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De Novo Transcriptome Analysis of Orange Jasmine (Murraya paniculata) Infected with Citrus Greening Disease

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DE NOVO TRANSCRIPTOME ANALYSIS OF ORANGE JASMINE (MURRAYA PANICULATA) INFECTED WITH CITRUS GREENING DISEASE

A Thesis

by

JEFFREY AQUINO GOMEZ

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ABSTRACT

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Citrus greening disease, also known as Huanglongbing, is one of the most devastating citrus diseases in the world. *Candidatus* Liberibacter asiaticus (CLas) is a non-culturable, phloem-limited bacterium that serves as one of two vectors for the transmission of any of the three kinds of greening disease. In cooler climates, the African variant transmitted by the African citrus psyllid (*Trioza erytreae*) is more prevalent. The Asian form transmitted by the Asian citrus psyllid (*Diaphorina citri*) where is predominant in warmer conditions. Recently has been discovered in Brazil the American form that's also transmitted by *Diaphorina citri*

Murraya paniculata is an ornamental citrus belonging to the *Rutaceae* family. *M. paniculata* is commonly used in traditional Chinese medicine to treat diarrhea, abdominal pain, stomachache, headache, edema, and blood clots. In addition, it was utilized as an anticonvulsant, expectorant, detoxicant, and local anesthetic. Bark and leaf extracts are stimulant, astringent, antinociceptive, anti-inflammatory, antidiarrheal, antitrypanosomally, anti-diabetic, anti-malarial, antibacterial, and antifungal, according to previous research.

Despite the fact that *M. paniculata* is a favoured host for the CLas vector *Diaphorina citri*, it has been reported that infected plants exhibit minimal to no symptoms.

DEDICATION

"The essence of what we are today, are the memories of yesterday"

-Ednita Nazario (1992)

My master's thesis is dedicated to my beloved parents, who always encouraged and supported me in pursuing my life's ambitions and objectives, and who shown me unconditional love, understanding, and support so that I may always remember where my true home was.

Who taught me the real meaning of love, and to be unconditional, no matter the outcome.
Myrna Gomez Perez
MY MOTHER

My definition of hard work, who taught me that patience with efforts lead to expected results.
Humberto Aquino Vega
MY FATHER

ACKNOWLEDGMENTS

Developing the transcriptome analysis of a de novo organism such as Orange Jasmine to uncover candidate genes that can aid in the management of citrus greening disease required not only my work and effort but also the advice, assistance, and support of a number of exceptional individuals. Not only did these scientists, colleagues, and friends contribute to the completion of this significant chapter in my life, but they also made my graduate experience an unforgettable adventure that provided me with invaluable personal and professional insights. I consider myself fortunate to have met everyone who contributed to my achievement.

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Second, I am immensely appreciative of Dr. Renesh Bedre's assistance and collaboration in offering his significant time and knowledge in bioinformatics and statistical analysis. I feel immensely privileged to work with him, as he is an exceptional scientist and a wonderful person. Dr. Maria Y. Cervantes, my job supervisor, not only encouraged me to obtain this degree, but also went above and beyond to accommodate my schedule for the courses and program requirements and provided me the chance to become a faculty member in the Biology Department at South Texas College.

Third, I'd like to thank a lifelong friend, an eminent researcher, a devoted professor, but most importantly, a great person. Dr. Ismael E. Badillo-Vargas, beyond all the work he was handling, was always available when I had questions or got stuck during the development of this project. Despite the fact that his life was cut short, his legacy, charm, and work inspired many more generations of scientists to come.

Fourth, I would want to thank the UTRGV Biology Department's faculty, lab coordinators, administrative personnel, and graduate students. Their wonderful contributions, which included access to equipment, time, assistance, and encouraging words, enabled me to complete most of my work and try again until I achieved my objective.

Last but not least, To Fabian, my furry son, who stayed at my side the long hours of writing and studying, keeping me warm and sane. You are an exceptional group!

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

1.1 Citrus Greening Disease

Citrus Greening Disease (CGD) is the most severe citrus disease and the major cause of citrus plant losses in the world. Also known as yellow dragon disease or Huanglongbing (HLB) the disease affects the general health of the tree as well as the fruit development, ripening, quality of the fruit as well as the juice, (Dala-Paula et al., 2019). Citric with HLB demonstrates symptoms such as: a smaller size, asymmetry, and a fruit greener than a normal one. In addition, observable changes on the tree leave presenting asymmetry, blotchy mottle symptoms not limited to more foliar symptoms that included yellow coloration with an enlarged lateral veins and midribs. The inversion of color has been present in fruits with yellowing from the end of the stem and fruits were small, lopsided, had stained vascular bundles at the end of the fruit axis, and contained aborted brownish-black seeds(Da Graca et al., 2015; Dala-Paula et al., 2019). The juice of symptomatic fruits has shown higher values of titrable acidity and lower values in soluble solids, solids/acid ratio, total sugars, and malic acid levels when compared with healthy fruits; furthermore, the taste of juice from symptomatic trees change, and this due to the increased production of secondary metabolites, the flavor is described as sour, salty/umami, metallic, lack of sweetness and fruity/orange flavor, musty and distinctly bitter(Dala-Paula et al., 2019).

Due to these consequences of the HLB infection in orchards around the world, these conditions are considered the most devastating citrus disease. The disease has affected citrus

crops in Afghanistan, Brazil, China, India, Indonesia, Malaysia, Mauritius, Mexico, Myanmar, Nepal, Pakistan, Paraguay, Philippines, Saudi Arabia, Sri Lanka, Thailand, the Ryukyu Islands and United States (Ángel et al., 2014; K. L. Manjunath, S. E. Halbert, C. Ramadugu, S. Webb, & R. F. Lee, 2008; Martínez-Carrillo et al., 2016; Salcedo et al., 2011; Suaste-Dzul et al., 2017).

