Clavibacter Detection and QTL Mapping of Resistance in Tomato

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Thesis for Master of Science in Plant Biotechnology
Specialization in Molecular Breeding and Phytopathology

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Abstract

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a gram-positive bacteria causing bacterial wilt and canker disease in tomato (*Solanum lycopersicum*). It is the most significant bacterial disease in tomato that causes a great deal of economic damage. International quarantine regulation in *Cmm* is being implemented to prevent disease outbreak. Due to the lack of resistant and tolerant cultivars, the most important strategy to control the disease is the use of pathogen free seeds and transplants. The aim of this study is to determine and map quantitative trait loci (QTL) for clavibacter resistance in a cross between S. *pimpinellifolium* and the susceptible cultivar "Moneymaker" and to fine map QTL regions of clavibacter resistance in *S. peruvianum* using SNP marker.

Wild accession of tomato like *S. pimpenellifolium* and *S. peruvianum* are known to harbour resistant traits against clavibacter. And in order to identify QTL for bacterial canker resistance, QTL mapping was done on F6 RIL population of *S. pimpinellifolium* x *S. lycopersicum* cv. Moneymaker. Five putative QTL for resistance were mapped. QTL derived from wilt disease scoring were found at chromosome 1, 7, 10 and 12 while the only QTL derived from bacterial quantification specific at the upper part of the plant was found in the region 66.6-86.8 cM of chromosome 7 where it overlaps with the region of the previous QTL found in chromosome 7 (65-85 cM).

From previous study using *S. peruvianum* (LA 2157) *x S. lycopersicum* cv. Solentos, three QTL conferring resistance to clavibacter was found in chromosome 5,7 and 9 using RFLP markers. Among the three QTL, it is believe that QTL in chromosome 7 may contain the most important resistance gene between the region flanked by the markers TG61 and TG 166. Five SNP markers were designed to fill up the gap between these two markers to be used for the analysis of the RIL lines of *S. peruvianum* (LA 2157) *x S. lycopersicum* cv. Solentos. Markers for the chromosome 5 and 9 have already been designed prior to the start of my experiment.

KEY WORDS: Clavibacter, Bacterial Canker, Single nucleotide polymorphism (SNP), Qualitative trait loci (QTL), Linkage map, Taqman PCR, Bead array, Fine mapping

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Chapter 1.General Introduction

A. INTRODUCTION

1.1 Tomato

Tomato (*Solanum lycopersicum*) is one of the most widely produced and consumed vegetables in the world. It ranks the second most important vegetables crop next to potato but it tops the list of canned vegetables. Global tomato production reported by 175 countries in 2009 was about 150 million tonnes produced on 4.3 million hectares(FAOSTAT Database, 2009).The leading top five tomato producing countries are the China, United States, India , Italy and Turkey (FAOSTAT Database, 2009) .

The cultivated tomato (*Solanum lycopersicum*) is a perennial diploid dicotyledon (2n=2x=24) with 12 chromosomes. The ancestor of the cultivated tomato came from the Peru-Ecuador area but the first domestication was believed to occur in Mexico during the Mesoamerican civilization (Sims, 1980; Harvey *et al*., 2002). Wild relatives of tomato are native to Andean region (Chile, Colombia, Ecuador, Bolivia and Peru) (Sims, 1980). There are two major complex/division in the genus *Lycopersicum* proposed by Rick (1976) which are the *lycopersicum* complex and *peruvianum* complex. The *lycopersicum* complex are composed of *Lycopersicum* species which are closely related to *S. Lycopersicum* and could easily be crossed with the commercial tomatoes namely: S.var. *cerasiforme*, S. *pimpinellifolium*, S. *cheesmannii*, S. *parviflorum*, S. *chmielewski, S. habrochaites*.The peruvian complex which include S. *chilense, S. peruvianum* (S.*peruvianum f.glandusulum* and *S. peruvianum var. Humifusum*) are wild species that are not compatible with the commercial tomatoes and can only be crossed as a pollen parent, producing seeds with low viability that needs embryo rescue (Taylor, 1986).

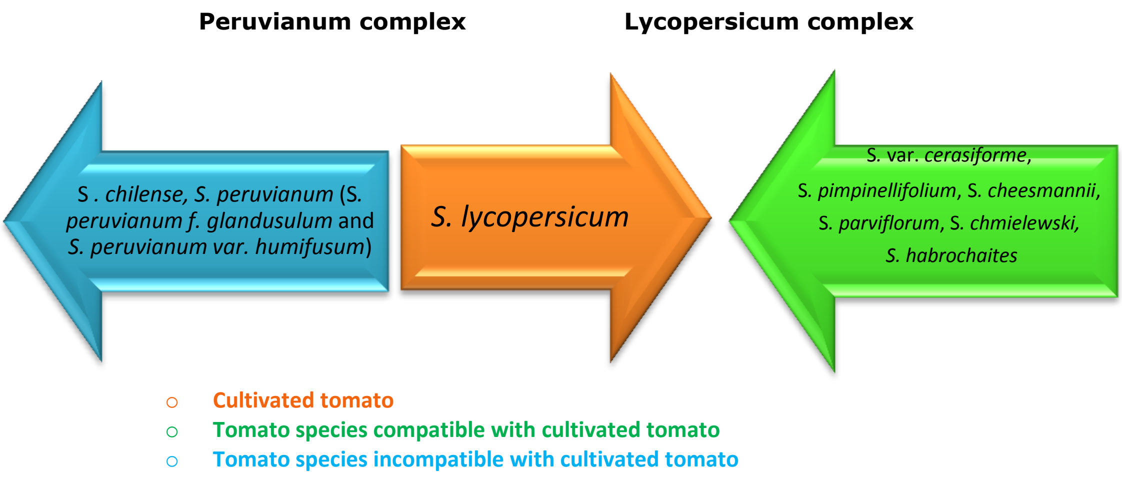

Figure 1. Phylogenetic Relationships among Tomato species

1.2 Bacterial Canker Disease in Tomato

The gram-positive bacterium *Clavibacter michiganensis subsp. michiganensis* (*Cmm*) is the causal agent of bacterial canker in tomato (Davis et al., 1984). The disease was first discovered in 1909 in Grand Rapids, Michigan, USA (Strider et al., 1969) but is currently reported in all tomato growing regions in the world. Random outbreaks occurs annually and may be more severe in dry years that has caused major economic losses in commercial tomato production worldwide(Carlton et al., 1994).

Bacterial canker disease appears in humid environments and is increasing where greenhouse-grown transplants are used to established field stands (Kabelka et al., 2002). The pathogen is seed transmitted and can survive in plant debris and soil for 2-3 years and on contaminated greenhouse structures for several months (Gleason et al., 1993, Luo et al., 2008). Secondary spread from plant to plant can be accomplished by means of water splashing, contaminated equipment and cultural practices (Tsiantos, 1987) were the Cmm can enter the plant through wounds or natural openings like the stomata.

Symptoms cause by *Cmm* may vary depending whether they develop from systemic or localized infections. Wilting is the most conspicuous symptom for systemic infection where the infection comes from seed or wounds directly from vascular tissue. Systematically infected plants as young seedlings will wilt and rapidly collapse compared to older plants were it will develop wilt symptoms gradually (Gleason et al., 1993). And the infection through vascular tissues shows yellow streaks development along stems of affected plants where stem cankers are formed. As the *Cmm* spreads from xylem to phloem dark brown canker appears that reveals extensive necrosis of pith and cortex leading to collapse (Gleason et al., 1993). Fruit symptoms begin as small, raised lesions surrounded by a white margin or halo referred to as birds-eye spots. Often, several lesions are formed where fruits touch in a cluster, giving the fruits a scabby appearance that reduces the fruit's fresh-market value (Carlton et al., 1994).

For localized infection where entry of the pathogen are through the broken trichomes or natural openings , early symptoms of the disease usually begin on lower leaves were leaflets margins become necrotic referred to as the "firing stage". As the necrotic margin gradually widens the entire leaves and branches wilt and die. Leaves and branches in the upper portions of infected plants wilt and die as the bacteria moves up through the stems(Carlton et al., 1994).

Figure 2. Bacterial wilt and stem canker symptoms cause by clavibacter

1.3 Detection Method of *Clavibacter michiganensis subsp. michiganensis* (*Cmm*)

The most important bacterial disease in tomato which has most significant economic lost is bacterial canker cause by *Cmm*. To prevent the outbreak of bacterial canker, international quarantine regulations for *Cmm* is being implemented in EU, USA, Canada, Israel and some other more countries which restrict importation and exportation (Hadas et al., 2005, Luo et al., 2008). Therefore, the use of pathogen-free seeds and transplant has become the most important strategy to control the disease (de Leon et al., 2006, Luo et al., 2008). But because of the long time it takes for the symptom to developed, differentiating healthy from infected plants during transplanting is difficult and could cause the undetected spread of the disease.

Conventional methods of detection and identification includes biochemical test, serological and culture assay, and metabolomics profiling (Zhao et al., 2007). However, these conventional methods have several limitations which include poor sensitivity, lack of specificity, laborious methods and take a long time for the results. The serological method like ELISA may not always be reliable because of cross reaction with other bacteria and limited sensitivity while plating on selective media are time consuming and can easily be affected by certain factors (Zhang et al., 2009). At present, the most common method of detection is the PCR-based method which is fast and sensitive. PCR-based method for the detection of *Cmm* in infected plants and seeds was first describe ten years ago (Dreier et al., 1995) but since *Taq* DNA polymerase is sensitive to factors present in biological samples and some chemicals used in DNA extraction can interfere with DNA amplification, many studies have been conducted to improve the reliability and sensitivity of this method.

1.4 Objectives of the study

The aim of this study is:

- to determine and map quantitative trait loci (QTL) for clavibacter resistance in a recombinant inbred line population derived from a cross between *S. pimpinellifolium* and the susceptible cultivar "Moneymaker"
- to do fine mapping on the QTL regions of clavibacter resistance in *S. peruvianum* using SNP markers

Chapter 2.QTL mapping of clavibacter resistance in *S. pimpinellifolium*

A. INTRODUCTION

2.1 QTL mapping

The framework for determining the location of the genes responsible for variation in plant growth and development is the availability of a complete genetic map. A large number of important phenotypes are influenced by quantitative trait loci which is why QTL mapping is becoming an important tool in life science. Using the genetic map, developed algorithms in analytical methods can make an accurate estimation of the location of the QTL by testing correlation between marker genotypes and quantitative phenotypes (Paterson, 1996). The most common population use for mapping QTL is F2, BC1 and recombinant inbred lines (RIL). F2 population are quick to make and contain all possible two alleles combination in a locus that could easily be used to determine the mode of gene interaction in a loci. The advantage of using RIL is its reproducibility, because individual in this population is homozygous and its genotype can be replicated many times with high fidelity.

2.2 *Solanum pimpinellifolium*

This species of tomato is indigenous to the lower-elevation coastal valleys of Peru (<1,000m). The fruit are small, approximately 1cm in diameter, and red in color (yellow varieties do exist). The fruit size gives it the common name of the 'currant tomato'. The foliage tends to produce a unique odor when crushed. Stems are often smooth and lack the hairs found on *S. lycopersicum*. It has been my observation that the stems are not as brittle either. All populations of this species are self-compatible. Some populations of this species differ considerably in morphology while others are highly uniform. Some populations are exclusively autogamous (self-pollinating) while others allow some out breeding due to exerted stigmas which project well beyond the anther cone (www.kdcomm.net).

Used of this species in breeding is practical because of its tendency to readily cross with cultivated tomato exhibiting a natural introgression. In fact it is probable that both species evolved from a common ancestor. There are thousands of PI's (Plant Introductions) of *S. pimpinellifolium*, of which many are used as donor for introgression breeding in *S. lycopersicum* resistance to several diseases like bacterial wilt and fusarium wilt.

Figure 3. *Solanum pimpinellifolium* plant structure

2.3 Taqman PCR for bacterial detection and quantification

The **TaqMan RT-PCR** method was named on the enzyme Taq Polymerase and the famous videogame PacMan which resembles the mechanism of the method (Taq Polymerase + PacMan = TaqMan). The *TaqMan RT-PCR* assay is based on Taq Polymerase 5´–3´ nuclease activity. During the process of hybridization, the enzyme cleaves a dual-labelled probe to the complementary target sequence and fluorophore-based detection. In real-time PCR methods, the fluorescence signal allows quantification of the accumulated product during the exponential stages of the PCR. The TaqMan RT-PCR significantly increases the specificity of the detection (*www.dnavision.com*).

The Taqman RT-PCR probe, which is labelled with two fluorescent dyes, is created within the amplicon defined by a gene-specific PCR primer pair. The 5' end is labelled with a reporter dye (usually 6-carboxy-fluorescein, FAM), while the 3' end is labelled with a second fluorescent dye (6-carboxy-tetramethyl-rhodamine, TAMRA). As long as the probe is intact, the emission of the reporter dye is quenched by the second fluorescence dye. During PCR, the probe anneals specifically between the forward and reverse primer to an internal region of the PCR product. The polymerase then carries out the extension of the primer and replicates the template to which the TaqMan® is bound. The 5' exonuclease activity of the polymerase cleaves the probe, releasing the reporter molecule away from the close vicinity of the quencher. The fluorescence intensity of the reporter dye, as a result increases. This process repeats in every cycle and does not interfere with the accumulation of PCR product (*www.premierbiosoft.com)*.

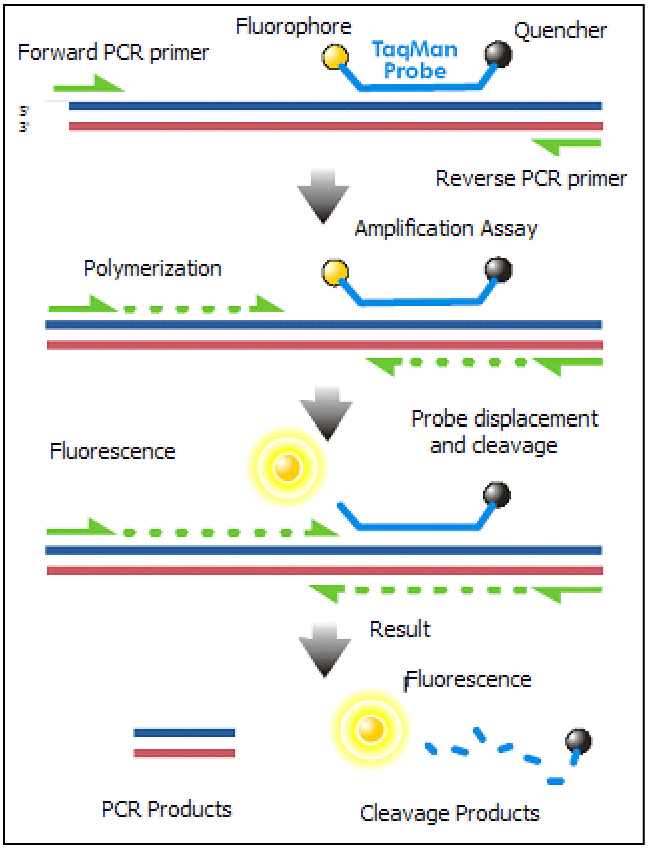

Figure 4.Taqman Probe Mechanism (*www. wikipedia.org*)

The specific software plays a very important role in the normalization of the raw data. The algorithm normalizes the reporter signal (Rn) to a passive reference. The standard deviation of the background Rn in the first few cycles then is multiplied by a default factor of 10 to determine a threshold. This calculation is made in order to define the threshold cycle (Ct) – the cycle at which this baseline level is exceeded (see the figure below).

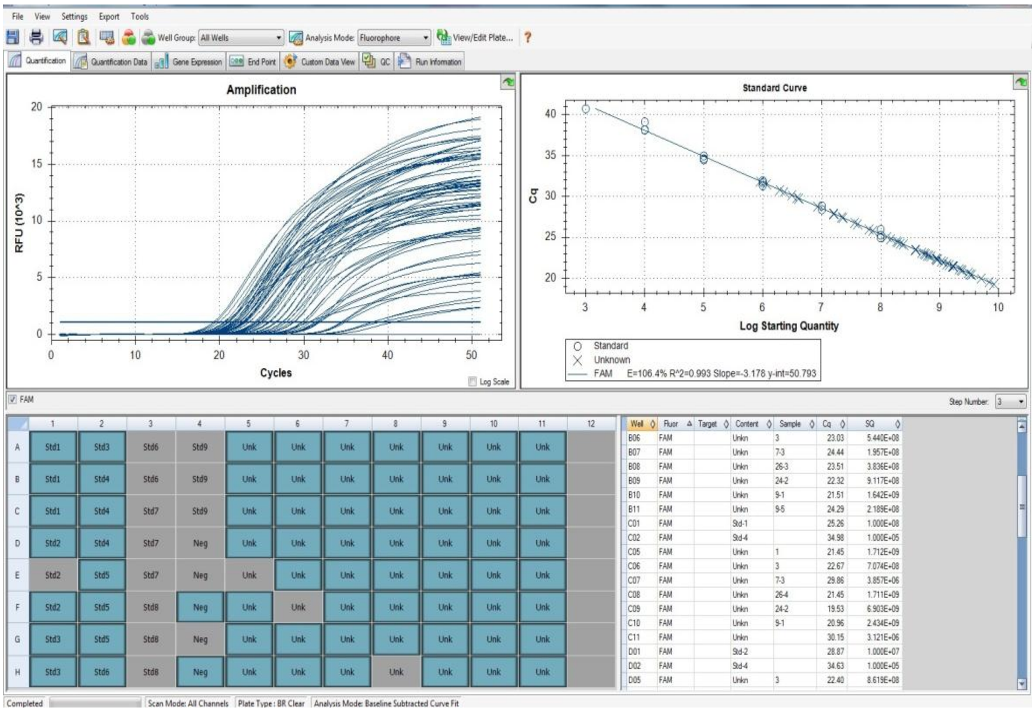

Figure 5.View of the Taqman PCR results showing threshold cycles, standard line, sample plates and sample quantification values

The absolute value of threshold cycle (Ct) depends on the initial template copy number, the efficiency of both DNA amplification and cleavage of the TaqMan probe. The Ct values of the samples are interpolated to an external reference curve constructed by plotting the relative or absolute amounts of a serial dilution of a known template vs. the corresponding Ct values.

