confirm that the following RGAs are present in the wild type of hops genome: EF464235, EF464283, EF464240, EF464295, RGA1 and RGA15. The following RGAs were not identified in these gels: EF464222, EF464228, EF464288, EF464230, RGA17, and Arabidopsis thaliana homolog.



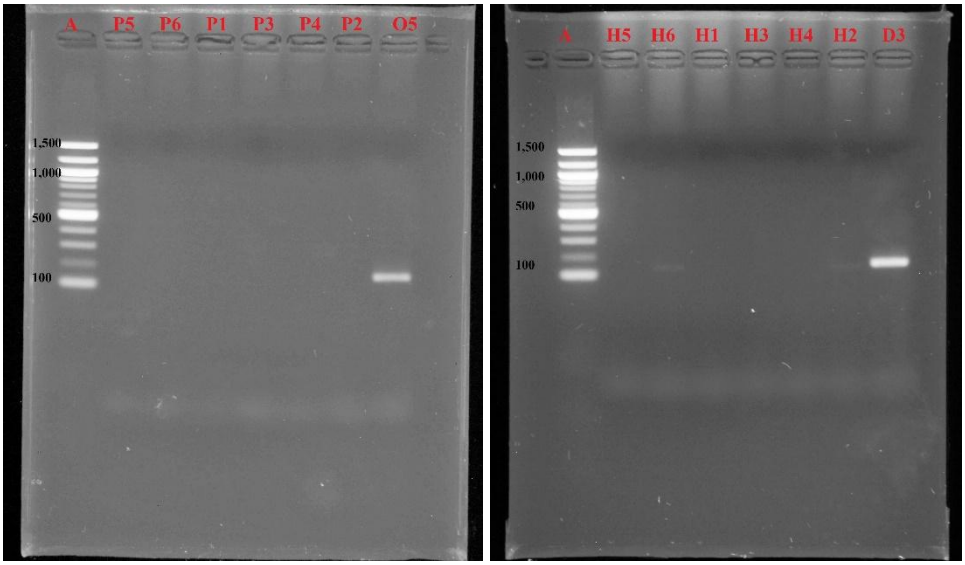
This figure consists of the two gels ran with DNA samples from the unknown Jenkins line of hops. These gels confirm that the following RGAs are present in the Willamette genome: EF464235, EF464240, EF464295, EF464222, EF464230, RGA1, RGA13, and RGA17. The following RGAs were not identified in these gels: EF464283, EF464288, and Arabidopsis thaliana homolog. The following RGAs were identified; however, these bands were very faint: EF464228 and RGA15

Second Run of Gel Electrophoresis

A second round of PCR and Gel Electrophoresis was performed for all primer pairs that were not identified in the first run of gels. This was done to determine if the RGA is in fact not present in a particular line of hops or if a mistake was made in the first run of gels. The primer combinations that showed up faint in the first run of gels were ran again. The main objective for the rerun was to determine if a mistake was made in the procedure in the first run that prevented a band from showing up.

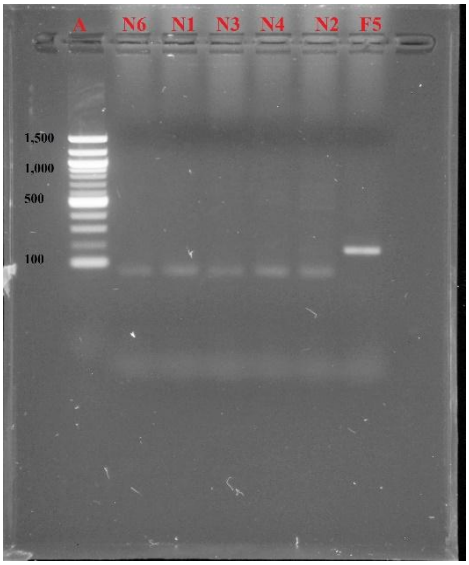


Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
G5	HB Trail	EF464228	RGA8 2/1d	RGA8 2/1d
A	100 BP ladder			
G6	Jenkins	EF464228	RGA8 2/1d	RGA8 2/1d
G1	Chinook	EF464228	RGA8 2/1d	RGA8 2/1d
G3	Cascade	EF464228	RGA8 2/1d	RGA8 2/1d
G4	Willamette	EF464228	RGA8 2/1d	RGA8 2/1d
C2	Zeus	EF464283	WT2-D9	WT2-D9 WTb