This citrus condition is caused by a vector-transmitted pathogen, the bacteria responsible is *Candidatus* Liberibacter asiaticus (CLas) a Gram-negative bacterium in the Rhizobiaceae family. The origin of CGD is not clear yet, despite the fact that the early reviews and observations of the disease in south China over a hundred years ago. Other reports and compiled data overlooked, pointed to the Indian subcontinental area as the origin, here where the first citrus was infected by CLas (Bové & Barros, 2006; Da Graca et al., 2015; Zhao, 1981)

1.1.1 Citrus Industry

The Texas citrus industry is a multimillion dollar business that is in charge of the supplies of fresh fruit to market, moreover the reputation for the Texas red grapefruit goes beyond United States borders (Setamou, Da Graca, & R.Prewett, 2012), but commercial citrus is mostly confined to the three southernmost counties of the state known as the Lower Rio Grande Valley (LRGV) Area. The first case of HLB reported in the United States was from Florida in 2005. The first case confirmed in Texas was in January 2012 on a Valencia sweet orange variety in a commercial orchard in San Juan, Texas ((Da Graca et al., 2015). Both cases were determined by real-time and conventional PCR essays using the partial sequence of 16S rRNA gene of the pathogen. Only one species of CLa causing HLB is currently confirmed in the USA. Screenings for CLa has showed negative results for the species of *Candidatus* liberibacter americanus and *Candidatus* liberibacter asiaticus(Shigenaga & Argueso, 2016)

1.1.2 *Candidatus* liberibacter

The term *Candidatus* indicates that it has not proved possible to maintain this bacterium in culture (Fagen et. al, 2014) consider a highly fastidious phloem-inhabiting Bacteria (Halbert, 2004). Currently exist three species: *Candidatus liberibacter africanus, Candidatus liberibacter americanus and Candidatus liberibacter asiaticus,* been the last one the responsible of the spreading of citrus greening disease in Asia and America due to its tolerance to higher temperature (Granados, 2018; Sarpa-Senasica, 2012).

The gram-negative bacterium *Candidatus* Liberibacter asiaticus is a causative agent of Huanglongbing disease (HLB) also known as citrus greening disease (Nadarash, 2014). *Candidatus liberibacter* is transmitted by two insects from Psyllidae family; *Diaphorina citri* in Asia, found in Brazil and Florida, and *Trioza erytreae* found in Africa (Hansen,2018).The *Candidatus* Liberibacter asiaticus is more heat tolerant, while the African strain, *Candidatus* liberibacter africanus is asymptomatic at temperatures above 30°C. Species of *Candidatus* liberibacter, infecting solanaceous plants has been identified and it was carried by another psyllid, a potato pest *Bactericera cockerelli* (Vereijssen, Smith, & Weintraub, 2018).

1.1.3 Pathogen Vectors

As previously mentioned, the HLB is a vector-transmitted pathogen, meaning that for the pathogen to infect the final host is necessary an intermediary that will carry the pathogen to the host. In the case of the African citrus psyllid an invasive insect that favors cool and moist conditions for optimal growth, such as the conditions the citrus crops in Africa become infected(Pérez-Rodríguez et al., 2019). On the other hand, conditions under citrus crops are infected in Asia are war as the bacteria are transmitted by *Diphorina citri* (Wu et al., 2018).

1.1.4 *Diaphorina citri*

Diaphorina citri, the Asian citrus psyllid Kuwayana, is a sap-sucking, Liviidae-family hemipteran insect. It is one of two confirmed citrus greening disease vectors (Lallemand, Fos, & Bové, 1986).It is widespread in southern Asia and has spread to other citrus-growing areas. It is a significant citrus pest in a number of countries because it transmits a devastating citrus disease known as greening disease or Huanglongbing. Several citrus industries in Asia and Africa have been decimated by this disease(K. á. Manjunath, S. Halbert, C. Ramadugu, S. Webb, & R. Lee, 2008). Prior to recent times, the Asian citrus psyllid was not detected in North America or Hawaii, however it was reported in Brazil (Catling, 1970; CosTA & do Brasil, 1942) In June 1998, the insect was discovered on the east coast of Florida, from Broward to St. Lucie counties, where it was presumably restricted to front-yard host plants. This pest had spread to 31 Florida counties by September 2000 (S. Halbert, 2009).

Citrus psylla is a common name for *Diaphorina citri*, but it is also frequently applied to *Trioza erytreae* (Del Guercio, 1918), the African citrus psyllid pest. Trioza erytreae should be referred to as the African citrus psyllid or the two-spotted citrus psyllid to prevent misunderstanding (the latter name is in reference to a pair of spots on the base of the abdomen in late-stage nymphs). These two psyllids are the only known vectors of the etiologic agent of citrus greening disease (Huanglongbing) and the only economically significant psyllid species on citrus worldwide. Six other species of Diaphorina have been reported on citrus, however they are non-vector species of limited relevance (S. E. Halbert & Núñez, 2004).

The mature psyllid has a body that is mottled with fawn and brown and a light brown head. It measures around four millimeters in length. It has a whitish, waxy secretion that covers it, giving it the appearance of being dusty. The forewings are black around the periphery and

have a pale gap towards the apex. The forewings are broadest at the rear and have a dark edging around the periphery. The antennae have a light brown color overall and have black tips. These characteristics differentiate it from the African citrus psyllid, which is superficially similar to it. When it is sucking sap, it will often assume a position in which its head is lowered, and its tail is up. Additionally, the antennae of a psyllid have ten segments, whereas the antennae of an aphid are typically comprised of only four or six segments. Psyllids do not have cornicles on their abdomens, but the majority of aphids have.(UF|IFAS, 2022)

The psyllid nymph goes through a total of five different moults. It is spotless on the abdominal region and has a hue that is somewhere between yellow and orange. The wing pads stand out, particularly in the later instars of the life cycle. The eggs have the shape of almonds and measure around 0.3 millimeters in length. They are wider at the bottom and becoming thinner as they move upward. In the beginning, they are a pale tint, but as they develop, they turn yellow and then orange before they hatch. The long axis is oriented in a direction that is perpendicular to the surface of the leaf. (UF|IFAS, 2022).