2.4 SNP Array

A SNP array is a type of DNA microarray which is used to detect single nucleotide polymorphisms (SNPs) within a population. A single nucleotide polymorphism (SNP) is a variation at a single site in DNA. It is the most frequent variation in the genome. As SNPs are highly conserved throughout evolution and within population, the map of SNPs serves as an excellent genotypic marker for research. The basic principles of SNP-array are the same as the DNA microarray which is the convergence of DNA hybridization, fluorescence microscopy and solid surface DNA capture. The three mandatory components of the SNP arrays are (1) the array that contains immobilized nucleic acid sequences or target; (2) one or more labelled probes; (3) a detection system that records and interprets the hybridization signal. The principle of the SolSjaak array used for genotyping follows the bead array platform of Illumina where 50-base oligonucleotide probes are synthesized, immobilized on beads, and pools of these beads are arrayed on glass slides. Each of these oligos anneals to the 50 bases flanking the SNPs present in a genomic DNA sample. Following hybridization, a single base extension reaction is performed in parallel for all SNPs being interrogated on the BeadArray. The reaction uses dideoxynucleotides and two-color dye chemistry to distinguish the two alleles for a given SNP. Therefore, at a given bead type, which is designed to interrogate a particular SNP, a homozygous individual will generate a fluorescent signal for only one of the two colors (e.g. GG = all green or TT = all red), while a heterozygous individual will generate approximately equal fluorescent signals of both colors (e.g. TG = red and green) (www.med.stanford.edu).

B. MATERIALS AND METHODS

2.1 *Solanum pimpinellifolium* population

The *S. pimpinellifolium* RIL population (F6) is a cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* consisting of 90 lines obtained by a single seed descent that was used for disease screening and genotyping.

2.2 Plant Material

S. pimpinellifolium RILs consisting of 90 lines with four replicates for each line were grown in the greenhouse up to a stage where it have six true leaves before inoculation with *Cmm*. Plants were left standing on the greenhouse for 2 months to allow for the symptoms to developed. Tomato stem cuttings from the upper, middle and lower part of the plant were collected from the RIL population and placed on the Bio-Reba bags for crushing with PBS buffer to extract DNA.

2.3 Inoculation of Bacteria

Cmm 542-strain were plated in TSA media and grown for 2 days prior to inoculation. *Cmm* was dissolved in PBS buffer and $OD_{600\ was}$ measured to adjust the concentration to 10^8/ml. Using a syringe small amount of *Cmm* was injected in the second stem node of the young tomato plant. Plants was covered with plastic to create a favourable environment for bacterial development and removed after one week.

2.4 Disease Scoring

Plants were grown under 100% relative humidity for 7 days to stimulate bacterial infection. Growth condition in the compartment was set at 20^0C, 16 hours of day length and 70% relative humidity. Disease symptoms like wilting, leaf necrosis and stem canker were recorded from 18 to 65 days of inoculation with 3-4 days interval. Visual assessment of the disease was based on the following scale: 0=no symptoms, 1= 25% of leaves are wilted, 2= 50% of the leaves are wilted, and 3= 75% of leaves are wilted, 4= wilted (dead).

2.5 Taqman Protocol

Cmm quantification was done using the Taqman PCR protocol. DNA was extracted from the plant samples using the Kingfisher processor together with QuickPick SML Plant DNA purification kit from Bio-Nobile followed by a purification step on a PVPP column. RT-PCR mix used in this protocol consisted of 12.5 µl Takara mix, 0.5 µl Rox II, 2 µl of combined F primer, R primer and probe with 4 µM, 4 µM and 1 µM ratio, and 10 µl of DNA template in total of 25 µl for every reaction. Bio-Rad CFX thermocycler was used and the PCR conditions are as follows: 95 ºC for 30 sec, 50 cycles at 95 ºC for 3 sec and 60 ºC for 25 secs. A seven fold serial dilution of the Cmm bacteria from $10^8 10^1$ was used as template to obtain a standard curve to quantify the amount of bacteria present in the samples using the cycle threshold data form Taqman PCR.

2.6 Solsjaak SNP array

Single Nucleotide Polymorphisms (SNP) data were searched in sequence of several tomato lines and six thousand SNPs were selected and send to Illumina. Bead Array based on this data was designed to represent 5528 SNPs (472 of the 6000 oligo's did not meet the quality standards of Illumina). All 5528 SNPs were named after their position in the SL2.30 version of the published tomato genome under the International Tomato Genome Sequencing Project (http://solgenomics.net/). Solsjaak SNP array was used to genotype the *S. pimpinellifolium* RIL population. Data from this genotyping were loaded to Genome Studio from Illumina to convert the data to a file that could be loaded and recognized by the mapping program like JoinMap 4.1 (Van Ooijen, 2006) software to construct the linkage map.

2.7 QTL Mapping

MapQTL6 software (Van Ooijen, 2006) was used to find new QTL region for resistance to Clavibacter. To use the software, 3 kinds of data file should be was prepared, loc.file which contain locus and genotype data, map. File which has the calculated order of the markers and qua.file which contains the trait data gathered from the population like symptoms scoring and bacterial quantification. This two file will be loaded to the program together with the genetic map prepared on Joinmap to calculate the QTL position on genetic linkage maps in our population by conducting search associations between segregating molecular marker patterns and segregating trait information.

C. RESULTS

2.1 Disease scoring and Cmm quantification

From the F6 *S. pimpinellifolium* RILs (*S. lycopersicum cv.* Moneymaker x *S. pimpinellifolium*) consisting of 90 lines with four plants per line that were maintained in the greenhouse, final phenotypic scoring of the infected plants were done after 65 days of inoculation. Plants were scored according to the scale mentioned in the methodology. The phenotypic scorings were done 2 summer seasons (summer1=60 lines, summer2=25 lines) and 1 winter season (28 lines) where 89 out of 90 lines were phenotype. Out of 89 lines, 32lines have an average score of zero; 8 lines have an average score of one, 18 lines have average score of two, 10 lines have average score of three and 17 lines died with a score of four. Among the 89 lines, 16 lines showed variation on the disease scoring among the four replicates. Eleven lines showed segregation which may be due to the high percentage of Moneymaker allele present in the genome based on the genotyping data. The other caused of variation was the scape which means that plants are less infected due to chance (Appendix 1).

After the final phenotypic scoring, fruits were harvested and the plants were cut down to collect stem cuttings that was used as samples for bacterial quantification. Each lines have four plants and three stem cutting were collected from each plant from the lower part near the inoculation point, middle part and upper part of the plant. Quantification of *Cmm* was done using Taqman PCR that gives threshold level values which was converted to amount bacteria by using a standard regression line generated from the serial dilution of the bacteria from 10^8 to 10^1 (Appendix 2).

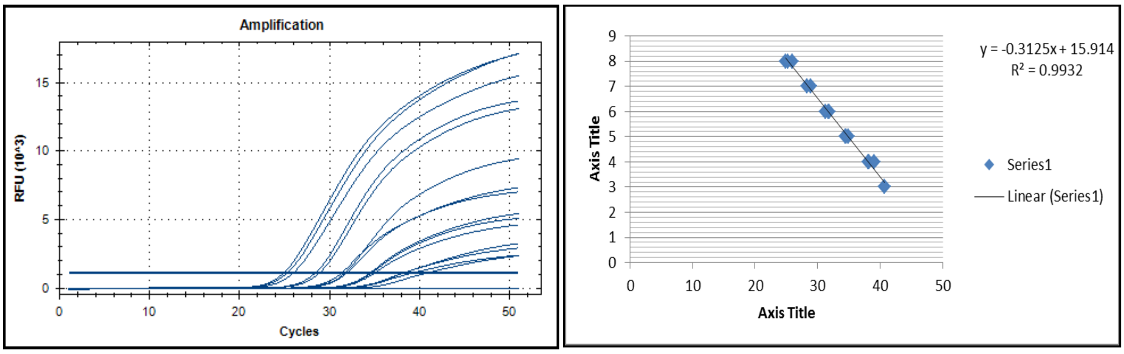

Figure 6. Standard regression line for *Cmm* bacterial quantification derived from serial dilution

By plotting the phenotypic score data against the bacterial quantification data, resistant and susceptible lines as well as tolerant and sensitive lines can be easily derived as shown from the figure below (Fig 7). The parent lines are the last 2 lines in the graph. Moneymaker shows high disease score as well as high bacterial count while the *S. pimpinellifolium* has zero disease score it still contain high amount of bacteria. Co-relation between disease score and bacterial quantification was also computed through regression statistical analysis (Appendix 6). Results from the analysis (Fig.8) illustrate that there is no correlation between the two traits, R=0.0027.

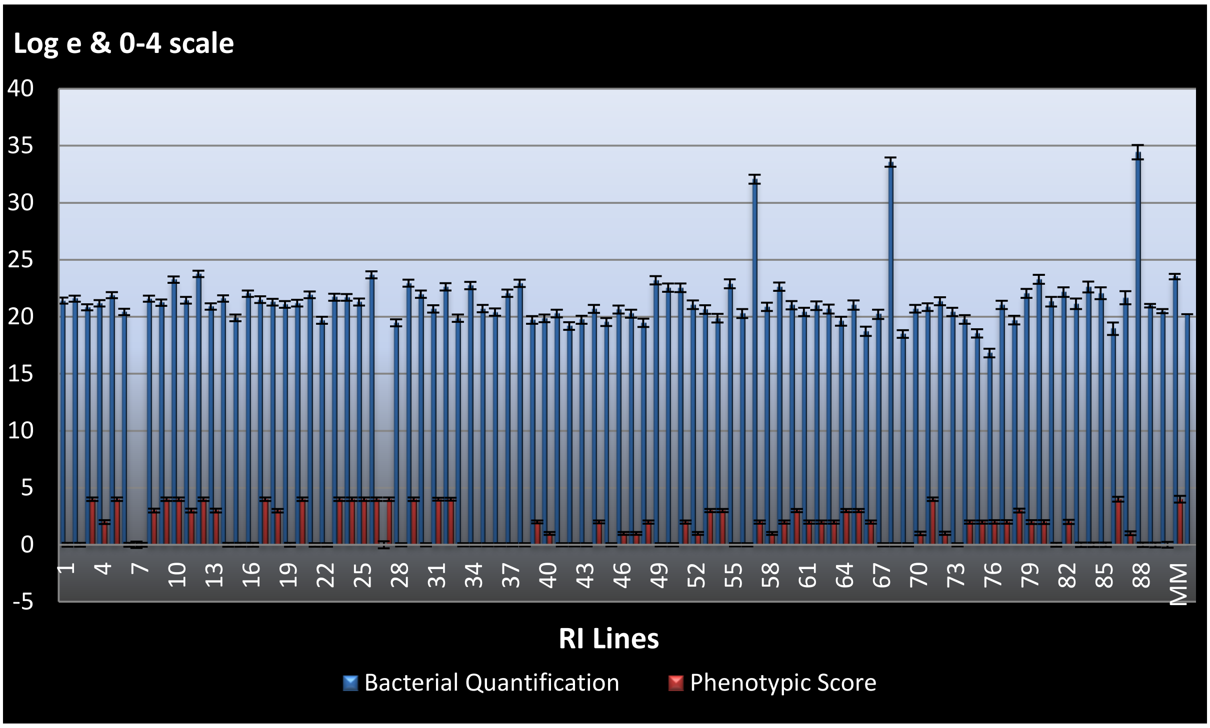

Figure 7. Plot of disease scoring against bacterial quantification of *S. pimpinellifolium* RIL population

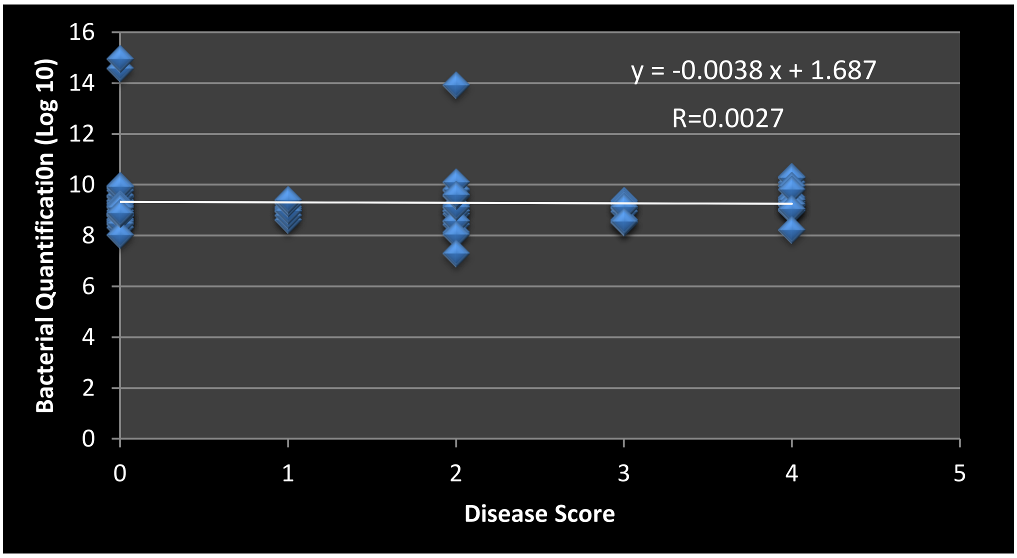

Figure 8. Co-relation between Disease score and Bacterial Quantification

Listed in the tables below are the ten most resistant/susceptible lines and the ten most tolerant/sensitive lines among the 90 lines in the *S. pimpinellifolium* RIL population. Resistant lines are lines having the lowest bacterial count regardless of the disease damages, and susceptible lines are the exact opposite. The most resistant line in the population is BU95695 which has the lowest bacterial count in the whole population at $1.99x10^7$cfu and the most susceptible line is BU95709 which have the highest bacterial count at $9.1x10^{14}$cfu. Tolerant lines are lines having low percentage of disease damages while having a high amount of bacterial titer and sensitive lines are the exact opposite. The most tolerant line is BU95709 with average disease score of 0 and average bacterial count of $9.10x10^{14}$ cfu and the most sensitive line is BU95707 which have an average phenotypic score of 4 and $1.71x10^8$ cfu average bacterial count.

Table 1. Ten most resistant and susceptible lines in *S. pimpinellifolium* RIL population

RESISTANT LINES					SUSCEPTIBLE LINES				
lines	Lower	Middle	Upper	Average	lines	Lower	Middle	Upper	Average
BU95695	5.67E+07	2.52E+06	4.95E+05	1.99E+07	BU95709	7.07E+09	2.75E+09	2.73E+15	9.1E+14
BU95680	2.97E+08	2.40E+07	2.03E+06	1.08E+08	BU95679	2.30E+09	1.35E+09	1.14E+15	3.8E+14
BU95693	1.82E+08	1.57E+08	1.40E+06	1.13E+08	BU95667	3.76E+09	4.99E+09	2.55E+14	8.5E+13
BU95677	3.33E+08	7.46E+07	6.05E+05	1.36E+08	BU95615	8.87E+09	8.25E+09	4.54E+10	2.08E+10
BU95707	1.49E+07	3.71E+08	1.28E+08	1.71E+08	BU95632	1.03E+10	1.24E+10	3.56E+10	1.95E+10
BU95650	1.45E+07	2.53E+08	3.75E+08	2.14E+08	BU95701	1.30E+10	1.89E+10	7.03E+09	1.3E+10
BU95656	3.36E+07	7.12E+07	7.48E+08	2.84E+08	BU95612	9.89E+09	8.09E+09	1.99E+10	1.26E+10
BU95635	6.51E+08	2.05E+08	2419999	2.86E+08	BU95657	1.22E+10	3.20E+09	2.06E+10	1.2E+10
BU95653	2.77E+06	1.97E+07	8.99E+08	3.07E+08	BU95636	6.19E+09	9.16E+09	1.21E+10	9.15E+09
BU95675	2.81E+09	5.43E+07	1.84E+06	3.32E+08	BU95645	5.23E+09	1.33E+10	8.85E+09	9.13E+09

Table 2. Ten most tolerant and sensitive lines in S. *pimpinellifolium* RIL population

TOLERANT LINES						SENSITIVE LINES					
Line	Ave DS	Lower	Middle	Upper	Average	Line	Ave DS	Lower	Middle	Upper	Average
BU95709	0	7.07E+09	2.75E+09	2.73E+15	9.10E+14	BU95707	4	1.49E+07	3.71E+08	1.28E+08	1.71E+08
BU95679	0	2.3E+09	1.35E+09	1.14E+15	3.80E+14	BU95638	4	1.55E+09	1.11E+09	2.47E+08	9.69E+08
BU95645	0	5.23E+09	1.33E+10	8.85E+09	9.13E+09	BU95603	4	1.44E+08	1.10E+09	2.10E+09	1.11E+09
BU95665	0	1.77E+10	7.23E+09	1.13E+09	8.69E+09	BU95682	4	2.86E+09	2.45E+08	2.64E+08	1.12E+09
BU95641	0	7.97E+09	8.83E+09	5.73E+09	7.51E+09	BU95625	4	3.01E+09	9.91E+08	7.47E+08	1.58E+09
BU95658	0	1.39E+10	2.85E+09	1.75E+09	6.17E+09	BU95611	4	9.1E+08	4.07E+09	4.09E+07	1.67E+09
BU95644	0	3.01E+09	6.77E+09	1.66E+09	3.81E+09	BU95631	4	8.95E+08	1.52E+09	2.84E+09	1.75E+09
BU95620	0	6.49E+09	4.49E+09	8.54E+06	3.66E+09	BU95621	4	4.02E+09	2.15E+09	3.30E+08	2.17E+09
BU95626	0	5.43E+09	1.17E+09	3.40E+09	3.33E+09	BU95630	4	1.87E+09	6.33E+08	5.48E+09	2.66E+09
BU95618	0	4.32E+09	1.88E+09	9.82E+08	2.39E+09	BU95628	4	3.18E+09	2.24E+09	*	2.71E+09