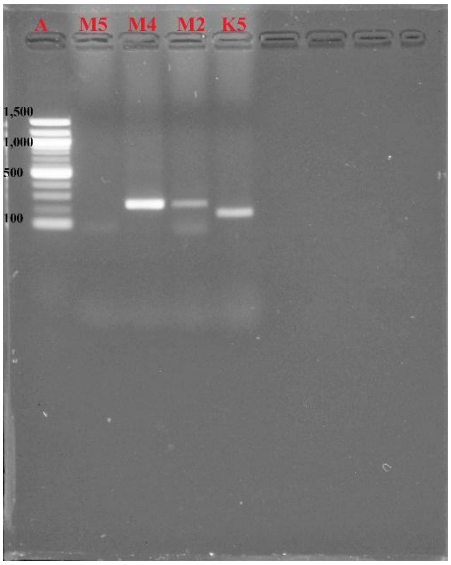


Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
P5	HB Trail	AT Homolog	WT3B-B12	WT3B-B12
P6	Jenkins	AT Homolog	WT3B-B12	WT3B-B12
P1	Chinook	AT Homolog	WT3B-B12	WT3B-B12
P3	Cascade	AT Homolog	WT3B-B12	WT3B-B12
P4	Willamette	AT Homolog	WT3B-B12	WT3B-B12
P2	Zeus	AT Homolog	WT3B-B12	WT3B-B12
O5	HB Trail	RGA17	WT2-A12	WT2-A12

Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
H5	HB Trail	EF464288	RGA8 WTa	RGA8 WTa
H6	Jenkins	EF464288	RGA8 WTa	RGA8 WTa
H1	Chinook	EF464288	RGA8 WTa	RGA8 WTa
H3	Cascade	EF464288	RGA8 WTa	RGA8 WTa
H4	Willamette	EF464288	RGA8 WTa	RGA8 WTa
H2	Zeus	EF464288	RGA8 WTa	RGA8 WTa
D3	Cascade	EF464240	WT1-D7 2/1spl	WT1-D7 2/1spl



Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
N6	Jenkins	RGA15	WT1-B8	WT1-B8
N1	Chinook	RGA15	WT1-B8	WT1-B8
N3	Cascade	RGA15	WT1-B8	WT1-B8
N4	Willamette	RGA15	WT1-B8	WT1-B8
N2	Zeus	RGA15	WT1-B8	WT1-B8
F5	HB Trail	EF464222	HL-RGA2	RGA2-2/1f

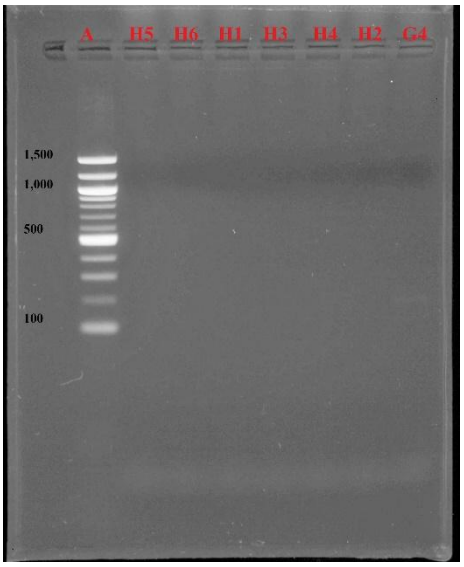


Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
M5	HB Trail	RGA13	WT3B-A7	WT3B-A7
M4	Willamette	RGA13	WT3B-A7	WT3B-A7
M2	Zeus	RGA13	WT3B-A7	WT3B-A7
K5	HB Trail	EF464230	RGA8 2/1g	HL-RGA8