Eggs are typically placed on the tops of emerging shoots, in the spaces between unfolding leaves and close to them. During the course of her life, which can span several months, a female can deposit as many as 800 eggs. Depending on the temperature and the season, the entire process of development might take anywhere from two weeks to seven weeks to complete (Husain, 1927). *Brachygastra mellifica* and *Tamarixia radiata* are two species of wasp that are frequently seen as parasitoids of *D. citri* (Reyes-Rosas, Loera-Gallardo, Lopez-Arroyo, & Buck, 2013). Lady beetles are another prominent group of predators in the ecosystem.

Diaphorina citri is found primarily in tropical and subtropical Asia, but it has also been documented in the following regions: Afghanistan, the Caribbean (including the Bahamas, the

Cayman Islands, Jamaica, the Dominican Republic, Cuba, Puerto Rico, plus interceptions from St. Thomas and Belize), Central America (Guadeloupe), China, Hong Kong, India, Indonesia, Malaysia, Mauritius, Mexico, Myanmar, Nepal, Pakistan, the Philippine Islands, Reunion Island, the Ryukyu Islands (Hall, Richardson, Ammar, & Halbert, 2013)

The first record of *Diaphorina citri* coming from the Near East was made when it was found in Saudi Arabia (Wooler, Padgham, & Arafat, 1974). *Trioza erytreae* can also be found in Saudi Arabia; however, it is most common in the eastern and highland regions of the country where the environment is more arid and harsh, whereas *Diaphorina citri* is widespread in the western and coastal regions that have a more temperate temperature.

This species can be found in the following states and territories in the United States and its territories: Alabama, Arizona, California, Florida, Georgia, Guam, Hawaii, Louisiana, Mississippi, Puerto Rico, South Carolina, Texas, and the United States Virgin Islands. Late in the month of May in 2008, specimens were found in the parishes of Jefferson and Orleans in the state of Louisiana. It wasn't until September 2, 2008 that the psyllid was found for the first time in San Diego County, California. In Yuma County, Arizona, on October 27, 2009, the psyllid was found there for the first time. The presence of the psyllid was confirmed by surveys carried out on April 21st, 2010, in the United States Virgin Islands (USDA 2010b).

1.1.5 Citrus Genome/Transcriptome related to CGD

The "central dogma" of molecular biology is a theory that outlines the transmission of genetic information from genes to the functions of cells and organisms. This theory was developed in the 1970s. This is accomplished through a two-stage process: first, DNA, which is the permanent, heritable, and genetic information repository, is transcribed by the RNA polymerase enzymes into RNA, which is a short-lasting information carrier; then, a subset of

RNA, mRNAs, and messenger RNAs, are translated into protein. DNA is the permanent, heritable, and genetic information repository. Therefore, the full collection of all RNA molecules found in a cell, a population of cells, or an organism is referred to as the transcriptome. Not all RNAs are translated into proteins; some serve structural functions, such as rRNAs in the assembly of ribosomes, while others are transporters, e.g., tRNAs, and still others serve regulatory functions, such as the siRNAs, short interfering RNA, or lncRNAs, long non-coding RNAs; these are not translated into proteins (Botchkareva, 2017). Nonetheless, these non-coding RNAs can and frequently do play a role in human diseases like cancer, cardiovascular, and neurologic disorders. While transcriptomics is most frequently applied to mRNAs, the coding transcripts, it also gives crucial information about the content of cell noncoding RNAs, such as rRNA, tRNA, lncRNA, siRNA, and others. The investigation of splice variants of the same gene in different tissues is addressed by specific methods.

Transcriptome Analysis is the high-throughput study of the transcriptome, the whole set of RNA transcripts produced by the genome, under specific conditions or in a specific cell. Transcription profiling, which examines changes in the activity of a cell as a whole, opposed to a single gene or a small number of genes, is utilized in numerous areas of research, such as illness diagnosis, biomarker development, risk evaluation of new therapies or environmental hazards, among others. The implementation of transcription profiling to loss- and gain-of-function mutants facilitates the detection of alterations associated with the gene mutation. Identifying the activities of genes has been facilitated by transcriptomic approaches in particular. Additionally, transcriptomics permits the discovery of pathways that respond to or mitigate environmental disturbances. Additionally, RNA-Seq can uncover disease-associated gene fusions, SNPs, and even allele-specific expression.

The most typical application of transcriptome analysis is to compare specific pairings of samples. Various external environmental conditions, such as hormonal influences or pollutants, may account for the variations. Typically, comparisons between normal and diseased states are made. In animals' diseases such as cancer, for instance, transcriptomics analyses address classification, pathogenic pathways, and even prognosis. Predictions of outcomes can provide gene-based criteria for predicting tumor progression and treatment response. These methods are already employed in customized medicine and cancer patient therapy.

At various phases of development, organisms and tissues can be molecularly characterized. Stem cell transcriptomes aid in the comprehension of cellular differentiation and embryonic development. Due of its broad approach, transcriptome analysis is an excellent tool for discovering therapeutic targets.

Recently, transcriptomics has become one of the most popular areas in biology. RNA-Seq is a high-throughput, high-sensitivity, and high-resolution technology for studying model and non-model organisms. Sequencing the transcriptome is also an important tool for investigating the genomes of plants, a topic on which there is minimal knowledge. The study of plant with high agricultural importance by means of transcriptomics can aid researchers in analyzing the functional genes and regulatory mechanisms of these crops and enhancing breeding, selection, and culture.

In an effort to understand more the HLB disease from a molecular perspective Wang (2016) HLB-tolerant "Jackson" grapefruit-like hybrid trees and HLB-vulnerable "Marsh" grapefruit trees following infection with HLB. 686 genes exhibited differential expression (DE) between the two cultivars. 247 genes were up-expressed, and 439 genes were down-expressed in citrus trees with tolerance. In addition, they discovered 619 genes whose expression of

alternative splicing isoforms varied significantly between HLB-tolerant and HLB-vulnerable citrus trees. Using two methodologies, the team examined the functional categories of DE genes and found that various pathways have been inhibited or activated in HLB-tolerant citrus trees, resulting in the activation of the citrus plants' innate resistance or immune. Using real-time PCR, have experimentally confirmed the expressions of 14 up-expressed genes and 19 down-expressed genes in HLB-tolerant "Jackson" trees and HLB-vulnerable "Marsh" trees. The majority of gene expressions agreed with the RNA-Seq findings. This work gave fresh insights on HLB-tolerance and beneficial direction for the future breeding of HLB-tolerant citrus.