*** No data**

2.2 Linkage Mapping

Linkage map was constructed using JoinMap 4.1 (Van Ooijen, 2006) with 2261 SNP markers and 90 individuals. After exclusion of identical markers and individuals which have high percentage of heterozygosity 728 SNP markers and 86 individuals were group using recombination frequency criteria. There are fourteen linkage groups constructed base on the F6 *S. pimpinellifolium* RIL population using the Monte Carlo maximum likelihood (ML) mapping algorithm (Appendix 7). Tomato has twelve chromosomes, each linkage group represent one chromosome. The extra two groups are extension of chromosome 1 and 8. The total length of the linkage map is 1174. 2 cM which is comparable to other published linkage maps of cultivated tomato: 1467.8 cM and 1422.7 cM (Shirasawa et al., 2010), 1002.4 cM (Sharma et. al., 2008)

2.3 QTL Mapping for Resistance

The software MapQTL (Van Ooijen ,2006) was used to find the QLT regions in *S. pimpinellifolium* F_6 population related to resistance to Cmm. MapQTL uses several types of data files containing different information. The disease scoring data and bacterial quantification data are used to construct aquantitative datafile (Appendix 3) and for the locus genotype datafile, data from the Solsjaak arraywas used to construct a mapfile where all loci in the segregating population were positioned in the respective chromosome . Interval mapping was utilized to identify marker interval on chromosomes containing QTLfor resistance to clavibacter. Permutaion test was done to identify the threshold LOD score for QTL detection. The LOD threshold value derived from disease scoring was 3.3 while LOD threshold from bacterial quantification is 3.0. To discriminate major QTLs with minor QTLs, advance mapping was also done by using MQM-mapping. Five putatative QTL regions were derived, four QTLs found on chromosome 1, 7, 10 and 12 could be involved in leaf wilting and QTL on chromosome 7 in regions between markers TG 149 and TG 438 related to the effect of the quantity of bacteria. Results of the QTL mapping are shown below (Fig.9-13). Presence of the QTLs were checked back to the selected lines (based on zero disease score and low bacterial count). Origin of the allleles in the QTL present on the lines were identified using the genotype data (Table 3). Among the selected lines, BU95666 contain all the QTL regions coming from the *S. pimpinellifolium.*

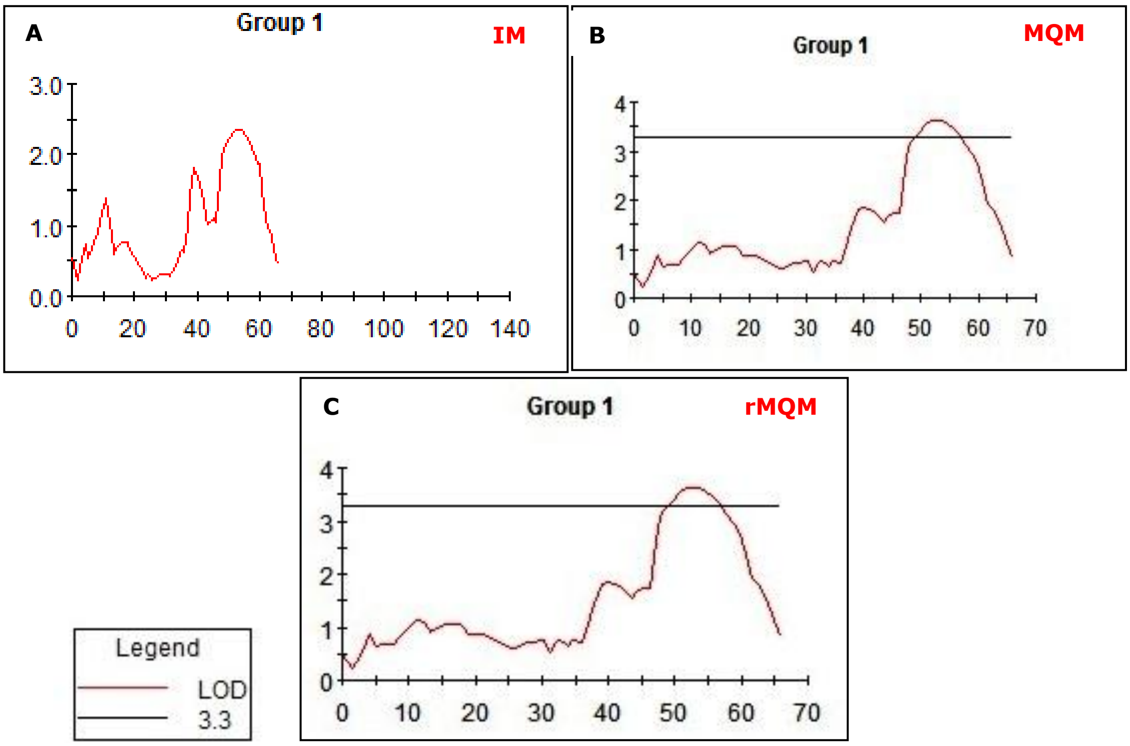

Figure 9. QTL found in chromosome one derived from wilt disease scoring with threshold value of 3.3 using: a) Interval mapping, b) Advance MQM mapping using co-factors from Chr.7,10 and 12, c) Restricted QTL mapping, does not use the co-factors.

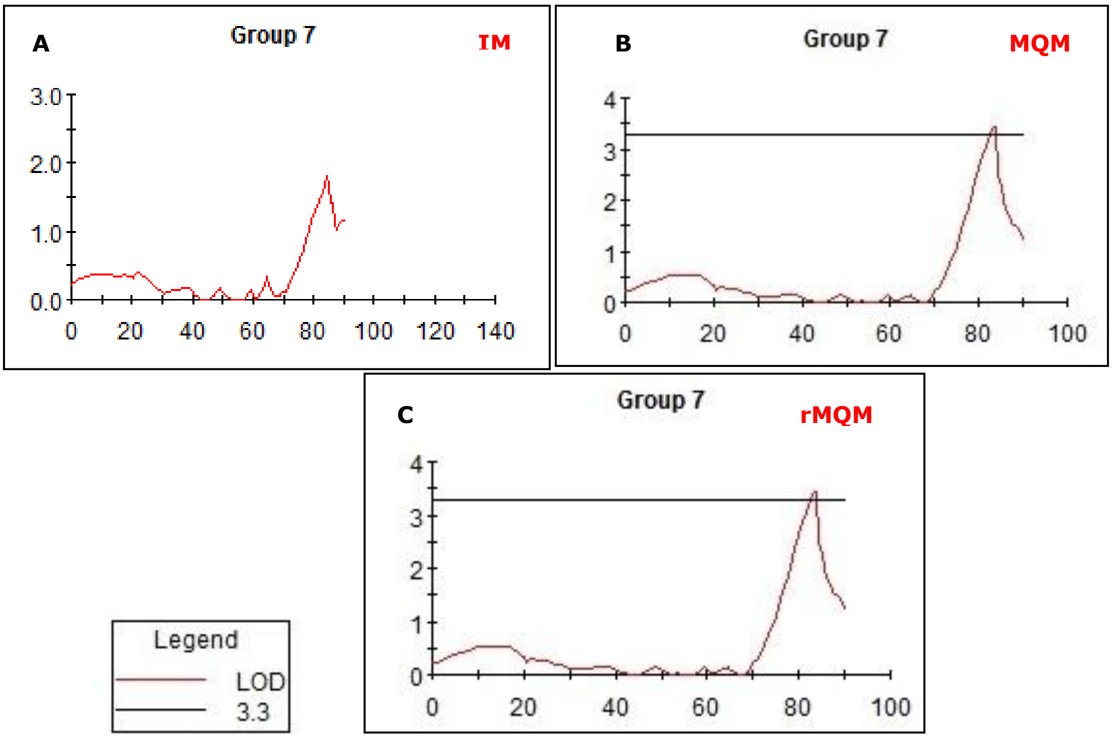

Figure 10. QTL found in chromosome one derived from wilt disease scoring with threshold value of 3.3 using: a) Interval mapping, b) Advance MQM mapping using co-factors from Chr.1,10 and 12, c) Restricted QTL mapping, does not use the co-factors.

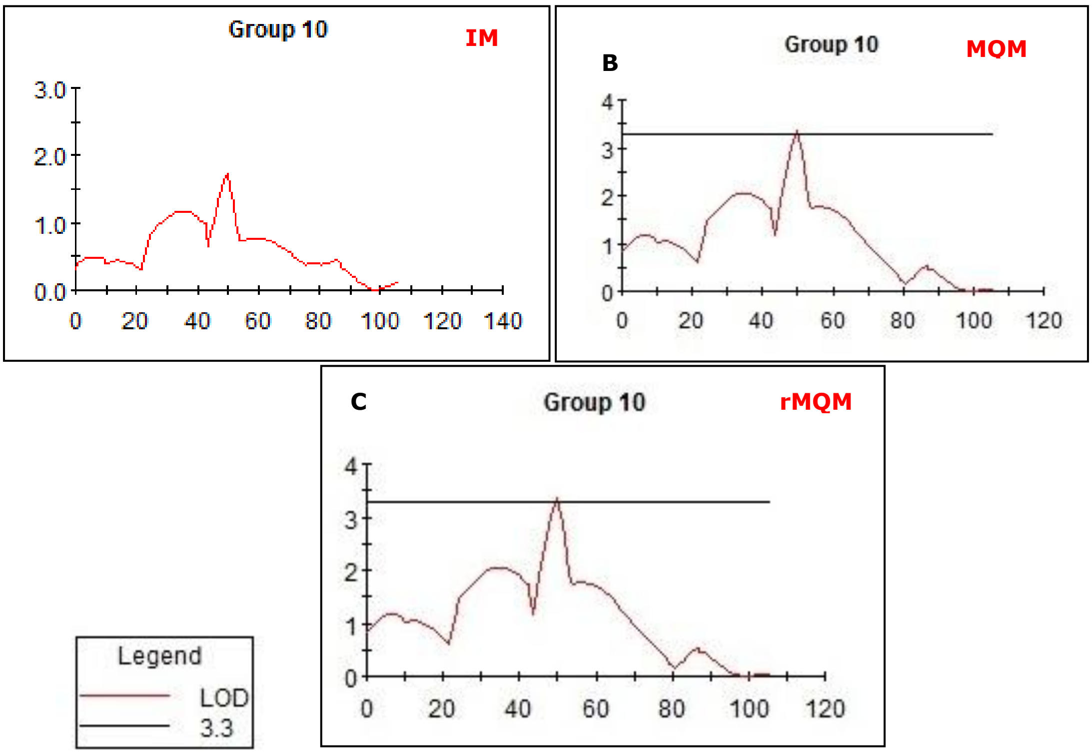

Figure 11. QTL found in chromosome one derived from wilt disease scoring with threshold value of 3.3 using: a) Interval mapping, b) Advance MQM mapping using co-factors from Chr.1,7 and 12, c) Restricted QTL mapping, does not use the co-factors.

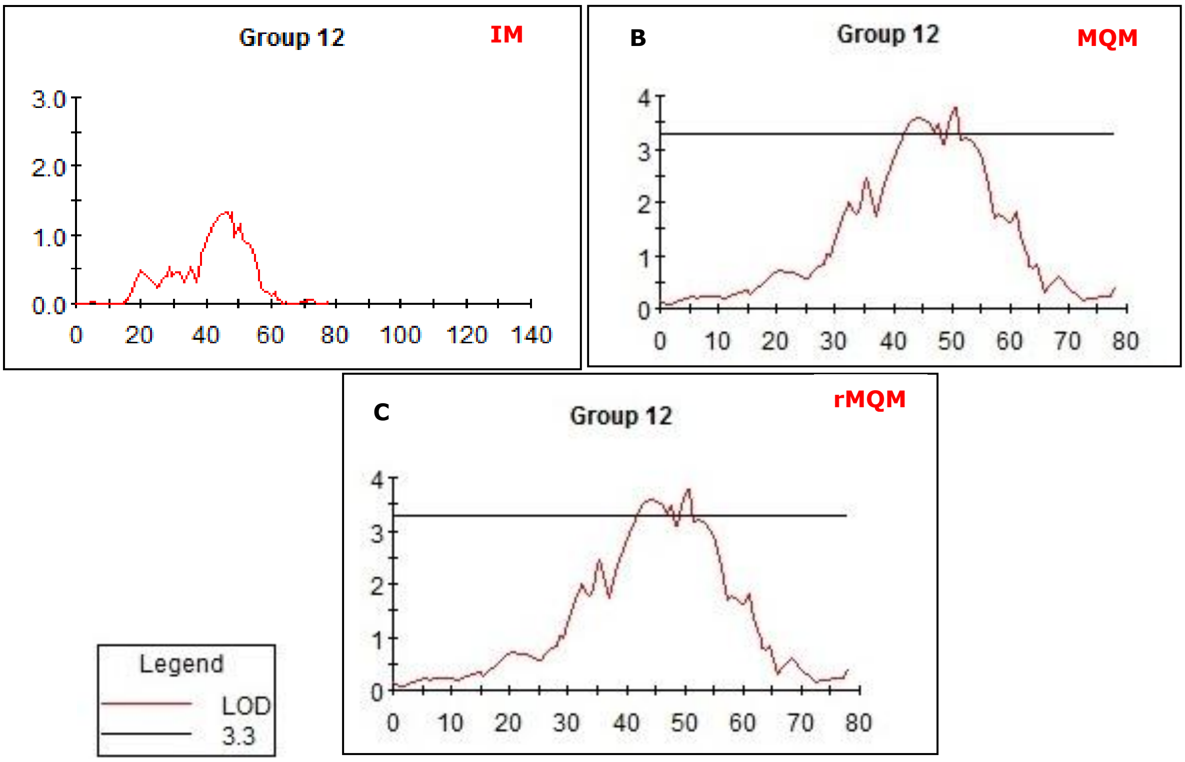

Figure 12. QTL found in chromosome one derived from wilt disease scoring with threshold value of 3.3 using: a) Interval mapping, b) Advance MQM mapping using co-factors from Chr.1,7 and 12, c) Restricted QTL mapping, does not use the co-factors.

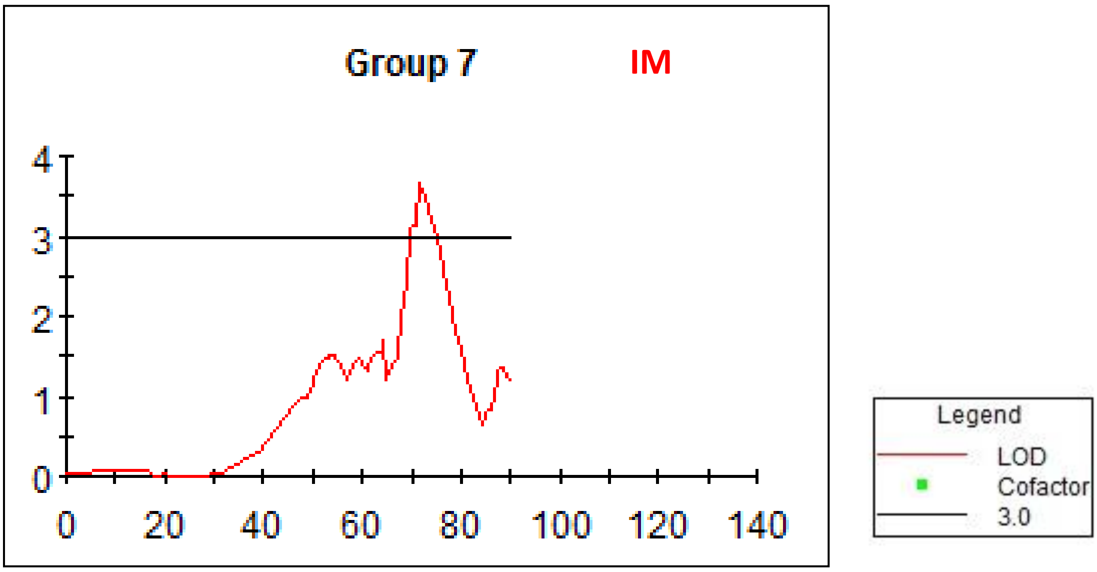

Figure 13. QTL derived from bacterial count specific in the upper portion of plant found on chromosome 7 with LOD score higher than threshold level (3.0).