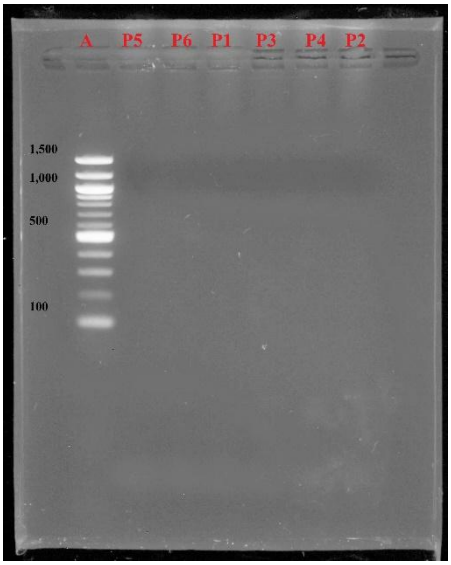
Gels and results for the second run

Third Run of Gel Electrophoresis

The third run of gel electrophoresis was performed for all primer pairs that had not shown up in the first and second runs. This was done to confirm that these RGAs were not being isolated during the PCR process.



Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
H5	HB Trail	EF464288	RGA8 WTa	RGA8 WTa
H6	Jenkins	EF464288	RGA8 WTa	RGA8 WTa
H1	Chinook	EF464288	RGA8 WTa	RGA8 WTa
H3	Cascade	EF464288	RGA8 WTa	RGA8 WTa
H4	Willamette	EF464288	RGA8 WTa	RGA8 WTa
H2	Zeus	EF464288	RGA8 WTa	RGA8 WTa
G4	Willamette	EF464228	RGA8 2/1d	RGA8 2/1d



Lane	DNA Sample	Target sequence	Forward Primer	Reverse Primer
A	100 BP ladder			
P5	HB Trail	AT Homolog	WT3B-B12	WT3B-B12
P6	Jenkins	AT Homolog	WT3B-B12	WT3B-B12
P1	Chinook	AT Homolog	WT3B-B12	WT3B-B12
P3	Cascade	AT Homolog	WT3B-B12	WT3B-B12
P4	Willamette	AT Homolog	WT3B-B12	WT3B-B12
P2	Zeus	AT Homolog	WT3B-B12	WT3B-B12

Gels and results for third run

Target Sequence	Primers S	High Bridge Trail sample	Jenkins	Chinhock	Cascade	Willamette	Zeus
EF464235	1	Yes	Yes	Yes	Yes	Yes	Yes
EF464283	2	Yes	Yes	Yes	Yes	Yes	Yes
EF464240	3	Yes	Yes	Yes	Yes	Yes	Yes
EF464295	4	Yes	Yes	Yes	Yes	Yes	Yes
EF464222	5	Yes	Yes	Yes	Yes	Yes	Yes
EF464228	6	Yes	Yes	Yes	Yes	No	Yes
EF464288	7	No	Faint	No	Faint/No	No	Faint
EF464230	8	Yes	Yes	Yes	Yes	Yes	Yes
RGA1	9	Yes	Yes	Yes	Yes	Yes	Yes
RGA13	10	Yes	Yes	Yes	Yes	Yes	Yes
RGA15	11	Yes	Yes	Yes	Yes	Yes	Yes
RGA17	12	Yes	Yes	Yes	Yes	Yes	Yes
Arabidopsis thaliana Homolog	13	No	No	No	No	No	No

The final table that identifies whether or not a degenerative primer pair is present, not present, or present but faint in a particular line of hops based on PCR amplification.