1.1.6 Orange Jasmine *(Murraya paniculata)*

Murraya paniculata (Also known as: Orange Jasmine, Orange Jessamine, China Box, Mock Orange and Murraya exotica) is a small tree native to South Asia, Southeast Asia, and Australia and a species of the Rutaceae family. It has smooth bark, pinnate leaves with up to seven egg-shaped to elliptical leaflets, white or cream-colored fragrant blooms, and oval, orange-red berries with hairy seeds. Carl Linnaeus formally described Orange Jasmine in Mantissa Plantarum in 1767, giving it the name Chalcas paniculata. (Linné, 1767; Mabberley, 2016). In his book, Descriptions of Malayan Plants [Malayan Miscellany], published in 1820, William Jack switched the name to Murraya paniculata. (Stone, 1978)

Considered an invasive tropical or subtropical shrub to small tree in portions of Australia, Hong Kong (China), Mauritius, Reunion, Bermuda, and Hawaii, United States. The species is classed as potentially invasive in Cuba, Florida (United States of America), and portions of Australia. Although this plant is labeled as invasive in Florida, it is considered possibly invasive due to its establishment in cultivated areas. Brazil, Vanuatu, Australia, and Bermuda are identified as being affected by it. Although *M. paniculata* has been identified as an invasive

species, nothing is known about its impact on natural ecosystems and native species. This species is one of the primary concerns since it is one of the hosts of *Diaphorina citri*, the vector of the pathogen that causes citrus greening disease or huanglongbing. In Australia, *M. paniculata* is recognized as an environmental weed, capable of developing in the understory of primary rainforests at low to moderate concentrations in various regions. To limit the undesired spread of this species, the use of non-seeding clones grown from cuttings is encouraged. In Cuba, *M. paniculata* is classified as a Category 2 possibly invasive species, which is characterized as "a species that has become naturalized and is expanding in some regions." In Florida, it is classified as a Category 2 species, which is defined as "an exotic that has risen in quantity or frequency without affecting plant communities."(Lieurance & Gettys, 2019)

The leaves are stimulating and astringent and are used to manage diarrhea, dysentery, and tooth and gum disorders; they are also effective against rheumatism, coughs, and hysteria (Chopra & Industrial, 1956; Ghani, 1998). The essential oils demonstrated notable anti-inflammatory and analgesic properties (Gupta, George, Singhal, Sharma, & Garg, 2010). And it exhibited ant amoebic properties(Sawangjaroen et al., 2006). The leaves and other tissues are used to treat diarrhea, dysentery, wounds, joint discomfort, and body aches (Parrotta, 2001), venereal disease and as an abortive(Kinoshita & Firman, 1996; Xiao & Wang, 1991). In addition to essential oils, Orange Jasmine tissues contain the indole alkaloid yuehchukene (Xiao & Wang, 1991) and at least eight highly oxygenated flavones(Kinoshita & Firman, 1996). Orange Jasmine leaves produce an oil rich in sesquiterpenes (l-cadinene), -sesquiterpene alcohol, and methyl anthranilate (Chopra & Industrial, 1956). The major components of the leaf oil were β-cyclocitral (22.9 %), methylsalycylate (22.4 %), trans-nerolidol (11.7 %), α-cubebene (7.9 %), cubenol (6.8 %), β-cubebene (5.8 %), and isogermacrene (5.7 %) (Olawore, Ogunwande,

Ekundayo, & Adeleke, 2005). The most abundant chemical was β-caryophyllene (24.1 %), followed by germacrene D (11.9 %) and bicyclogermacrene in decreasing proportions (11.8 %). There have been reports of the chemical composition of the leaf oil of *M. paniculata* of Asian or Australian origin (Garg and Nigam (Aziz et al., 2010; Narkhede, Ajmire, & Wagh, 2012; Poonpaiboonpipattana, 2013). *M. paniculata*, on the other hand, contains an abundance of caryophyllene oxide, which possesses antifungal properties(Yang, Michel, Chaumont, & Millet-Clerc, 1999).

1.1.7 Plant response and pathway involved in diseases

It is imperative that global food production be increased in light of the expanding human population and the diminishing amount of land suitable for agricultural use (Ali et al., 2017). Pathogens, such as bacteria, fungi, nematodes, oomycetes, and viruses, as well as insect pests, are examples of biological dangers to the nation's food supply. These factors, when added together, are responsible for roughly 30 percent of the total loss in pre- and post-harvest agricultural output throughout the world (Bebber & Gurr, 2015). All plant pathogens consistently present a challenge to the immune system of the host plant (Z. Liu et al., 2017). As a result, in order to protect themselves against a wide variety of infections, plants have developed a wide variety of defense mechanisms that involve the activation or suppression of a number of genes (Huot, Yao, Montgomery, & He, 2014; Ali Noman, Shah Fahad, et al., 2017; Zaynab et al., 2017). Cascades of pathogen-associated molecular patterns, also known as DAMPs (host danger-associated molecular patterns), are recognized by plants as successive stages of pathogen assault (Dana et al., 2017; Liston & Masters, 2017; Z. Liu et al., 2017; Santoni et al., 2015). Pathogens, such as bacteria and fungi that cause PAMPs or DAMPs, are recognized by cell-surface PRRs (pattern-recognition receptors), such as FLS2, EF-Tu, LYK5, and CERK1(Li, Lu, & Shan, 2014;

Newman, Sundelin, Nielsen, & Erbs, 2013; Park, Caddell, & Ronald, 2012; Trdá et al., 2015). Both PAMP and DAMP are responsible for the activation of PTI (PAMP-triggered immunity), which includes the stimulation of PR (pathogenesis-related) gene expression, the deposition of callose, the formation of ROS (reactive oxygen species), and the buildup of SA (salicylic acid) (Jwa & Hwang, 2017; Withers & Dong, 2017).