Chromosome 7

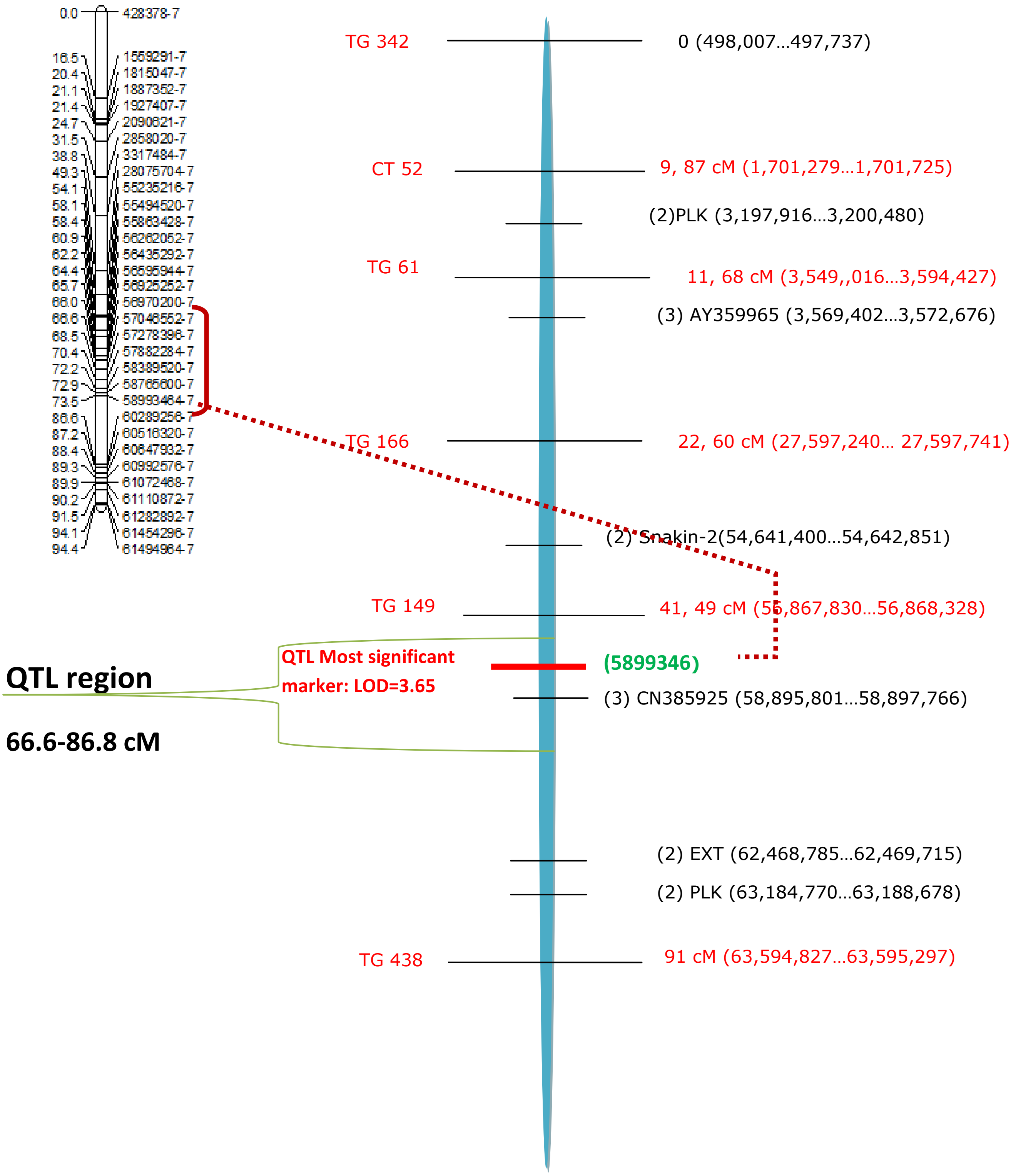

Figure 14.Genetic linkage map of chromosome 7 showing QTL region derive from bacterial quantification (The most significant marker is very close to Patatin-like gene (CN385925) which maybe one of the genes responsible to program cell death upon pathogen infection)

Table 3. Confirmation of the genotype of QTLs in the selected lines

	Genotype of QTL regions in chromosome:								
Good lines	**1**	**7**	**10**	**12**	**Bad lines**	**1**	**7**	**10**	**12**
BU95635	a	h	b	h	BU95607	b	a	a	b
BU95627	b	b	a	b	BU95612	b	a	a	a
BU95640	a	b	a	b	BU95615	b	a	a	b
BU95619	a	a	b	b	BU95628	a	b	a	b
BU95643	a	b	a	b	BU95630	b	a	a	b
BU95649	a	b	a	a	BU95632	h	a	a	a
BU95650	a	a	a	b	BU95636	a	b	a	a
BU95651	a	a	h	b	BU95639	b	a	b	a
BU95678	b	b	a	a	BU95659	h	b	b	h
BU95653	a	b	b	b	BU95667	b	a	b	b
BU95666	b	b	b	b	BU95669	a	b	b	b
BU95680	a	b	b	a	BU95701	b	a	b	a

Legend: a=Moneymaker
b=*S. pimpinellifolium*
h=Heterozygous
* Good lines were selected based on zero disease score with low bacterial count
* Bad lines were selected based on highest disease score with high bacterial count

To confirm the presence of the QTL region in the RIL population as shown in Table 3, twelve lines were selected where the disease score was zero and the quantity of the bacteria was low (good lines). For comparison twelve lines was also selected where the disease score was the highest as well as quantity of bacteria (bad lines). A genotype of **a** means the region on the chromosome was from Moneymaker, genotype **b** means the region on the chromosome was from *S. pimpinellifolium* while genotype **h** means heterozygous. Among the lines selected, only two lines shows the expected results. The line BU95666 from the good lines, have a genotype **b** in all QTL region which means this could be a good candidate for a resistant line compared to BU95632 from the bad line where almost all QTL regions have a genotype **a** showing susceptibility to the disease. With regards to the other lines selected, the QTL regions showed genotype variations.

D. DISCUSSION

S. pimpinellifolium is a wild species of tomato which carry resistance to several diseases like bacterial wilt and fusarium wilt. This species can be easily cross with the cultivated tomato because it exhibit natural introgression to *S. lycopersicum.* Results from this study show the existence of resistant and tolerant lines within the population. Resistant lines are lines where the bacterial quantity was the lowest and susceptible lines are lines where bacterial quantity was the highest. In determining the tolerant and susceptible lines the phenotypic scoring was taken into consideration. Tolerant lines are line which show very low score for symptoms but has a high bacterial quantity while sensitive lines shows very high score of symptoms but low bacterial quantity. Among the 90 lines 32 lines shows tolerance to the disease (28% of the population).

Two kinds of disease resistance exist, the qualitative resistance and the quantitative resistance. Qualitative resistance is controlled by a single dominant gene that determine if the plant is susceptible or resistant to the pathogen as shown in the gene –for –gene interaction or "guard" model (Coaker, 2003). On the other hand, the quantitative resistance can either be controlled by a multiple gene that produce small but continuous phenotypic effect or a large gene that has a large environmental influence which produce resistance with continuous variation (Coaker, 2003, Michelmore, 1995). In this study we are able to find five putative QTLs in our *S. pimpinellifolium (*F6) RIL population with the use of the genetic map and data from the wilt disease scoring and bacterial count which could be considered as quantitative resistance.

Genotype data from Solsjaak array which contains 5528 SNP markers that was analysed in Genome Studio from Illumina was used to make the linkage map. Using the JoinMap 4.1 (Van Ooijen, 2006) software genetic map was constructed by grouping 728 SNP markers on 86 individual*s of S. pimpinellifolium (*F6) RIL population using recombination frequency criteria and Monte Carlo maximum likelihood (ML) mapping algorithm. Fourteen linkage groups were generated with the two extra groups belonging to chromosome 1 and 8. The extra group and much shorter length of the genetic map could be explained as the recombination hotspot. These are regions in a genome that exhibit elevated rates of recombination or where the crossing over is concentrated, relative to a neutral expectation. This is the reason why the software recognizes it as a separate grouping thus, the formation of the extra linkage group.

The traits used in the analysis were the average disease score which could be related to wilting and the quantity of *Cmm* in three different parts of the plants (lower, middle and upper) which is related to the trait limiting bacterial growth. Co-relation between these two traits was tested through regression statistical analysis which results to an R= 0.0027 that indicate the two traits are not co-related. Thus, high titer colonization of the xylem is not responsible for the development of the wilt disease (Eichenlaub et. al., 2011). And since the response of tomato to *Cmm* infection and the mechanism associated with the development of the disease symptoms is not yet known, I can only speculate that wilting is a product of a basal defence triggered by the detection of *Cmm*, but this defence reaction is either too late or too weak to prevent disease development and has nothing to do with bacterial titer. On the other hand, disease development due to bacterial titer could be related to quorum sensing. Quorum sensing in bacteria involves production of signalling molecules called inducers that can be detected by the receptors that activates transcription of certain genes. As the population of the bacteria grows, the concentration of the inducer passes a threshold causing more inducer to be synthesized making the receptor to become fully activated.

Activation of the receptor induces the up-regulation of other specific genes, causing all of the cells to begin transcription at approximately the same time giving the bacteria “licence to kill”.

In mapping the QTL *S. pimpinellifolium* F_6 population were used consisting of 90 lines where two quantitative traits (wilting and limiting bacterial growth) were taken into consideration. These two traits are measured by using a disease scoring scale for wilting and Taqman PCR for bacterial quantification. For mapping QTL there is a high possibility of getting false-positive results because of the independent or unlinked genetic markers throughout the genome. Thus, perhaps one of the most important factor to consider for the analysis is establishing the threshold (LOD score) for determining the statistical significance and this is done by doing the 1000 x permutation test using the MapQTL 6 program. The permutation test resulted in LOD threshold values of 3.3 and 3.0 for disease score and bacterial quantification respectively across the genome. Four putative QTLs related to the wilting trait were found on chromosome 1, 7, 10 and 12 using the advance mapping (MQM) by taking into account the co-factors. These co-factors are the markers flanking the QTL which have the highest LOD score/significance. Inclusion of this markers as co-factors has considerably raised the LOD values of the other co-factors reaching the threshold level which means that the co-factors from the four chromosome has an additive effect on the significance of the regression analysis. In the analysis of the bacterial quantification trait, only one QTL was found on chromosome 7, this QTL was only specific to the data taken from the upper portion of the plant.

Positioning this QTL derived from the bacterial quantification in a linkage map of chromosome 7 was constructed based on the three studies conducted on the effect of *Cmm* infection on gene expression (Fig.11) we can hypothesize that maybe one of the gene responsible for limiting bacterial growth is the gene CN385925 (patatin like protein3) which based from recent studies in Arabidopsis plays an essential role in cell death execution in resistance to pathogen (La Camera et al., 2009,). Results from this studies show that over expression off this gene promotes necrotic symptoms mimicking the mutant *vascular-associated death 1 (vad1*) which prove that this gene is an integral component of plant cell death machinery. Another study on potato and sweet pepper which is a relative of tomato shows that patatin-associated esterase activity in potato is relevant to plant-pathogen interaction where patatin is involved in the release of arachidonicacid which is a potent elicitor of phytoalexin (antimicrobial substance) (Stermer and Bostock, 1987). Also wax synthase that co-purifies with patatin are responsible for the synthesis of hydrophobic layers on leaves and other plant organ to create physical barrier against pathogens (Vancanneyt et al., 1989).

CHAPTER 3. Fine Mapping of Clavibacter Resistance in *S. peruvianum*

A. INTRODUCTION

3.1 Breeding for clavibacter resistance

Genetic diversity is a necessary element for crop improvement. Most cultivated tomato exhibits uniformity as a result of years of breeding and selection at the expense of genetic diversity. If a trait of interest is not available in the gene pool, wild cultivars are potentially a rich source of allelic variation for genetic studies and cultivar development (Miller and Tanksley 1990; Williams and St. Clair 1993; Tanksley and McCouch 1997)

In tomato, the self-incompatible species like *S. peruvianum* carries as much genetic variation as all *S. Lycopersicum* varieties combined (Miller and Tanksley 1990). Sources of disease resistance in most commercial varieties originated from the cross with elite varieties to the wild species. These introgression efforts have increased the amount of genetic variation in commercial varieties of tomato relative to heirloom varieties (Williams and St.Clair 1993).

Several sources of partial resistance to *Cmm* have been genetically identified in *Solanum* species (Sandbrink et al., 1995, Francis et al., 2001). QTLs controlling *Cmm* resistance have been fine-mapped from *S. histurium* using interval mapping (Coaker and Francis, 2004) but still resistance mechanisms remain elusive. *Cmm* virulence targets *in planta* are also unknown and multiple hypotheses exist to explain *Cmm* virulence mechanisms (Jahr et al., 1999). A greater mechanistic understanding of resistance to *Cmm* will contribute to basic scientific knowledge and may enable more efficient disease control strategies.

Although sources of resistance to bacterial canker have been identified, the development of commercial cultivars has been slow and reliable control against the disease is lacking (Gleason et al. 1991).

The breeding strategy, limited pathogen knowledge, and limited knowledge about the genetic basis of resistance may all play a role in slow cultivar development through traditional breeding methods. Recent studies aimed at characterizing the genetic basis of resistance from *Solanum* germplasm suggest that resistance is quantitative and involves multiple loci (Poysa, 1993, Sandbrink et al., 1995, Kabelka et al., 2002). A successful breeding strategy will require genetically characterized Cmm sources of resistance that can be easily crossed to *S. lycopersicum*, and breeding populations that will enable the introgression of quantitative traits.

3.2 *Solanum peruvianum* Source

S. peruvianum is a small wild tomato from coastal deserts of Peru and Chile. The plant grows to about 50 centimetres. It has a velvety feel on its leaf and stem because of many short dense hairs. There is considerable variation in leaf morphology, plants from the north have leaf parts with deep lobes and teeth around the edges, but plants from the south have leaf parts that have almost no lobes. (www.eu-sol.net). Flowers are wide and bright yellow and the fused stamens curve sideways. Fruit is about 1-1.5 cm in diameter, round, green to greenish white with a dark green or purple stripe from the apex to base at fruit maturity. Among the tomato species it is the most genetically and morphologically diverse. But crossing *S. peruvianum* to *S. lycopersicum* is rarely successful. Attempts frequently result in embryo or flower abortion. As more lines have been evaluated, a few have produced at least one seed (www.kdcomm.net/~tomato). *S. peruvianum* has exhibited a number of agriculturally important traits like resistance to early blight, leaf mold, fusarium wilt, septoria leaf spot and nematodes.

Figure 15. *Solanum peruvianum* plant structure

3.3 QTL Fine mapping methods

The identification and mapping of loci affecting quantitative or qualitative traits can also be achieved through the use of molecular markers. These loci can then be monitored during introgression or selection programs. Molecular markers have great potential for reducing linkage drag in chromosomal regions linked to the gene being introgressed from wild species and selecting for rare recombinants that will bring genes or alleles into a favourable linkage phase. The most common populations for QTL analysis are F2 families, recombinant inbred lines, or backcross lines (BC1) populations. Although these populations provide balanced allele frequency for genetic analysis, considerable intercrossing and backcrossing is likely required to develop suitable material for varietal improvement when working with wild germplasm (Tanksley and Nelson, 1996)

B. MATERIALS AND METHODS

3.1 F2 population and Backcross population

Fifty F2 *S. peruvianum* x *S. lycopersicum* cv. Moneymaker lines were sown and grown for several weeks. Twenty lines were selected based on the presence of QTL regions among the 50 lines to be backcross to produce F2BC1 seeds. Using the pollen from the selected 20 plants, 874 backcrosses were made.

3.2 Embryo rescue

Seeds gathered (5384 seeds) from the fruits produce during the backcross were sown in 2 kinds of culture media which consisted of 3 concentration of gelrite. Media 1 consisted of MS+ GA3 (0, 35mg/l) +BAP (0, 2mg/l) +6% sucrose prepared in 0%, 2 % and 0.5% gelrite. Media 2 consisted of MS+kinetin (0, 2mg/l) +GA3 (0, 35mg/l) +6% sucrose also prepared in 0%, 2 % and 0.5% gelrite. Plantlets that germinated from this media were acclimatized and transferred to the greenhouse. Genomic DNA was extracted from this plants and genotype using Light Scanner PCR and KASpar technology. Selected hybrid plants were crossed with each other and backcross to *S. lycopersicum* cv. Moneymaker to produce the F3BC1.

3.3 Genotyping using KaSPar Technology

The KBiosciences Competitive Allele Specific PCR SNP genotyping system (KASPar) is a novel homogeneous fluorescent genotyping system developed at KBiosciences. KASPar offers the simplest, most cost effective and flexible way to determine SNP genotypes. This method relies on the discrimination power of novel form of competitive allele specific PCR to determine the alleles at a specific locus within genomic DNA for SNP typing. The system is comprised of two components, the Assay Mix (three unlabelled primers; this is the SNP specific component of the system) and the Reaction Mix (all other components required including the universal fluorescent reporting system).The genotyping process is as follows: (1) Design of assay by primer picker software (2) Array samples in microtitre plate (3) Make assay mix from designed oligos (4) Male reaction mix from kit components and assay mix (5) Dispense reaction mix over samples (6) Seal plate (7) Thermocycle (8)Read plate in fluorescent plate reader (9) Plot and score data. Genotypes can be determined according to the sample clusters and by adding a passive reference can lead to more accurate data calling (www.KBioscience.com).

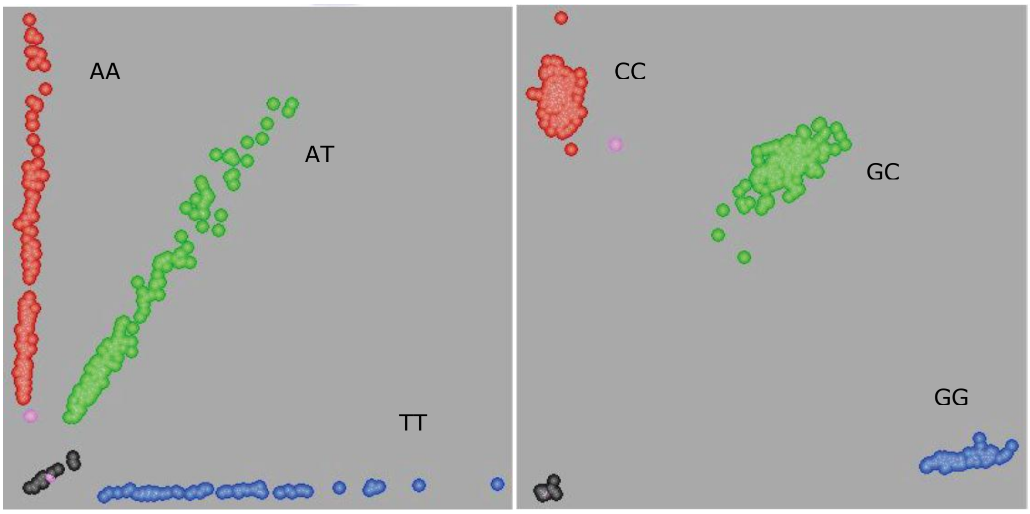

Figure 16.Grouping genotype data using KBioscience Kluster caller software. (www.KBioscience.com)

3.4 Genotyping using Light Scan PCR

The LightScanner instrument is an air thermocycler capable of automatically analysing samples for the presence of targeted nucleic acid. It combines real-time, rapid thermocycling technology and high resolution melting (HRM) and is capable of 20 minute PCR protocols. The Light Scanner instrument is configured for three-color detection for real-time and single-colour detection for high resolution melting (HRM).The LightScanner is the first system to use High Resolution Melting, or HRM analysis for mutation scanning and genotyping. Real-Time PCR results can be generated using a variety of labelled probe chemistry including SYBR® Green, hydrolysis probes, HydProbes®, and others. Optimal high resolution melting (HRM) results are generated using LCGreen® Plus dye.