Root Analysis

Clipping Name	Length	Number of leaves	Size of largest leaf L*W	Hormone yes or no	Root Environment (light or dark)	Location	Number of Roots	Root Length
RG5F	21.4	4 FULL	1.7*2.0.	no	dark	greenhouse	0	n/a
RG7E	18	2 FULL 2 EMERGING	1.4*1	no	dark	greenhouse	0	n/a
RG9B	25.6	4 FULL 2 EMERGIN	3.6*3.1	no	dark	greenhouse	0	n/a
HB4	27.9	2 FULL 2 BROWN	2.8*2.43	no	dark	humid	0	n/a
RG5A	23.4	2 FULL 4 EMERGING	1.5*1.5	no	dark	humid	0	n/a
RG7I	23	6 FULL 2 EMERGING	4.7*3.5	no	dark	humid	0	n/a
RG7A	13	4 FULL 2 EMERGING	2.1*2	no	dark	humid	0	n/a
RG9D	16.8	4 FULL	2.0*2.6	no	Light	greenhouse	0	n/a
RG7G	22	6 FULL	4.6*3.8	no	Light	greenhouse	3	0.1
HB3	24.6	2 FULL 6 DEAD	2.0*1.2	no	Light	humid	0	n/a
RG7C	28.6	4 FULL	3.9*3.4	no	Light	humid	0	n/a
RG9F	29.1	6 FULL 4 EMERGING	3.2*3	no	Light	humid	0	n/a
RG5E	18.6	4 FULL 2 EMERGING	3.1*2.5	no	Light	humid	0	n/a
RG7J	25.4	4 FULL 2 EMERGIN	2.6*2.2	yes	dark	greenhouse	0	n/a
RG5C	18	1 EMERGING	1.1*.0.5	yes	dark	greenhouse	0	n/a
RG7H	26.4	3 FULL	1.8*1.4	yes	dark	greenhouse	0	n/a
RG9A	26.8	5 FULL	4.3*4.8	yes	dark	greenhouse	0	n/a
HB2	20.1	2 FULL 2 EMERGING	1.1*0.6	yes	dark	humid	0	n/a
RG9C	24.4	6 FULL	3.9*3.6	yes	dark	humid	0	n/a
RG9F	17.3	4 FULL 1 EMERGING	3.4*3.1	yes	dark	humid	0	n/a
RG7K	23.9	4 FULL	2.9*2.6	yes	Light	greenhouse	0	n/a
RG7D	18.4	4 FULL	2.4*2	yes	Light	greenhouse	0	n/a
RG5D	21.1	5 FULL	3.0*2.4	yes	Light	greenhouse	0	n/a
RG9E	29.6	6 FULL	2.0*2.4	yes	Light	greenhouse	0	n/a
RG7B	30.2	3 FULL 2 EMERGING	1.3*1.5	yes	Light	humid	0	n/a
HB1	28.8	3 BROWN	1.2*1.1	yes	Light	humid	0	n/a
RG5B	11.9	4 EMERGING	.4*.8	yes	Light	humid	0	n/a

Results of root analysis

DISCUSSION

Propagation

Hope plants grow best in soil with good drainage, a temperature of 25°C, and a pH between 5.0 and 5.8 While the average temperature of Virginia during hop growing season is usually higher than 25°C, we found that the clay that makes up most of the soil in Virginia can support hops growth. It has good drainage, and its pH, although not prime, can easily be altered by commercial fertilizers. For our leaf propagation trials, we were unable to obtain any growth. This may be due to the environment that we kept the cuttings in. According to Clark and Toogood, “the greenhouse is not the best place for these cuttings as they are likely to be scorched by the bright light” (1990). They also stress that if a greenhouse must be used, the cuttings must be kept in a shaded area, which they were not, and that the cuttings must not be exposed to water to prevent scorching, which they were.

Isolating Mildew

We had some success in isolating mildew through several methods. We were able to isolate several fungal-like colonies with our broth method, however we were unable to test them to see if they were truly downy and powdery mildew. Future testing should be done to see if mildew isolation can be performed through this method, as it is a method to propagate a large amount of mildew in a short amount of time in a contained area. We were also able to propagate fungal like colonies on agar plates with a high degree of success. Unlike the broth tests, we knew we had isolated downy mildew on these tests due to the blue bacteria that we observed. However, our research ended before we were able to perform any further tests. Had we more time, we would have selected two plates that best represented Powdery and Downy mildew. We would have then picked four non-infected hops plants and had four leaf clippings, including stems, taken. Cotton swabs, saturated in sterile water, would have been used to transfer the mildew from the plants to the leaves. The cuttings would have been placed in a 250ml beaker of water and left inside a growth chamber at 28°C and observed until infection was apparent.