However, along the course of evolution, a large number of pathogens have developed effector proteins that are employed for inhibiting PTI, which has led to the phenomenon known as effector-triggered susceptibility (ETS) (de Wit, 2016; Gouveia, Calil, Machado, Santos, & Fontes, 2017; Schuebel et al., 2016). As a defense mechanism, the plant secondary immune response, often referred to as effector-triggered immunity (ETI), is activated in response to the pathogen. Moreover, in this never-ending arms race, plants make use of new resistance (R) proteins in order to identify the effectors and set off ETI responses (Kushalappa, Yogendra, & Karre, 2016). R proteins in the body typically cause a reaction that is both more robust and more specific, such as a hypersensitive response (HR) (Bashir et al., 2013). An HR brings an infection under control by resulting in cell death at the infected locations and therefore limiting the proliferation of the pathogen. As a consequence of defense and counter-defense between hosts and pathogens, plants have evolved new R proteins to differentiate and combat novel effectors (Bigeard, Colcombet, & Hirt, 2015; Fawke, Doumane, & Schornack, 2015; Henry, Toruño, Jauneau, Deslandes, & Coaker, 2017). This has allowed plants to remain resistant to a wider variety of effectors.

The sRNAs are non-coding RNA molecules that range in length from 20 to 30 nucleotides (nt), and they are responsible for controlling the expression of eukaryotic genes through the process of RNA silencing (Islam, Zaynab, Qasim, & Wu, 2017; X. Liu, Hao, Li,

Zhu, & Hu, 2015; Patil, Zhou, & Rana, 2014). Both microRNAs and small interfering RNAs, or siRNAs and miRNAs, are considered to be important groups of sRNAs found in plants. (Achkar, Cambiagno, & Manavella, 2016; Cui, You, & Chen, 2017; D'Ario, Griffiths-Jones, & Kim, 2017; MacFarlane & R Murphy, 2010; Noman & Aqeel, 2017; Ali Noman, Muhammad Aqeel, et al., 2017; Rolle et al., 2016) microRNAs range in length from 21 to 24 nucleotides and are produced from RNAs that have poorly base-paired hairpin structures.

It is possible that RNA-dependent RNA polymerases are required in order to produce siRNAs, which are derived from long strands of double-stranded RNA (dsRNA) (Katiyar-Agarwal & Jin, 2010). Several other sub-classes of siRNA have been found in plants, including lsiRNAs (long siRNAs), nat-siRNAs (natural antisense transcript-derived siRNAs), hc-siRNAs (heterochromatic siRNAs), and ta-siRNAs (trans-acting siRNAs)(Huang, Yang, Lu, & Zhang, 2016). sRNA-mediated gene regulation in hosts or pathogens is mostly the result of post-transcriptional gene silencing (PTGS) or transcriptional gene silencing (TGS) (Méndez, Ahlenstiel, & Kelleher, 2015). Both microRNAs and siRNAs have the potential to trigger post-transcriptional gene silencing (PTGS) either through the cleavage or degradation of messenger RNA (mRNA) or through the RNA-induced silencing complex (RISC) (Martienssen & Moazed, 2015; A. Noman et al., 2017). On the other hand, transcriptional gene silencing (TGS), which results in either the methylation of DNA, the alteration of histones, or the modification of chromatin, is often mediated by siRNAs and also by some particular miRNAs (Moazed, 2009; Sampey et al., 2012). The biogenesis of various small RNAs is a complicated and species-specific process. Despite the fact that certain processes are shared throughout all small RNAs, many other steps are unique to individual small RNAs. There is a wealth of information available(Bloch, Węgrzyn, Węgrzyn, & Nejman-Faleńczyk, 2017; Guleria, Mahajan, Bhardwaj,

& Yadav, 2011; Martienssen & Moazed, 2015; Moazed, 2009; Sampey et al., 2012; Tripp et al., 2017; Yu et al., 2017; Zhang, Wu, Li, & Wu, 2015) about the biogenesis of the numerous sRNAs. Therefore, as a result of the enormous role that sRNAs play in plant immunity, we have compiled a list of the targeted tasks that sRNAs play as key participants in mediating plant development when it is subjected to the effects of pathogens. We discuss current developments in the field of sRNA research, focusing in particular on the explanation of their function as a dynamic network in modulating plant protection against pathogens.

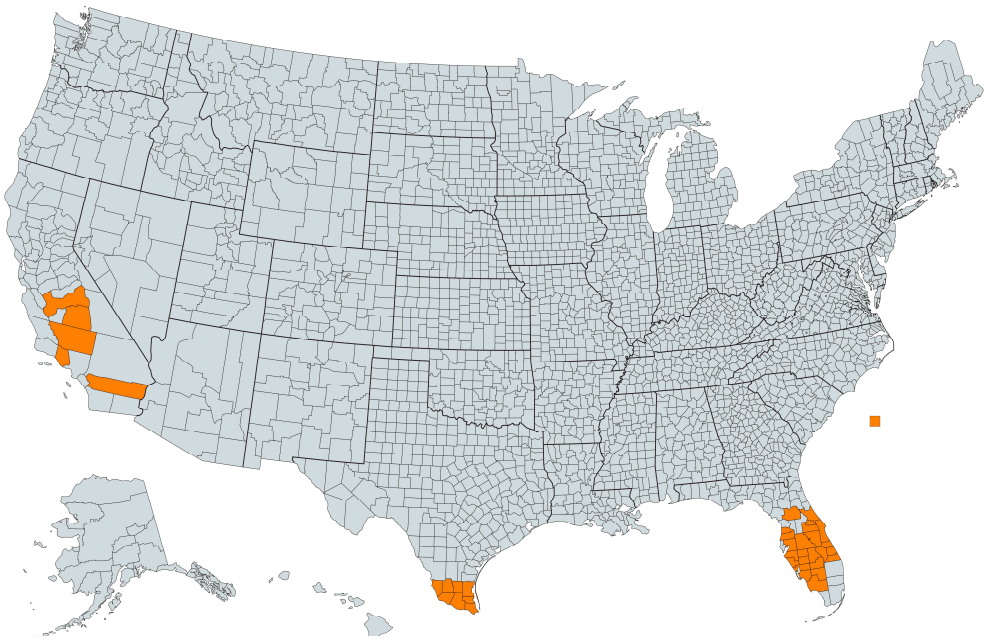

Figure 1.1 States where CGD has been confirmed. In the figure above can be appreciated the states and counties in which the Citrus Greening Disease has been confirmed by the USDA.