DNA from *S. lycopersicum* cv. Moneymaker and *S. peruvianum* (LA2157) was used as reference for genotyping. The PCR were performed in a total volume of 10 µl with each reaction containing 30-40ng of DNA template, 1µl of PCR buffer solution (10x), 1µl dNTP (10µM), 1µl Taq polymerase, 1µl LC green, 1 µl forward primer (5µM), 1µl reverse primer (1µM) and 1µl Probe (5µM) (see list below).

Before amplification 15µl of oil was added to each well. Amplification condition were 94˚C for 30 secs followed by 55 cycles 30secs 94˚C, MT for 30 secs, 72˚C for 30 secs and after cycling reaction 30 secs at 94˚C. Afterwards the PCR plate will be loaded to the Light Scanner system from the Idaho Technology Inc.

Table 4. LightScan probes for the QTL regions in chromosome 5, 7 and 9 with the corresponding melting temperature in the parent species

Probes	PCR MT	MM MT	Peruv MT
Ch5			
TG432	62	66	71
CT167	62	74	70
TG363	61	71	66
TG23	62	60	69
TG524	61	69	61
Ch7			
CT 52	61	70	65
CT52	61	66	70
TG61	62	61	69
TG166	61	70	66
TG149	61	66	70
Ch9			
C2_At35..	61	67	72
TG254	61	70	66
TG18	62	65	69
TG223	61	75	70

3.5 Primer and Probe Design

Primers were designed according to the position of the markers in the QTL region. Fragments of 500-600bp were taken from the region between the markers using 'Editseq' software and blasted to the Sol genome database to see if it is a unique region. PRIMER 3 software was used to design the forward and reverse primers based on the chosen 500bp region. One primer pair was designed on every 1Mbp in the between markers. Primer the shows single band after PCR analysis using dna template from *S. lycopersicum* cv. Solentos and *S. peruvianum* (LA2157) were sequence and analysed for presence SNPs using 'Seqman' software. A unique fragment of 150bp containing the SNP was selected to be sent to the company for probe design.

C. RESULT

3.1 Genotyping of *Peruvianum* RIL

Twenty lines were selected based on the existence of the QTL regions from the 50 (F_2) *S. peruvianum* x *S. lycopersicum* cv. Moneymaker lines that was sown. Using the pollen from the selected 20 plants, 874 backcrosses were made. Seeds gathered (5384 seeds) from the fruits produced during the backcross were sown in 2 kinds of culture media which consisted of 3 concentration of gelrite. Twelve plants were generated from the embryo rescue and grown under greenhouse condition. Light scan PCR was used to genotype the 12(F2BC1) plants. In LightScan PCR, results are shown as peaks according to the melting point of the probes. Sample of the results are shown below for the probe TG 61 were the *S. peruvianum* (LA2157) parent is the yellow line having a melting peak at 61˚C and *S. lycopersicum* cv. Moneymaker is the green line having a melting peak at 68˚C. The hybrid plants are the represented by the red lines having 2 melting peaks approximately at same temperature of the both parents (Fig.14). Genotyping of the 12 (F2BC1) plants are shown in Table 4 and 5 were 6 plants are hybrids. Genotyping using KASpar technology was also tested on the 9 plants out of the 12 (F2BC1) plants and results are shown on Table 6. In this genotyping, results are shown as the nucleotide present at the certain position in the chromosome using SNP markers and presence of a SNP indicates a hybrid plant. The result of the 2 genotyping method both confirm that plant 2, 4, 7, and 9 are hybrids. This result was used as the basis for crossing the plants between each other and backcrossing to *S. lycopersicum* cv. Moneymaker.

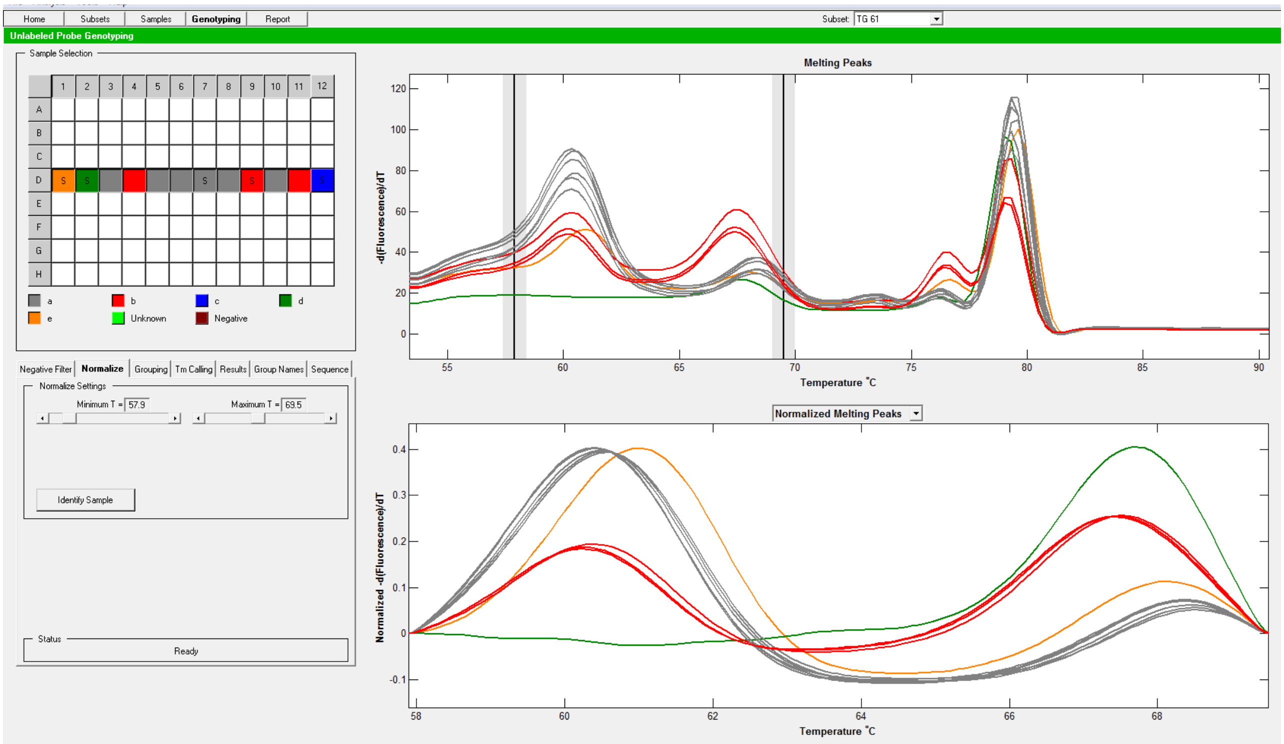

Figure17. Melting point peaks and grouping using LightScan PCR (Yellow line=Solentos parent, Green line=Peruvianum parent, Grey line=samples with alleles from Solentos, Red line= heterozygous)

Table 5. Genotyping of the 12 F2BC1 plants derived from embryo rescue using LightScan PCR at 61˚C annealing temperature.

Plant	CT 52-71	TG 166	TG 149	C2....53580	TG 254	TG 223	TG 363	TG 524
1	a	a	a	a	a	x	x	a
2	h	a	a	h	a	h	a	a
3	a	a	a	a	h	x	x	a
4	a	x	b	h	a	x	h	h
5	a	a	a	a	a	x	x	a
6	a	a	a	x	a	x	x	a
7	h	a	a	h	a	h	x	a
8	a	a	a	a	a	x	x	a
9	h	h	b	a	a	x	x	h
12	x	h	b	x	b	x	x	h
13	x	a	h	x	b	x	x	h
15	x	h	x	x	x	x	x	h

Legend:

probe for chr5
probe for chr9
probe for chr7
hybrid

a= *S. lycopersicum* cv. Solentos
b= *S. peruvianum* (LA 2157)
h=heterozygous
x=no data

Table 6.Genotyping of the 12 F2BC1plants derived from embryo rescue using LightScan PCR at 62˚C annealing temperature.

Plant	TG 61	TG 18	TG 254	TG 432	TG167	TG 23
1	a	a	a	a	a	a
2	h	b	b	h	a	a
3	a	a	a	a	a	a
4	a	b	b	h	a	h
5	a	a	a	a	a	a
6	a	a	a	a	a	a
7	h	b	b	h	a	a
8	a	a	a	a	a	a
9	h	a	a	a	a	h
12	x	b	x	x	a	a
13	h	h	x	h	a	h
15	h	h	x	H	a	h

Legend:

probe for chr5
probe for chr9
probe for chr7
hybrid

a= *S. lycopersicum* cv. Solentos
b= *S. peruvianum* (LA 2157)
h=heterozygous
x=no data

Table 7. Genotyping of F2BC1 *S. peruvianum* lines using KASpar technology

Chromosome	5	5	5	5	5	6	6	6	6	6	7	7	7	8	8	8	9	9	9	9	
Position	1860303	6461255	20527748	43744992	59785880	1625550	13419477	34766972	40596644	42863540	2090621	55235216	58659004	807232	56347200	60044540	6758111	21284240	55638296	64308952	
DNA \ Assay	seq-md53	seq-md75	seq-md13	seq-md18	seq-md45	seq-md87	seq-md77	seq-md35	seq-md55	seq-md41	seq-md45	seq-md60	seq-md77	seq-md80	seq-md85	seq-md10	seq-md89	seq-md15	seq-md27	seq-md435	
G1.1554	G:G	T:T	T:T	C:C	?	T:T	T:T	C:C	G:G	C:C	G:G	G:G	C:C	C:C	T:T	A:A	C:C	G:G	C:C	G:G	
LA1401	G:G	T:T	T:T	C:C	?	T:T	T:T	C:C	G:G	C:C	G:G	G:G	C:C	C:C	T:T	A:A	C:C	G:G	C:C	G:G	
Lyc4	G:G	T:T	Bad	C:C	?	?	T:T	C:C	G:G	C:C	G:G	G:G	C:C	C:C	T:T	?	C:C	?	T:T	G:G	
MB	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	
MM	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	DUPE	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	
Yusuf1	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	no hybrid
Yusuf2	G:T	C:C	C:C	T:T	?	C:C	C:T	C:T	G:A	T:T	A:A	A:A	C:A	?	C:C	G:G	C:T	T:T	T:T	T:T	hybrid
Yusuf3	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	?	T:T	T:T	no hybrid
Yusuf4	G:T	C:T	C:C	T:T	?	C:C	C:T	C:T	G:A	T:T	A:A	G:G	C:A	?	?	G:G	C:T	T:T	T:T	T:T	hybrid
Yusuf5	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	no hybrid
Yusuf6	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	no hybrid
Yusuf7	T:T	C:C	C:C	T:T	?	C:C	C:T	C:T	G:A	T:T	A:A	A:A	C:A	?	C:C	G:G	C:T	T:T	T:T	T:T	hybrid
Yusuf8	T:T	C:C	C:C	T:T	?	C:C	C:C	T:T	A:A	T:T	A:A	A:A	A:A	T:T	C:C	G:G	T:T	T:T	T:T	T:T	no hybrid
Yusuf9	T:T	C:C	C:C	T:T	?	C:C	C:T	T:T	A:A	T:T	A:A	G:G	C:A	T:T	?	G:G	C:T	T:T	T:T	T:T	hybrid

3.2 Primer and marker design for fine mapping

Three QTLs for resistance derived from the cross between *S. lycopersicum* cv. Solentos and *S. peruvianum (*LA 2157) were mapped to chromosome 5,7 and 9 (Van Heusden et. al., 1999). In this study we design markers on the region of the QTL on chromosome 7 between the RLFP markers TG 61 and TG166. There are approximately 24 Mbp gap between this two markers or distance of 11cM. One primer pair was designed for every 1Mbp gap between the two markers. Twenty four primer pairs with 23-25 bp in size were designed to amplify a product size of 500-600bp (Appendix 5). DNA template from *S. lycopersicum* cv. Solentos and *S. peruvianum (*LA 2157-BU2002023) were used to test the primers by doing PCR. From the 24 primer pairs, 11 primer pairs (5, 7, 8,11,12,13, 14, 17, 19, 20 & 22) showing single polymorphism on the two parents were selected as shown on the gel picture below. PCR products of the selected primers were cleaned and sequence. Afterwards, sequence data were analyzed using "Seqman software" from Lasergene to identify SNPs. A sequence of 150bp with the SNP were selected and blasted to the solgenome to check for the uniqueness of the region and if it has the right location in chromosome 7. Five SNP marker were designed binding to the unique region in chromosome 7 between the RFLP markers TG61 and Tg166 (Table 7).

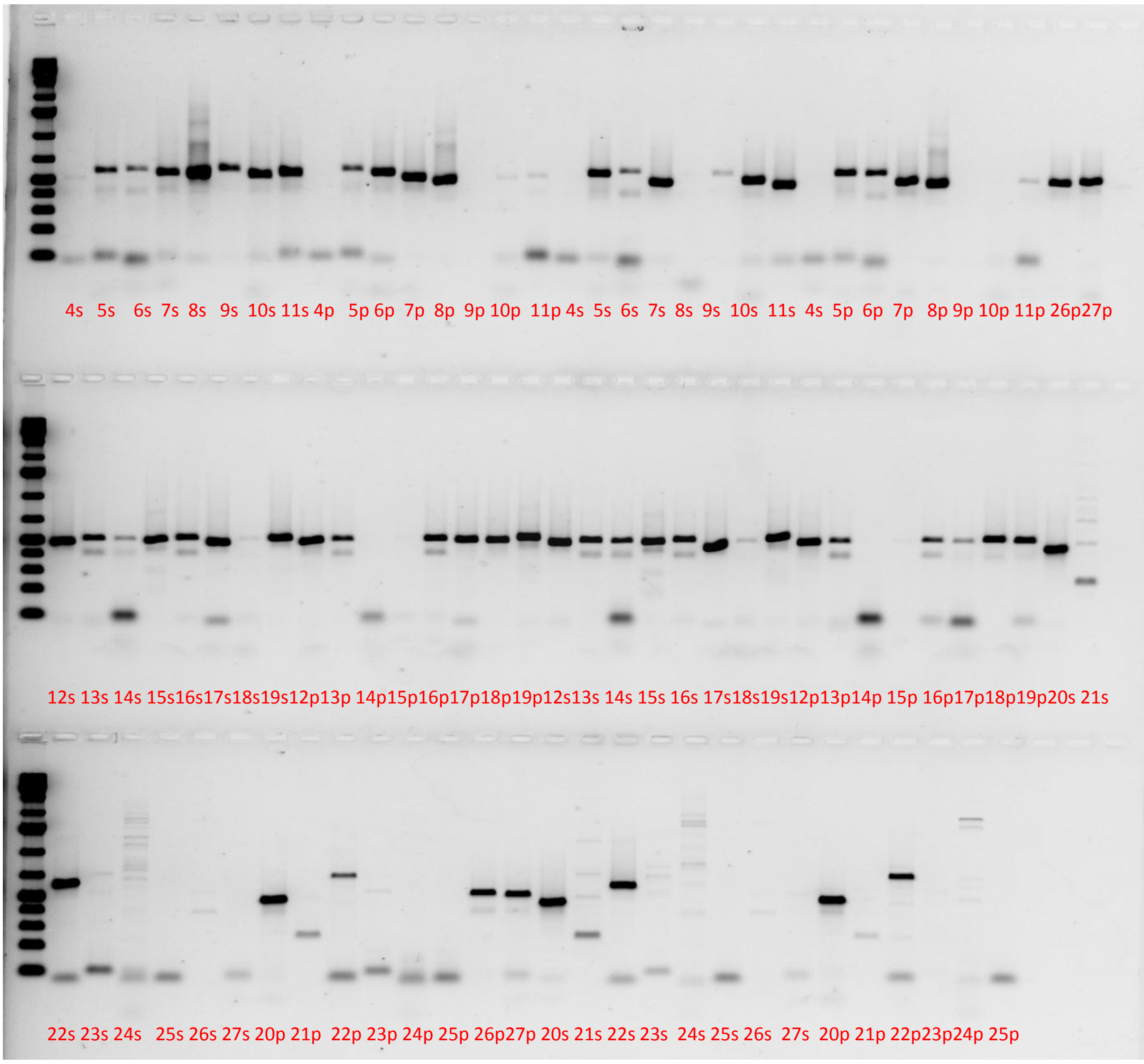

Figure18. PCR gel picture of the 24 primers using DNA template from *S. lycopersicum* cv. Solentos and *S. peruvianum (*LA 2157-BU2002023)