We also observed a high degree of success with infecting plants by directly rubbing leaves with downy mildew on the leaves of hops plants in the greenhouse. While four out of five plants showed signs of infection within the first three days, this did not spread to the rest of the plant. This leads us to believe that the temperature inside the greenhouse, which tended to range between 28°C and 36°C during the day, hindered the spread of the mildew. One study showed that downy mildew favors cooler temperatures between 18°C and 26°C. In that same study, downy mildew was less likely to grow when exposed to temperatures between 26°C and 30°C (Gilardi et al, 2016). In the future, we would keep our test plants at room temperature to encourage downy mildew growth.

Resistance PCR/Gels

We had a lot of difficulty in getting consistent results with our electrophoresis gels. Some gels, however, gave us some results that should be looked at more closely in the future. When running primer 11, both Cascade and Zeus showed quadruple bands. This is interesting, as it does not appear with any other primers, and no other varieties of hops show quadruple bands. In the future, these genes should be sequenced to see if they have resistance characteristics.

Root Analysis

These results are in no way counterproductive. We have a new hypothesis to test for growing clean plants from clean mother clippings. This is very important because of the clean plant network. The commercial implications of hops without an infection are astronomical. The ability to quickly and effectively grow hops from clean stem clippings is paramount in the growing craft beer industry. The goal is to continue research to determine the most effective way to root stem clippings of the hops plant. We are thinking that the more mature parts of the stem clippings are able to root faster than the top of the shoots due to the meristem not being present. The meristems job is to grow the plant so without that the plant is able to focus production on making new root material rather than continuing to grow. The observations that the rooting hormone improved the amount and quality of roots was one that we tested only a few times but noticed a severe difference and began using all tests with the rooting hormone. The rooting hormone improved the quality and quantity of roots which in turn led to plants growing faster once they were planted in pots with potting soil in the greenhouse. The clippings that rooted without the hormone when planted into pots took longer to start growing.

I would like to continue doing rooting experiments in the growth chamber and green house during the winter months. I would like to continue testing whether or not the larger leaves have a direct correlation with the success of the stems rooting. I would also like to continue testing to see if there is a

better soil medium to root the clippings in. I would like to develop a better agar solution to grow the fungal colonies on. I would like to be able to run genetic testing on the fungi as well. This way we can more reasonably conclude which fungi make up the fungal profile of the hops plant. I would like to independently test the roots and the leaves as well as the stems. I would also like to move into testing the growing of plants from seeds. This is to determine the rate of successful seed germination and to determine if there is a way to predict if a plant will be clean or infected.

Analogues

Based on the similar results of the degenerative primer PCR amplification throughout all of the lines of hops tested, we can conclude that the PCR amplification of the RGAs was a success. Since the three primer pairs, EF464288, EF464288, and *Arabidopsis thaliana* homologue, which did amplify in the PCR process are common for most of the hop lines, one of two conclusions can be made. The first is that these RGAs were not amplified because the correct PCR conditions were not correct or because the RGAs were not present in the hop genome. Another conclusion can be made, is that the target DNA was in fact amplified; however, the DNA template was too small to show up on the gel electrophoresis.

A possible hypothesis based on the results is that these RGAs are partial pieces of a possible larger R gene. This hypothesis could be tested by performing a RNA extraction for a particular line of hops. The RNA would then be reverse transcribe to form a complementary DNA (cDNA) library. This would allow for us to perform a southern blot using the same degenerative primers to determine whether or not the RGAs line up within the genome. This information would allow us to speculate on the possible location of an R gene or genes within the hop genome. Testing this hypothesis would allow us to be one step closer to possibly discovering an R gene within the hop genome, that would eventually lead to the manual mutation of the gene to raise the plant's resistance to powdery and downy mildews.

Root Analysis

We were unable to determine any definitive results. We were also unable to reasonably conclude if there was any difference in the fungus and infected and non-infected plant.

Acknowledgements

We would like to thank the Hampden-Sydney College Honors Council and Virginia Foundation for Independent Colleges/ Carilion Undergraduate Science Research Program for their financial support in allowing us the opportunity to pursue this research. We would also like to thank my advisor, Dr. Michael Wolyniak, for his vast amount of knowledge on molecular biology and answering any and all the questions we had during the research process. We would also like to thank Mrs. Jennie Jenkins for her aid in obtaining materials.

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