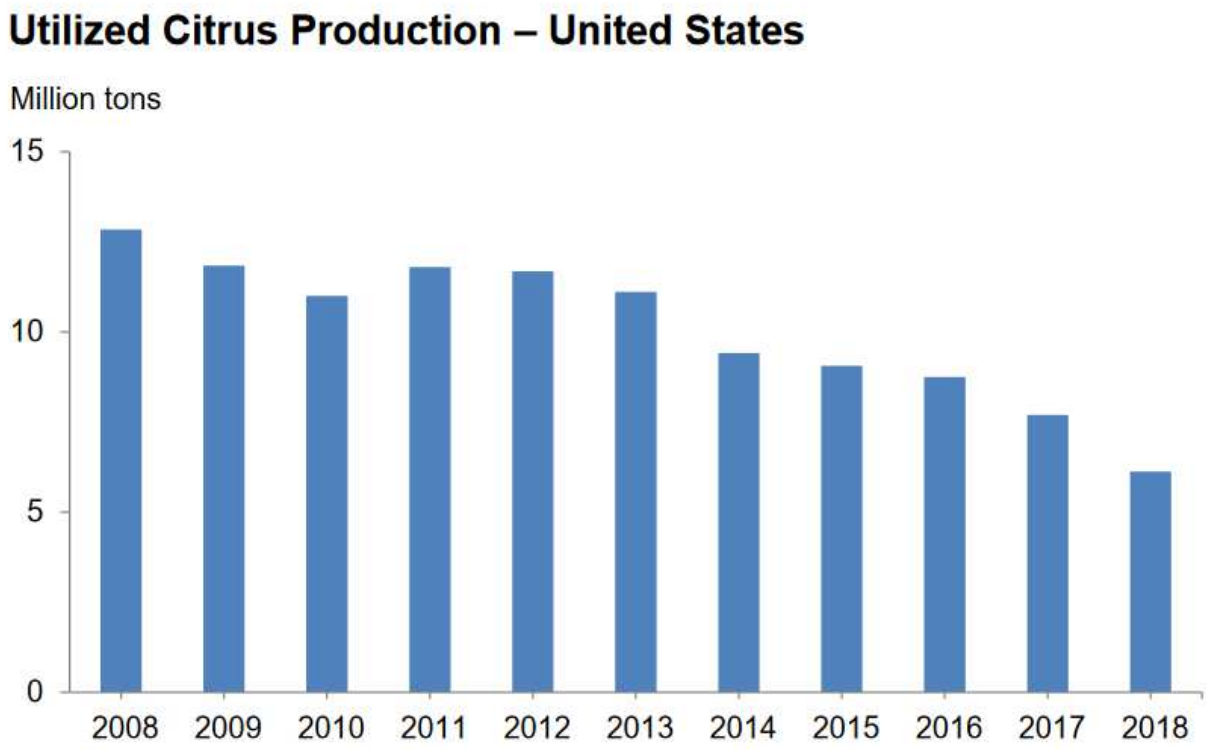

Figure 1.2 Utilized Citrus crop production in USA. The above figure depicts the annual million tons of citrus, produced in the United States from 2008 to 2018 as stated by the USDA in the 2018 Citrus fruits summary (2018).

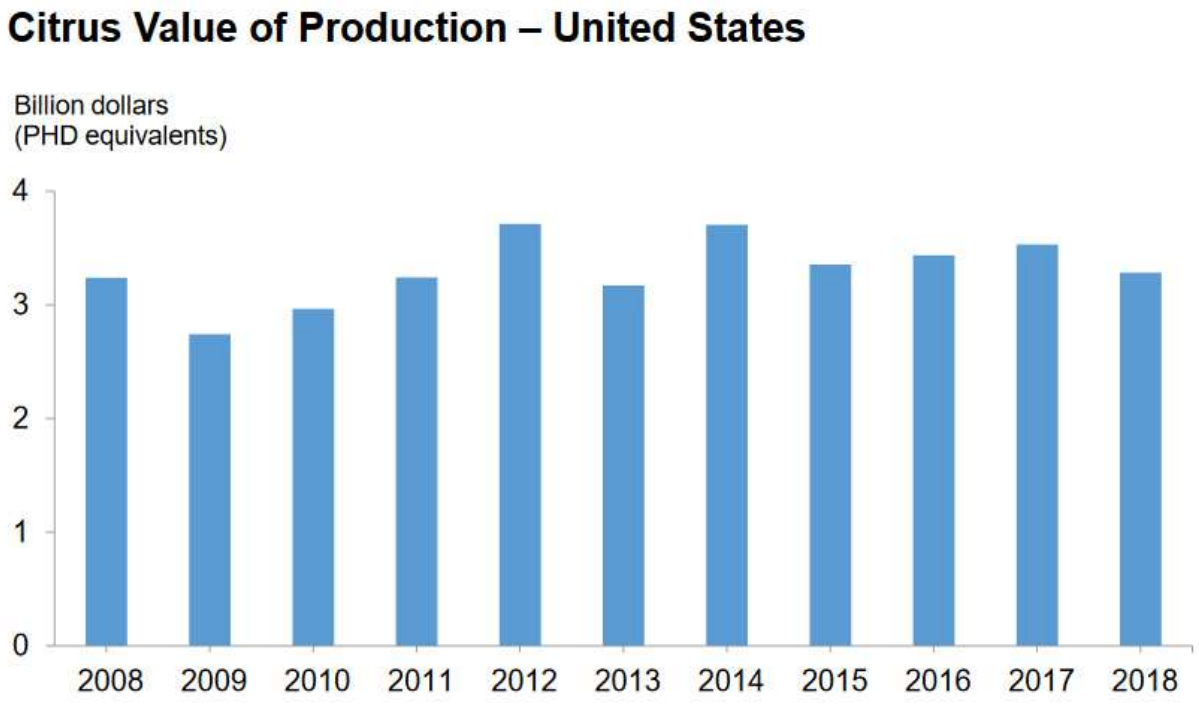

Figure 1.3 Citrus Value of Production in USA. The figure above depicts the value of citrus production in the United States in billions of dollars between 2009 and 2018, as stated by the USDA in the 2018 Citrus fruits summary (2018).