Table 8. SNP markers in chromosome 7 between TG61 and TG166

Position	**Sequence**
Chr7-7008127	CTTGCATGTTTACCATACTTAGTGCATTCAATGTGCTAATTCAATTTATTGTTCTATTCCTATAAGTGTAGGTGTCA**[A/T]**GTGAGGAGTCTCTTAGAAGC[G/A]AAGCTTGAGAATAACATTCTCTCAAGAAGACTTGTTATGTCCTCATATGAT
Chr7-13014753	GAGAAATTTGAAACTAATGAGTTTAATACAAAGAACTCAATACTAATTTGAAACTAACTATGTATGAAAATAA**[G/T]**CCTCTAACTTACTAGA[A/T]ATACT[T/G]GGACAAGCCCCCAATTATGTCTAGCAAGTAATGAAACAAAATGACTAAATGTA
Chr7-14011748	AAGGATCAATTGGTAGATTCTATGACTTGTCTAATGTTTGAGTACATTGATCTTGAAAAA**[T/C]**CCAATTCT[T/G]AGTTAAAAGAAACAACAAAGCTATGCTAGAAAACACTCATCAACTAGATTCTTGTAACCTGAACTGAAA[G/A]TTTAAAATGAAGTGTGATT
Chr7-19096799	TTTTATCTACATTGCTGACAACAAAAAGCGTTGTGATTGTAACTTTTTCGAGATGCAT[T/G]CTTG[C/T]TTATTCAAGATGG**[A/C]**AATGTTGTTGTTCTGCAGAATTTGGATACCCTTAAGGCACCATTTATCAACTGGTTGGCAGATCAAATGCAC
Chr7-20367764	TAATGGTAACTTATTGACATCAATGGTGAAAAATAATTTTAn[C/T]GGGGGTTCAAATGATGGAAGATGGTTATATTTGCTACTGAT**[A/T]**AAAACTTTGACAAAAATTGAACCATGATATTTAAGAACTTGAAGGGCCTTGTCACGGCAATAGA[C/T]AATGA

D. DISCUSSION

Sources of resistance to *C. michiganensis* subsp. *michiganensis* (*Cmm*) have been identified in wild relative species like *S. pimpinellifolium* and *S. peruvianum* but the best characterized source of resistance is found in *S. peruvianum* LA2157 (van Heusden et al., 1995, Kabelka et al., 2002). Three QTLs for resistance derived from the cross between *S. lycopersicum* cv. Solentos and *S. peruvianum (*LA 2157) were mapped to chromosome 5, 7 and 9; combination of any two of the QTLs conferred a high level of resistance to *Cmm* (Van Heusden et. al., 1999). In this study, plants derived from the backcross of *S. lycopersicum* cv. Moneymaker (F2BC1) were genotype to identify which QTL was introgress in each line and to confirm and assess level of resistance to *Cmm* in the *S. lycopersicum* genetic background. Among the three QTL mapped in *S. peruvianum* (LA2157), it is believe that the QTL in chromosome 7 may contain the most important resistance gene between the RFLP marker TG61 and TG166 (Van Heusden et. al., 1999), and fine mapping of this region may give us insight to what genes are involve in the resistance. Developing more markers in this region and determining the exact order of these markers are important in finding the resistance gene. In the study of Van Heusden et al, 1999, he tried to design markers in between the QTLs but failed. This study is a follow-up of the previous study using SNP markers. Single nucleotide polymorphism (SNPs) are the most abundant source of variation in the genome, therefore they can be a valuable basis for developing molecular markers among closely related lines (Shirasawa et al., 2010). From this study 5 SNP markers have been designed to narrow down the gap between the TG61 and TG166 flanking the resistance QTL. This markers will be sent to a Van Haeringen Laboratories o be further evaluated and shortened to 50bp to be used in future analysis of the resistance gene in chromosome 7.

To give us more insights to the possible genes that may be involve with the resistance to *Cmm*, genetic linkage map of RFLP markers on chromosome 5,7 and 9 containing the resistance QTLs were constructed. List of genes which are up regulated and down regulated during Clavibacter infection were taken from the results of three published studies: **[1]** Gene Expression Analysis during Interaction of Tomato and Related Wild Species with *Clavibacter michiganensis subsp. michiganensis* (Lara-Ávila et al., 2011) **[2]** Tomato Transcriptional Changes in Response to *Clavibacter michiganensis subsp. michiganensis* Reveal a Role for Ethylene in Disease Development (Balaji et al., 2008) **[3]** Silencing of Host Basal Defence Response-Related Gene Expression Increases Susceptibility of *Nicotiana benthamiana* to *Clavibacter michiganensis* subsp. *michiganensis (Balaji et al., 2011).* The genes were plotted against the constructed linkage map to see which genes are might present in our

QTL region that may be involve with resistance (Fig. 16, 17 &18). With this information we can make assumption on the possible mechanism of reaction of host plant to *Cmm*.

Chromosome 5

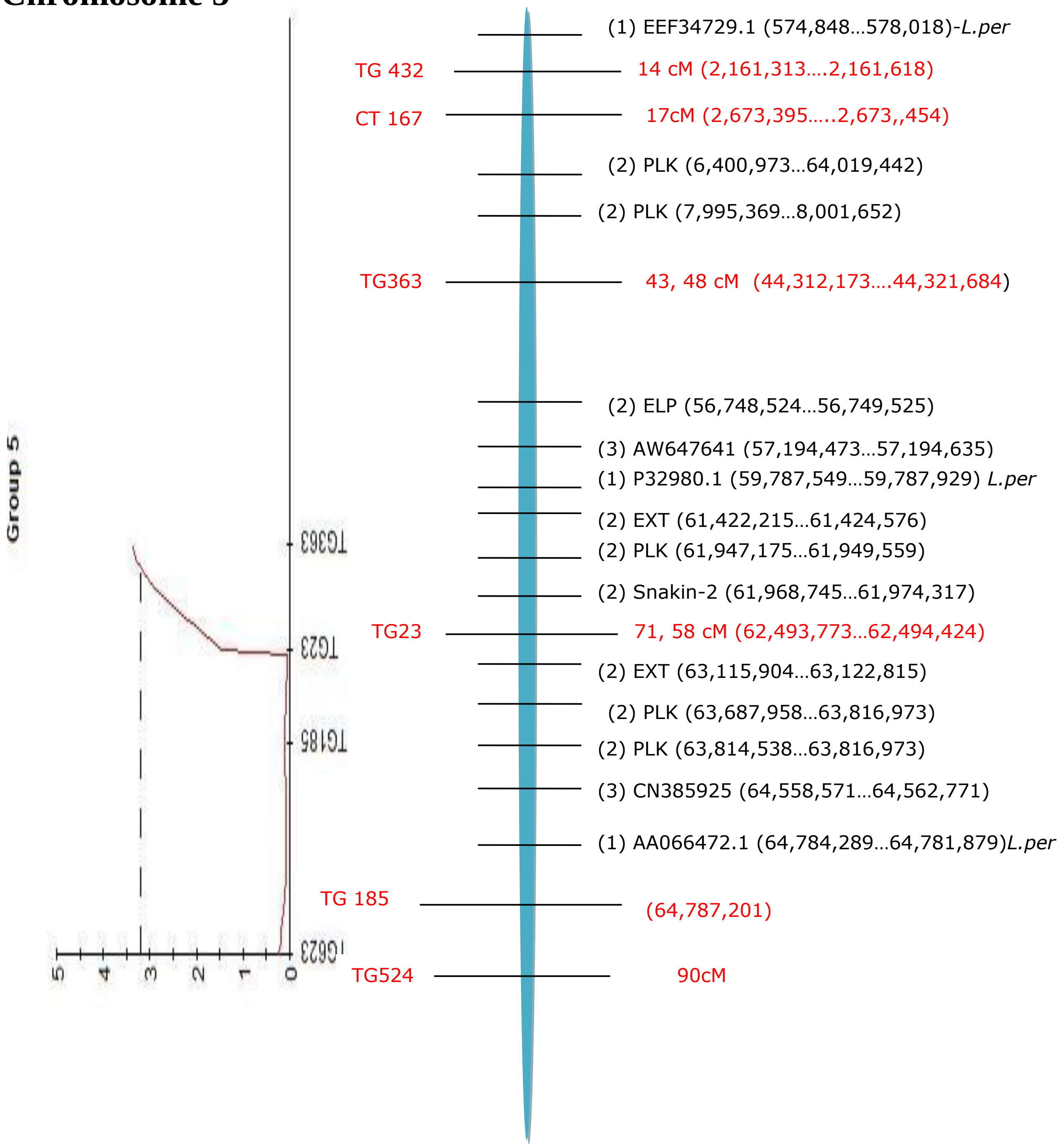

Figure 19. Genetic linkage map of RFLP markers on chromosome 5 showing up regulated and down regulated genes from the 3 published papers mentioned above plotted against the resistance QTL region

Chromosome 7

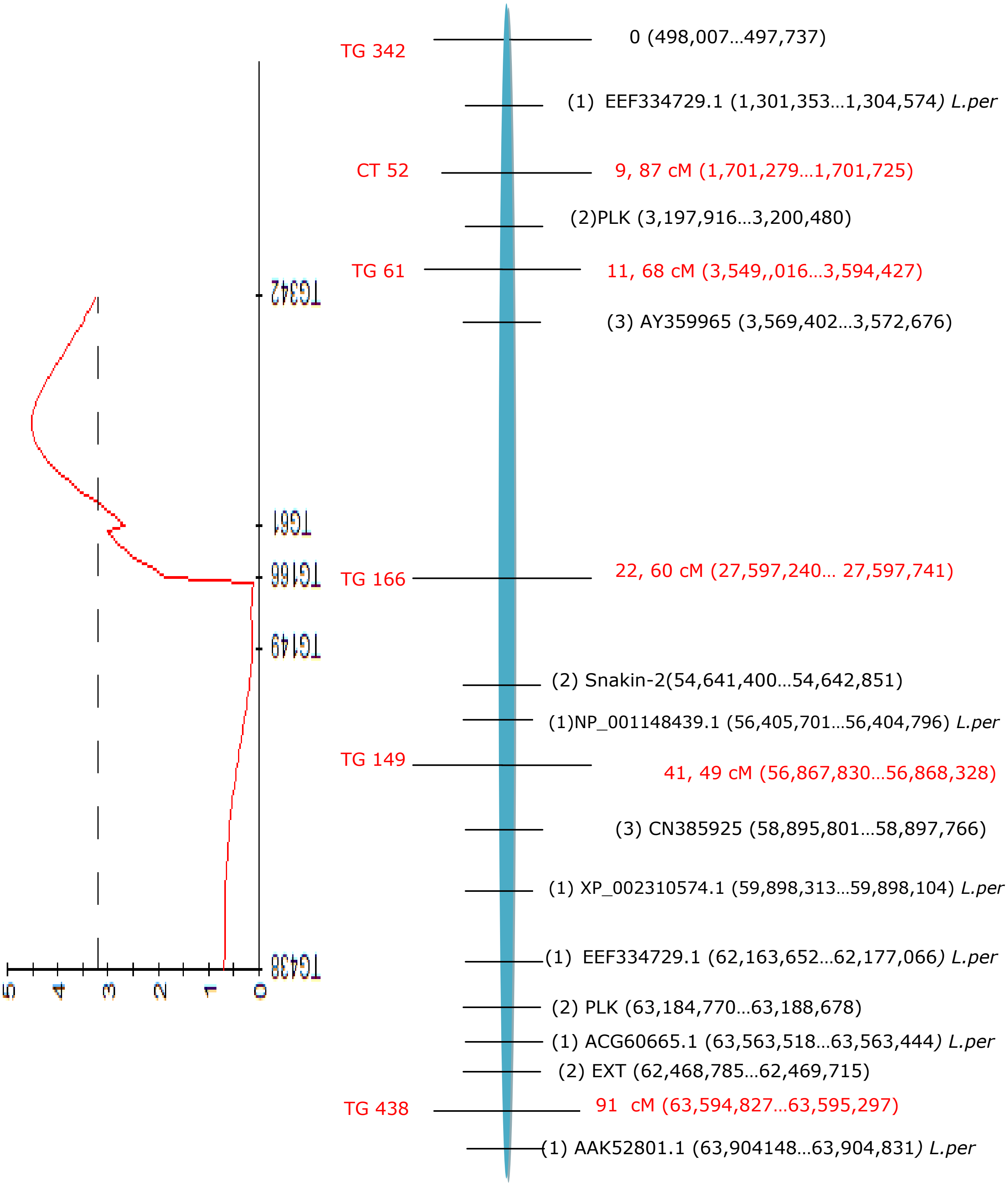

Figure 20.Genetic linkage map of RFLP markers on chromosome 7 showing up regulated and down regulated genes from the 3 published papers mentioned above plotted against the resistance QTL region

Chromosome 9

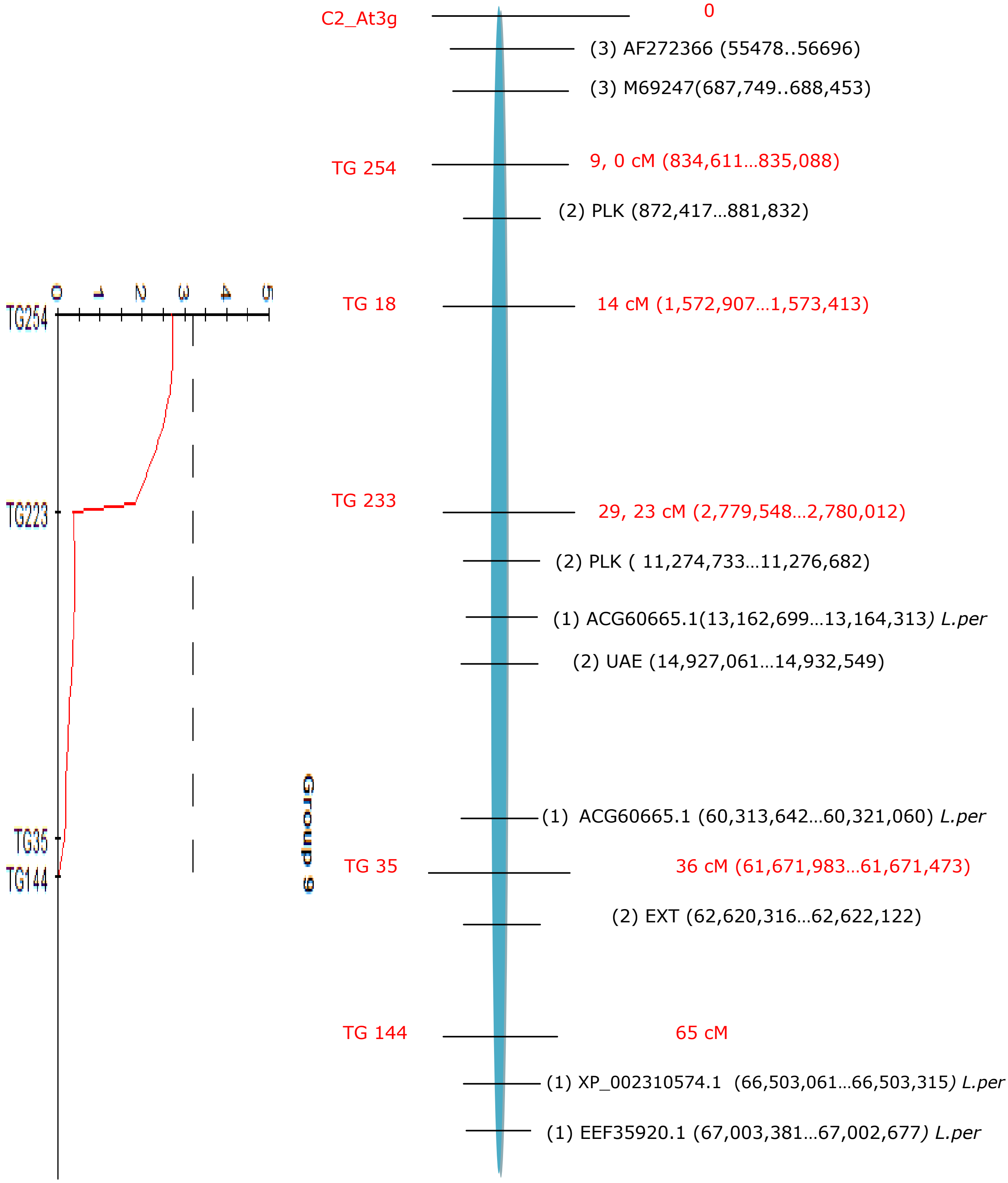

Figure 21. Genetic linkage map of RFLP markers on chromosome 9 showing up regulated and down regulated genes from the 3 published papers mentioned above plotted against the resistance QTL region

FINAL CONCLUSION

A linkage map was constructed through JoinMap 4.1 with 2261 SNP markers and 90 individuals F6 *S. pimpinellifolium* RIL population. Total length of the map generated was 1174. 2 cM with 14 linkage groups. The extra two groups are extension of chromosome 1 and 8 because of recombination hotspots.

Through the wilt disease score data and bacterial quantification using Taqman PCR, a total of 5 putative QTLs were mapped using MapQTL software. Four QTL which are derived from the wilt disease scoring data were found on chromosome 1, 7, 10 and 12 using the advance mapping (MQM). One QTL which was generated from the bacterial quantification was found on chromosome 7 on the same region as found for the previous trait. This QTL was only specific to the data taken from the upper portion of the plant. Furthermore, by using bioinformatics and published articles, a putative candidate gene was observed that maybe involve in the resistance. Through statistical analysis, the two traits are found to be not correlated (R=0.0027). Data on the wilt disease scoring might be related to basal defence triggered by the detection of *Cmm* while *the bacterial quantification might be related to quorum sensing which is a mechanism used by bacteria through production of signalling molecules to gain the silence to kill or produce infection.*

In the fine mapping of QTL for resistance to clavibacter in *S. peruvianum* located at chromosome 5, 7 and 9, five SNP markers were design on chromosome 7 to narrow down the gap between the TG61 and TG166 flanking the resistance QTL. This marker will be sent to Van Haeringen Laboratories to be further evaluated and shortened to 50bp to be used for bead array analysis of the peruvianum lines. For QTL in chromosome 5 and 9, no further analysis was done because the SNP markers were already designed between regions flanking the QTLs before the start of my study.