CHAPTER II

MATERIALS AND METHODS

2.1 Plan Materials and HLB Graft inoculation

The *Murraya paniculata plants,* were obtained from the USDA-APHIS station at Moore Air Base in Edinburg, Texas. Twelve plants were used in total. Experiments were conducted in a Biosafety Level 2 (BSL-2) Laboratory at the USDA-APHIS facility in Edinburg, Texas. Seedlings of Murraya paniculata (Orange Jasmine) were graft-inoculated using side grafting. Dr. Madhurababu Kunta from the Texas A&M University-Kingsville Citrus Center in Weslaco, Texas, collected the infected tissue and carried out the grafting. Three treatments will be applied: plant not grated not infected (BT), plant grafted not infected (C), and plant grafted and infected (H)The inoculated seedlings were housed in mesh cages per treatment (three samples per treatment) in a BSL- 2 greenhouse at a regulated temperature.

2.1.1 RNA Extraction

RNA was extracted from collected young leaves for each plant before grafting, and 1 week, 5 weeks, and 13 weeks after grafting. The young leaves were collected at each treatment time in liquid nitrogen and RNA extraction was performed using RNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) in the BSL-2 Lab. The quantify and quality of the RNA samples were measured using a Nanodrop ND-1000 spectrophotometer. RNA-Seq libraries were developed

following the Illumina mRNA-sequencing sample preparation protocol (Illumina Inc., San Diego, CA). Libraries were prepared.

2.1.2 Sequencing

Each library's quality was evaluated using a BioRad Experion (BioRad, Hercules, CA). BGI performed the high-throughput sequencing utilizing HiSeq2000 (Illumina, San Diego, CA). Libraries were sent Novogen Corporation (USA).

2.1.3 RNA-Seq Data Processing and Analyzing

To begin, we trimmed adaptor sequences and removed low-quality reads (Q20) from the raw Illumina reads. Using the Citrus Genome Database (http://www.citrusgenomedb.org/), we mapped each sample's reads to the C. clementina reference genome (Version 182) using Star (Dobin et al., 2013) with a maximum intron 5000 bp (–alignIntronMax 5000). SAMtools was used to sort and index the alignment bam files (Li et al., 2009). We used htseq-count from HTSeq to count the number of uniquely mapped reads for each gene (Anders et al., 2014).

To ensure high-quality readings for further analysis, the 100-bp single-end raw Illumina sequencing reads were filtered. Adapter contamination and low-quality, ambiguous, and uncalled nucleotide bases were removed from the raw readings. More than five percent of uncalled bases and an average quality of 20 bp were removed from the readings in the 5' to 3' direction with a window size of five base pairs.

Perl and Python scripts were used to construct an in-house pipeline that filtered and trimmed raw readings for analysis. Trinity (Grabherr et al., 2011) was used to construct the high-quality reads de novo, and the reads were assembled using settings such as k-mer size 25, minimum contig length 200 bp, and min kmer cov 2. Each of the six libraries has its own

assembly procedure. After assembling the overlapping k-mers into linear transcripts, clusters of overlapping transcripts were generated. From these overlapping transcripts, we were able to get the transcripts for alternatively spliced forms and paralogous genes. Using the Louisiana State University's SuperMike-II High Performance Computing facility, which has 16 CPUs, all bioinformatics data was analyzed. The total number of unigenes was determined by removing any transcripts that were precisely the same (i.e., identical). "Unigene" refers to the longest transcript from an alternatively spliced isoforms cluster, whereas "transcript" refers to the individual sequence assembly.

A Bioconductor software called DESeq (Anders and Huber, 2010) was used to identify differentially expressed (DE) genes in HLB resistant and HLB susceptible citrus plants. DESeq was used to normalize the raw counts of each gene to account for differences in sequencing depth across samples. The DESeq method used an adjusted p-value (FDR) threshold of 0.1 to identify genes that were differently expressed across citrus trees that were resistant to HLB and those that were susceptible.

2.1.4 Transcriptome Assembly from scratch and annotation of the reference genome

In order to build the merged clean reads, we used Trinity (Haas et al., 2013) with the default parameters but with the "normalize reads" option chosen to combine all nine samples into a single pseudo sample. BLAT (Kent, 2002) and GMAP (Kent, 2002) were used to map the assembled contigs to the reference genome (Wu and Watanabe, 2005). PASA was used to verify and consolidate the alignments (Haas et al., 2003). We next used the transcriptome alignments to update the original genome annotation, such as adding UTRs, changing splicing, expanding gene models, merging spliced genes, and finding isoform genes using the PASA program.

Figure 2.1 Plant grafted with CGD inoculum. Above can be observed the plants grafted with the diseases at week 1 (a.), and week 12 (b.).