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Appendix 1. Phenotypic Scoring of *S. pimpinellifolium* RIL population

Plant no.	Accession No.	Season	plt1	plt2	plt3	plt4	Average	Comments
MoneyMaker		Summer 2	4	4	4	4	4	
Pimpinellifolium		Summer 2	0	0	0	0	0	
601	BU95601	winter	0	0	0	0	0	
		summer 2	0	0	0	0	0	
602	BU95602							deleted
603	BU95603	winter	4	4	4	4	4	
		summer 2	2	1	0	0	0.75	segregating
605	BU95605	winter	2	2	2	3	2.25	
		summer 2	2	2	3	3	2.5	
607	BU95607	winter	4	0	4	4	3	escape
608	BU95608	winter	0	0	0	0	0	
		summer 2	0	0	0	0	0	
609	BU95609							no data
610	BU95610	winter	2	4	3	2	2.75	
611	BU95611	winter	4	3	4	3	3.5	
612	BU95612	winter	4	4	4	4	4	
614	BU95614	winter	1	2	0	0	0.75	segregating
		summer 2	3	3	3	3	3	
615	BU95615		4	4	4	4	4	
617	BU95617	winter	4	3	3	1	2.75	segregating
		summer 2	4	0	0	4	2	segregating
618	BU95618	winter	0	0	0	1	0.25	
		summer 2	0	0	0	1	0.25	
619	BU95619	winter	0	0	4	0	1	segregating
		summer 2	0	0	0	0	0	
620	BU95620	winter	0	0	0	0	0	
621	BU95621		4	4	4	4	4	
622	BU95622	winter	2	3	3	4	3	segregating
		summer 2	0	0	x	x	0	
624	BU95624	winter	0	0	0	0	0	
		summer 2	0	0	1	0	0.25	
625	BU95625	winter	4	4	4	4	4	
626	BU95626	winter	0	0	0	0	0	
		summer 2	0	0	0	0	0	
627	BU95627	winter	0	0	0	0	0	
628	BU95628	winter	4	4	4	4	4	
630	BU95630	winter	4	4	4	4	4	
		summer 2	4	4	4	4	4	
631	BU95631	winter	4	4	4	4	4	
632	BU95632	winter	4	2	4	4	3.5	escape
633	BU95633	winter	3	4	3	4	3.5	segregating
635	BU95635	winter	0	0	3	2	1.25	segregating
		summer 2	0	0	0	0	0	

636	BU95636	winter	2	4	4	3	3.25	escape
637	BU95637	winter	2	4	0	4	2.5	segregating
638	BU95638	winter	3	4	4	4	3.75	escape
639	BU95639	winter	3	4	4	4	3.75	escape
640	BU95640	summer 1	0	0	0	0	0	
641	BU95641							deleted
642	BU95642	summer 1	0	0	0	0	0	
643	BU95643	summer 1	0	0	0	0	0	
644	BU95644	summer 1	0	0	0	0	0	
645	BU95645	summer 1	0	0	0	0	0	
646	BU95646	summer 1	2	2	2	2	2	
648	BU95648	summer 1	1	1	1	1	1	
649	BU95649	summer 1	0	0	0	0	0	
650	BU95650	summer 1	0	0	0	0	0	
651	BU95651	summer 1	0	0	0	0	0	
652	BU95652	summer 1	2	2	2	2	2	
653	BU95653	summer 1	0	0	0	0	0	
654	BU95654	summer 1	1	1	1	1	1	
655	BU95655	summer 1	1	1	1	1	1	
656	BU95656	summer 1	2	2	2	2	2	
657	BU95657	summer 1	1	1	1	1	1	
658	BU95658	summer 1	0	0	0	0	0	
659	BU95659							deleted
660	BU95660	summer 1	1	1	1	1	1	
663	BU95663	summer 1	3	3	3	3	3	
664	BU95664	summer 1	3	3	3	3	3	
665	BU95665	summer 1	0	0	0	0	0	
666	BU95666	summer 1	0	0	0	0	0	
667	BU95667	summer 1	2	2	2	2	2	
668	BU95668	summer 1	1	1	1	1	1	
669	BU95669	summer 1	2	2	2	2	2	
670	BU95670	summer 1	3	3	3	3	3	
671	BU95671	summer 1	2	2	2	2	2	
673	BU95673	summer 1	3	3	3	3	3	
674	BU95674	summer 1	2	2	2	2	2	
675	BU95675	summer 1	2	2	2	2	2	
676	BU95676	summer 1	3	3	3	3	3	
677	BU95677	summer 1	2	2	2	2	2	
678	BU95678	summer 1	0	0	0	0	0	
679	BU95679	summer 1	0	0	0	0	0	
680	BU95680	summer 1	0	0	0	0	0	
681	BU95681							deleted
682	BU95682							deleted
684	BU95684	summer 1	1	1	1	1	1	
691	BU95691	summer 1	0	1	0	0	0.25	
692	BU95692	summer 1	2	2	2	2	2	

693	BU95693	summer 1	0	0	0	0	0	
694	BU95694							deleted
695	BU95695	summer 1	2	2	2	2	2	
696	BU95696	summer 1	0	0	4	2	1.5	segregating
697	BU95697	summer 1	3	3	3	3	3	
700	BU95700	summer 1	2	2	2	2	2	
701	BU95701	summer 1	2	2	2	2	2	
702	BU95702	summer 1	0	0	0	0	0	
703	BU95703	summer 1	2	2	2	2	2	
704	BU95704	summer 1	0	0	0	0	0	
705	BU95705	summer 1	0	2	0	4	1.5	segregating
706	BU95706	summer 1	0	0	0	0	0	
707	BU95707	summer 1	4	4	4	4	4	
708	BU95708	summer 2	2	2	0	0	1	segregating
709	BU95709	summer 2	0	0	0	0	0	
710	BU95710	summer 2	0	0	0	0	0	
711	BU95711	summer 2	2	0	0	0	0.5	

Appendix 3. Quantitative Data File used for QTL mapping

ntrt = 8
nind=84
miss = *
DS ;Disease score
BL ;Bacteria quantification (lower)
BM ;Bacteria quantification (middle)
BU ;Bacteria quantification (upper)
BAW ;Bacteria quantification Average
BHIG ;Bacteria quantification Highest
BLOW ;Bacteria quantification Lowest
ID ;number

DS	BL	BM	BU	BAW	BHIG	BLOW	ID
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4	21.14198	21.1995	22.60426	21.89575	22.60426	21.14198	607
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*	*	*	*	*	*	*	609
3	21.51626	21.76707	21.46043	21.59037	21.76707	21.46043	610
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4	23.01479	22.81389	23.71399	23.25908	23.71399	22.81389	612
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4	22.90594	22.83348	24.53878	23.76014	24.53878	22.83348	615
3	21.66327	20.41674	19.39901	20.89498	21.66327	19.39901	617
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4	22.11455	21.48762	19.61515	21.49611	22.11455	19.61515	621
3	21.77409	21.48873	19.32084	21.28402	21.77409	19.32084	622
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4	*	*	*	*	*	*	633
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	0	16.48966	19.3489	19.74244	19.18227	19.74244	16.48966	650
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	2	20.85429	20.57244	20.64314	20.69726	20.85429	20.57244	652
	0	14.83436	16.79613	20.61679	19.54287	20.61679	14.83436	653
	1	16.77042	21.27139	20.74307	20.64321	21.27139	16.77042	654
	1	19.81693	20.41538	20.47224	20.27542	20.47224	19.81693	655
	2	17.33004	18.081	20.43291	19.46542	20.43291	17.33004	656
*		23.2247	21.88642	23.74856	23.20817	23.74856	23.2247	657
	0	23.35515	21.77058	21.28288	22.54242	23.35515	21.28288	658
	1	22.06352	19.59625	15.32844	21.04741	22.06352	15.32844	660
	3	21.69404	18.05251	15.3986	20.6231	21.69404	15.3986	663
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	2	21.14198	20.23224	18.25463	20.42067	21.14198	18.25463	671
	3	22.12198	16.16689	16.41077	21.02925	22.12198	16.16689	673
	2	21.49799	21.21185	18.18875	20.98031	21.49799	18.18875	674
	2	21.75645	17.81003	14.42528	20.67762	21.75645	14.42528	675
	3	19.9893	20.05773	14.54306	19.62072	20.05773	14.54306	676
	2	19.62365	18.12765	13.31298	18.72867	19.62365	13.31298	677
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	0	21.55617	21.02337	34.6698	33.5712	34.6698	21.02337	679
	0	19.50924	16.99356	14.52355	18.49464	19.50924	14.52355	680
	1	22.21841	20.77206	18.79224	21.35719	22.21841	18.79224	684
	0	21.45563	18.61953	16.18575	20.41887	21.45563	16.18575	691
	2	20.84548	16.63092	14.82347	19.76393	20.84548	14.82347	692
	2	19.01952	18.87176	14.15198	18.54702	19.01952	14.15198	693
	2	17.85328	14.73977	13.11231	16.80648	17.85328	13.11231	695
	2	21.76354	20.97791	17.44251	21.04959	21.76354	17.44251	696
	3	20.62013	19.04126	14.97553	19.71192	20.62013	14.97553	697
	2	23.14807	16.20427	16.14765	22.05133	23.14807	16.14765	700
	2	23.28822	23.66243	22.67345	23.28642	23.66243	22.67345	701
	0	22.2687	20.39198	16.83594	21.31632	22.2687	16.83594	702
	2	22.5889	22.18883	21.3221	22.15914	22.5889	21.3221	703

	0	22.00974	20.66352	14.7516	21.14297	22.00974	14.7516	704
*		22.36226	16.66622	23.41789	22.61875	23.41789	16.66622	705
*		22.38149	22.55425	14.53335	22.06631	22.55425	14.53335	706
	4	16.51687	19.73171	18.66754	18.95893	19.73171	16.51687	707
	1	19.15784	22.09445	21.99863	21.66963	22.09445	19.15784	708
	0	22.67913	21.73487	35.54308	34.44447	35.54308	21.73487	709
	0	21.87384	14.80876	20.39198	20.98067	21.87384	14.80876	710
	0	21.15505	20.55366	11.01863	20.49346	21.15505	11.01863	711

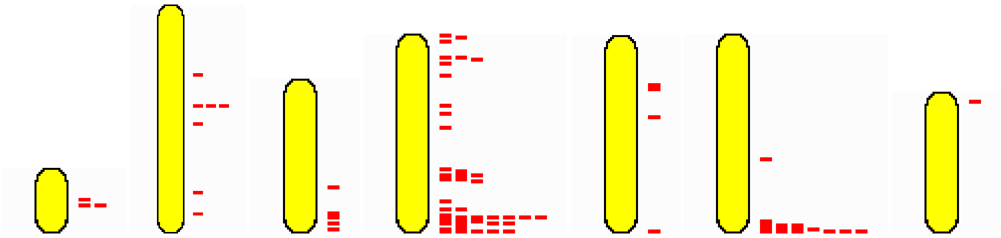
Appendix 4.List and position of up regulated genes during Clavibacter infection from recently published papers

Gene Expression Analysis during Interaction of Tomato and Related Wild Species with *Clavibacter michiganensis subsp. michiganensis*

José Pablo Lara-Ávila &Maria Isabel Isordia-Jasso &
Rosalba Castillo-Collazo & June Simpson &
Ángel Gabriel Alpuche-Solís

Transport- EEF35920.1

228 regions match (Per 2157 & 2172)



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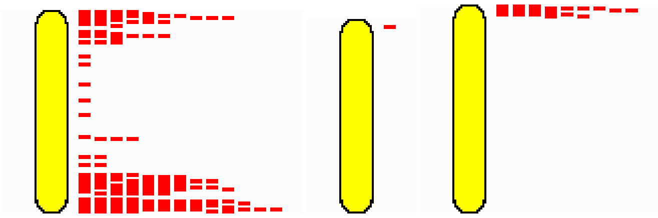


7 8 9

Defence-AAD33072.1- Chr02: 39,317, 754...39,319,043

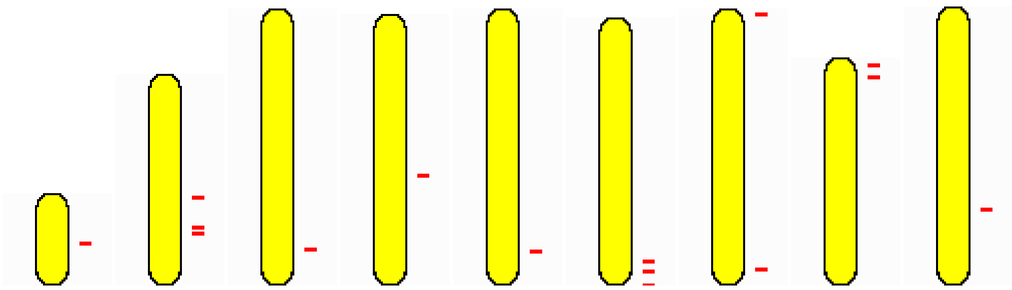
Signalling- NM_128064.2- Chr08:810489...810415

Energy-NP_001148439.1- Chr03:538199...59545706

Tomato Transcriptional Changes in Response to *Clavibacter michiganensis subsp. michiganensis* Reveal a Role for Ethylene in Disease Development

Vasude van Balaji, Maya Mayrose, Ofra Sherf, Jasmine Jacob-Hirsch, Rudolf Eichenlaub, Naim Iraki, Shulamit Manulis-Sasson, Gideon Rechavi, Isaac Barash, and Guido Sessa*

ELP- 15 regions match



0 2 3 4 5 8 10 11 12

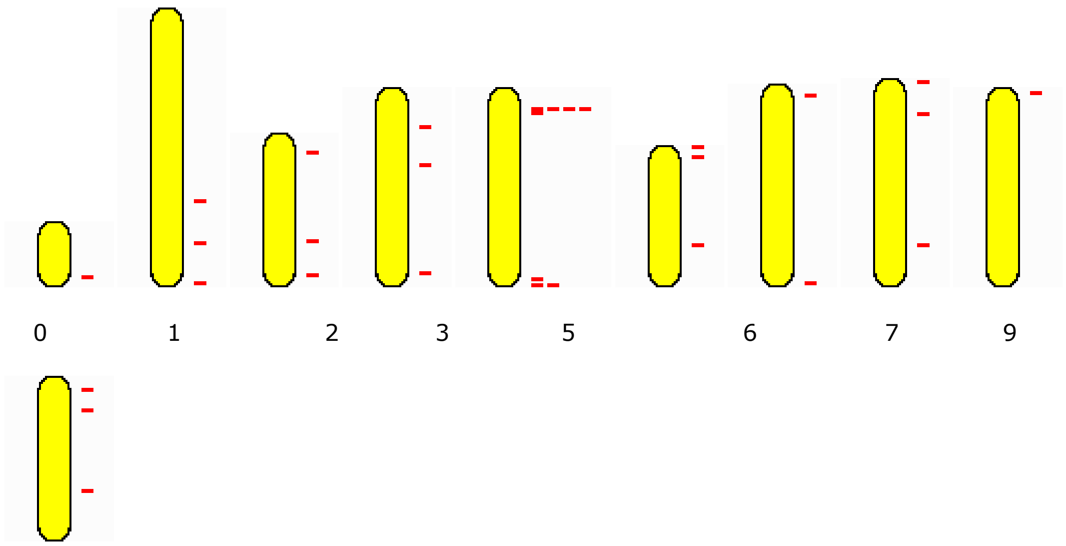
Snakin-2

genomic annotations: 1.24 kbp from **SL2.40ch11:8,383,387..8,384,626**

UAE- 5.489 kbp from **SL2.40ch09:14,927,061..14,932,549**

DES -SL2.40ch01:87953424..87955263

PLK-31 regions match



Silencing of Host Basal Defence Response-Related Gene Expression Increases Susceptibility of *Nicotiana benthamiana* to *Clavibacter michiganensis* subsp. *michiganensis*

Vasude van Balaji, Guido Sessa, and Christine D. Smart

HMGR-SL2.40ch02:40531424..40528275

U89256-SL2.4ch02:36,900,742..36,901,507

AJ133600-Chr01:1,017,302..1,018,625

AY093595- Chr08: 61020894..61021790

M69248-Chr00:18119571..18121696

AY093595-Chr08:61,020,894..61,021,790

Appendix 5. Primers used for the designing markers between TG 61 and TG166

4F-agcccaactataaaaaggcataact
4R- tttttaacaccgctagaaaaagttg
5F- attgacgagaaagctaaactcattg
5R- tacgtgttgtaaatcccaattttct
6F- cttcttgagaaatgggctacagata
6R- attaggtctgtttttgaccctcaat
7F-tgagcggtctcttcatatcttattt
7R- ggaatgaaagcttttcacaagaata
8F-acaaaatggtcacatcataatcctt
8R-tagagtagatccggacaaaattgaag
9F-cctacatatttctaacccgaagacc
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12F- tttggttactgaaatgaggtgtttt
12R-ttgaatggtcacatcataatccata
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14F- tttatggagtgggtgttagttccta
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25R-tactcctcgtgataaactctccaac
26F-tctgaaagttgggtaaagaaagaga
26R-tattaatgtccactagcacgtctca
27F-agacaatagtggagggaaagaaaat
27R-aatccttaacattattggagcaaca

Appendix 6. Statistical analysis of co-relation of disease scoring and bacterial quantification

SUMMARY OUTPUT

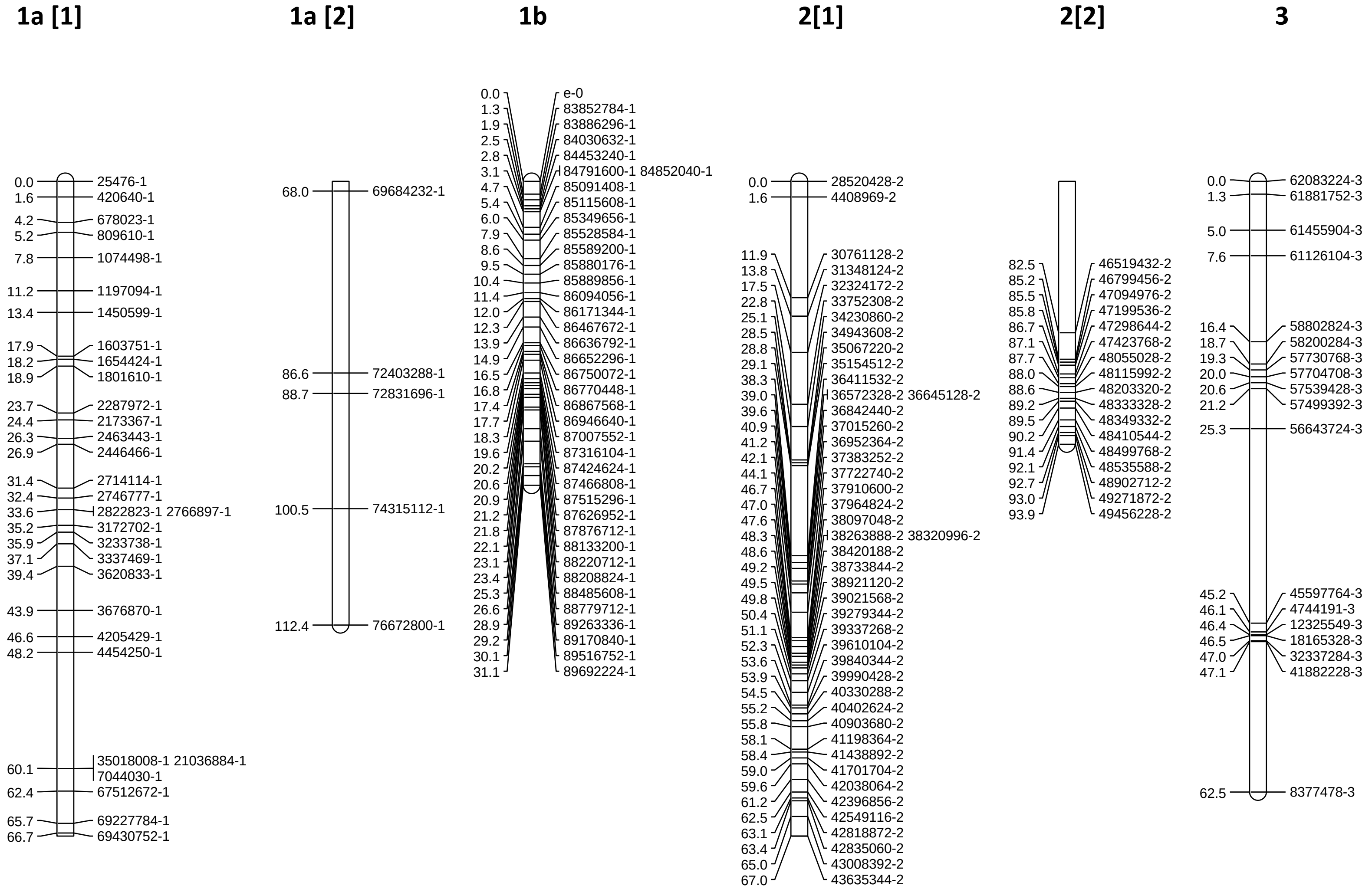
Regression Statistics	
Multiple R	0.00277858
R Square	7.72051E-06
Adjusted R Square	-0.01189695
Standard Error	1.57182515
Observations	86

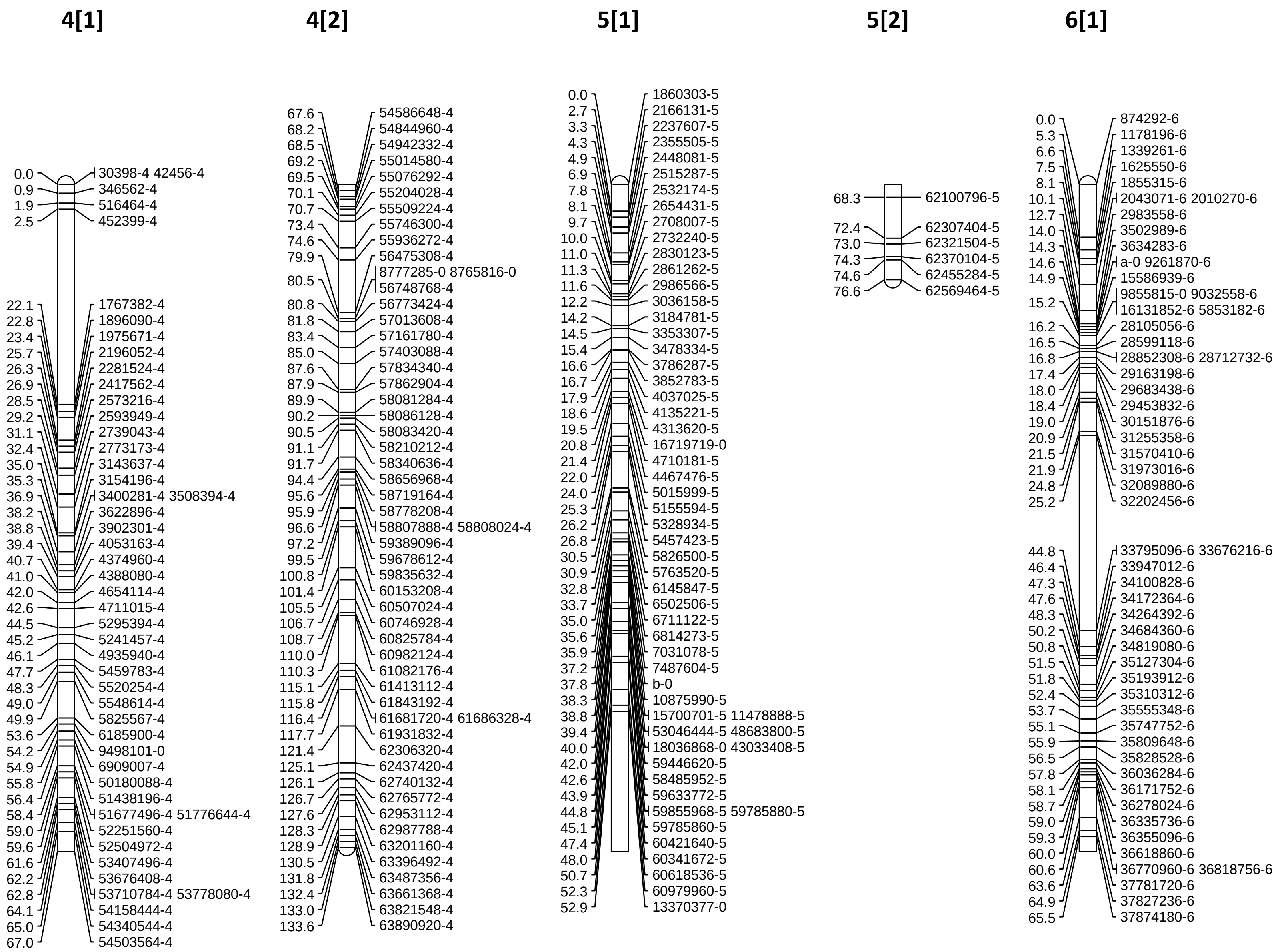
ANOVA

	df	*SS*	*MS*	*F*	*Significance F*
Regression	1	0.001602	0.001602	0.000649	0.979743505
Residual	84	207.5333	2.470634		
Total	85	207.5349			

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*	*Lower 95.0%*	*Upper 95.0%*
Intercept	1.686696224	1.405574	1.200005	0.233511	-1.108441738	4.481834	-1.10844	4.481834
X Variable 1	-0.00382093	0.150039	-0.02547	0.979744	-0.302190256	0.294548	-0.30219	0.294548

Appendix 7. Tomato Genetic Map (*S. pimpenellifolium* x Moneymaker)

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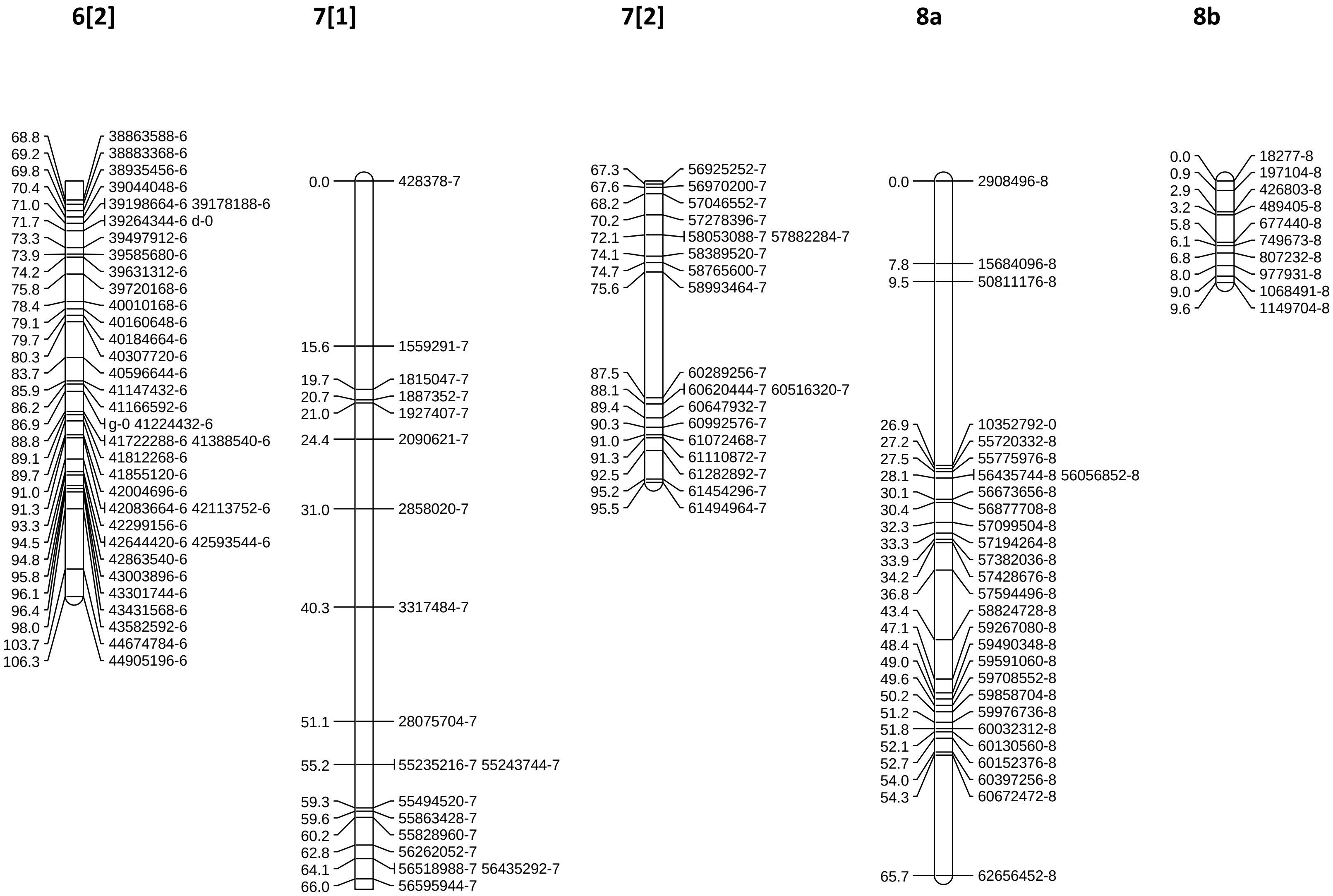
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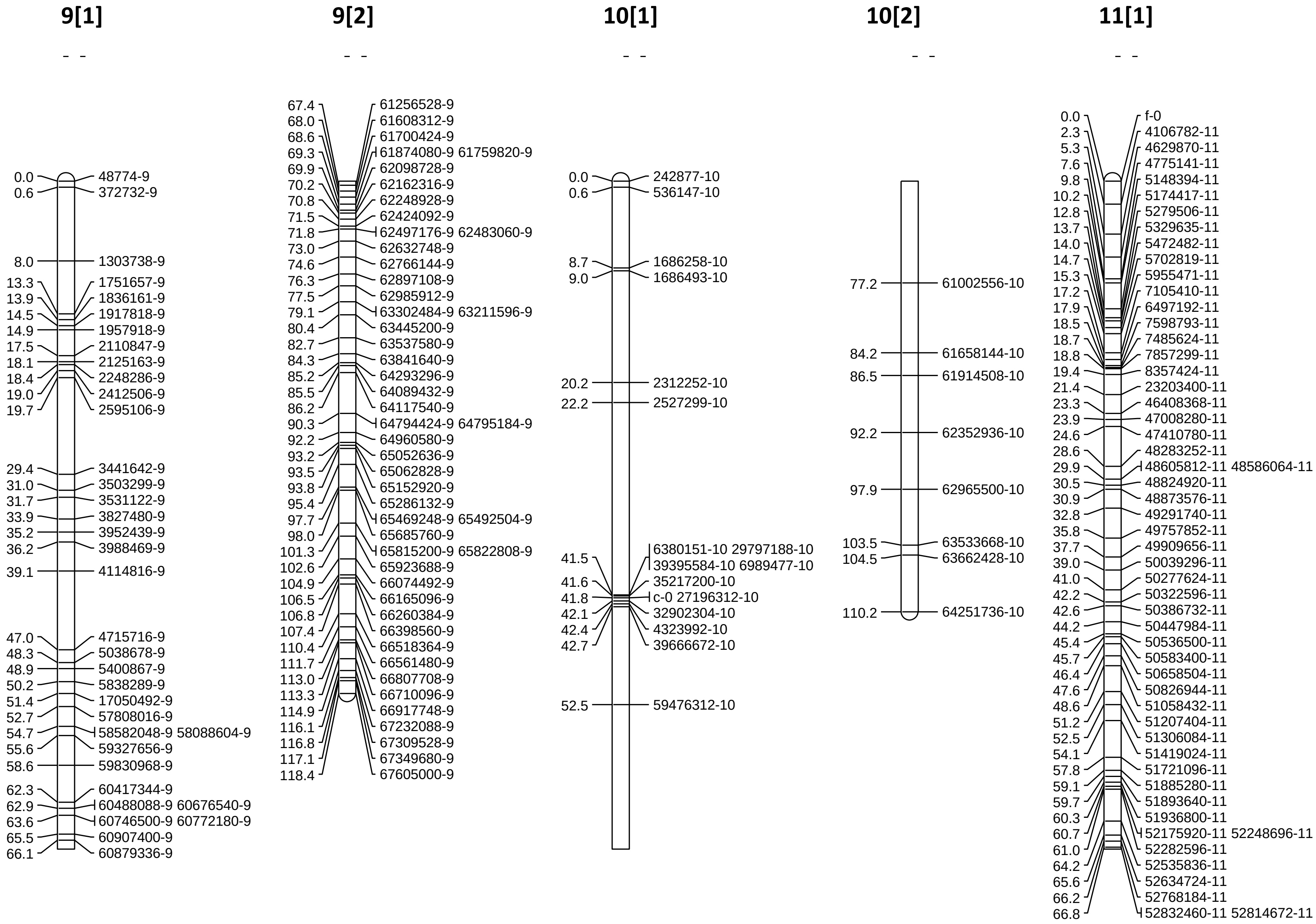
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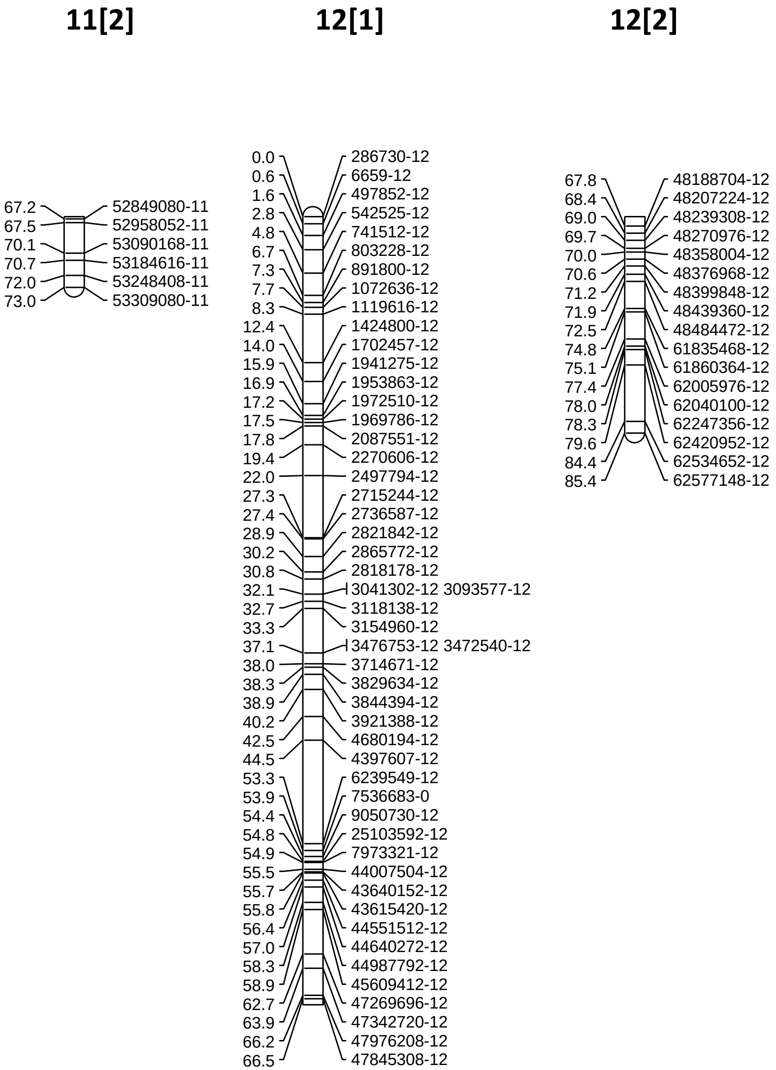
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