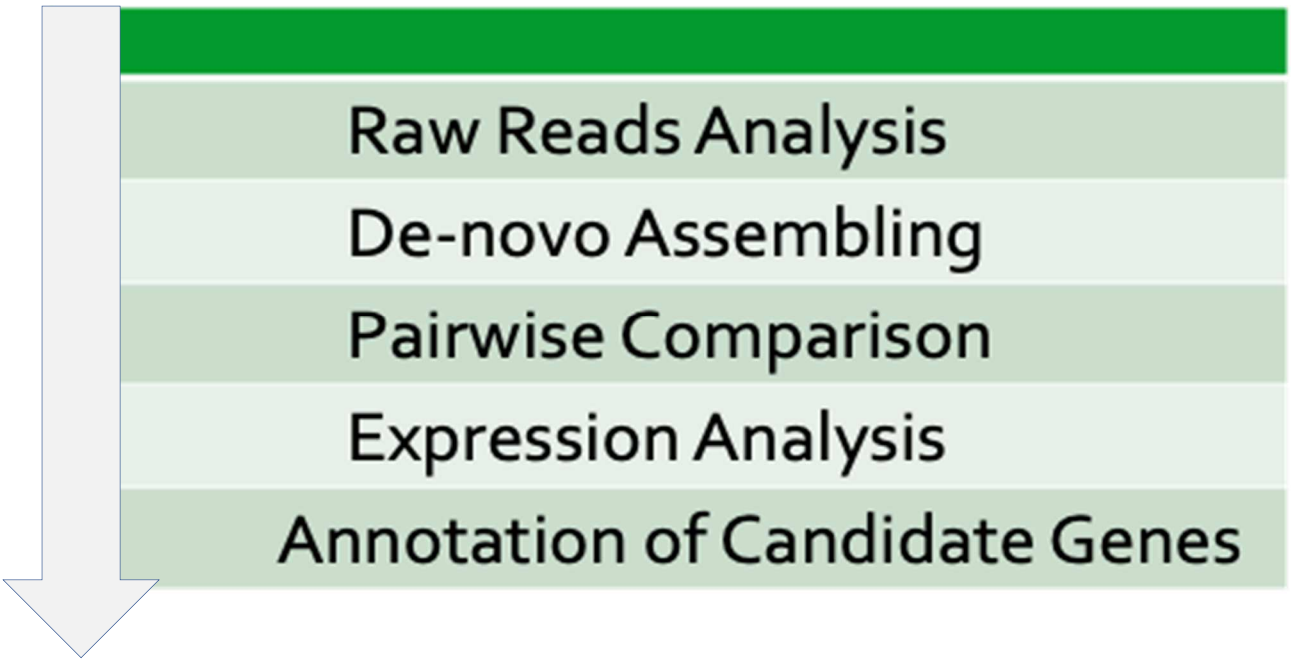

Figure 2.2 Bioinformatic analysis flowchart. The graph depicts the consecutive steps performed to process and analyze the data.

CHAPTER III

RESULTS

3.1 Visualization of the Transcriptomic Data of a plant to interpret Differentially Expressed Genes (DEGs)

The Experiment includes three separate treatments at two distinct timeframes, namely comparisons of the control group to the grafted non-infected group, the grafted non-infected group to the grafted infected group, and the grafted infected group to the control group at the 4th and 12th week. For the fourth week, the samples were designated BT4, H4, and C4, while for the twelve-week, the samples were designated BT12, H12, and C12. Deseq2 analysis was used to acquire transcriptomic data. Therefore, the log2Fold change value, adjusted p-value, base mean value, and p values were determined**.**

3.1.1 Illumina Sequencing, Quality Control, and Alignment

Sequencing of the six libraries BT4, H4, and C4 for the fourth week, while for the twelve-week, the samples were designated as BT12, H12, and C12 have generated 150,306,515 reads of 150 bp long resulting in a total of 15.0 Gbp sequence data. The average quality of the reads in the libraries after filtering was >30. There was a negligible amount of adapter/primer contamination in the reads.

3.1.2 De Novo Sequence Assembly

The high-quality reads from each of the six libraries from different experimental conditions were assembled independently into transcripts with a length of more than 200 bp. Trinity has a

better resolving power than others in identifying alternatively spliced transcripts, and thus produces fewer duplicates and chimeric transcripts. There were six contigs named Contig N10, Contig N20, Contig N30, Contig N40, and Contig N50 consists median contig lengths. These results strongly supported a high-quality transcriptome assembly of the citrus plant.

3.1.3 Functional Annotation

Out of the 6702 total unique transcripts were assigned functions based on their similarity to Citrus clementina and Lupinus albus protein database. The remaining un-annotated sequences were searched against NCBI nr and UniProtKB plant databases.

3.1.4 Identification of Differentially Expressed Genes (DEGs) in Response to Citrus Green disease

Statistically significant differentially expressed genes (DEGs) were calculated using a combination of Log fold to change (lf2c) and P-value criteria based on mapping of the reads against the Citrus genome. In Orange Jasmine leaves tissue at four weeks, 1265, 832 and 396 genes were up-regulated ($\log2FC \geq 2$ and $P < 0.05$) under BT vs C4, BT vs H4 and C4 vs H4 conditions, respectively (Figure 3.1). On the other hand, 247, 123 and 869 genes were down-regulated ($\log2FC \leq -2$, $P < 0.05$) under same experimental conditions, respectively. Similarly, leaves tissue at twelve weeks of infection, 688, 3848 and 89 genes were up-regulated under BT vs C12, BT vs H12, and DE C12 vs H12 conditions, respectively, whereas, 861,984 and 209 genes same experimental conditions, respectively (Figure.3.2). Thus, identification of the specific category of highly up-regulated genes from different clusters, and characterization of their biochemical response would provide potential candidates for functional characterization through genetic manipulation toward the improvement of resistance.

3.1.5 Heatmaps

The heatmaps were created using the genes' adjusted p-values and Log2fold change values. The Trinity id accession genes were displayed on the right side of the heatmap on the y-axis. (Figures 3.3,3.4,3.5,3.6,3.7 and 3.8). Heatmap showing differentially expressed genes (DEGs) of 4th-week Citrus in response to infection.The up-regulated genes (log2FC> = 2 and P<0.05) and down-regulated genes (log2FC< = -2 and P<0.05) are represented by dark brown and light color, respectively. Genes with similar expression profiles were clustered together by hierarchical clustering.

3.1.6 VENN Diagrams

A Venn Diagram was created to depict the relationship between the three datasets at different time frames. The number of genes present in all three comparisons of the Control group, Grafted infected group and Grafted non-infected group was shown by a Venn diagram of these three sets. The distributions of DEGs under different weeks are shown in threeway Venn diagrams for week 4 (Figure 3.9) and week12 (Figure 3.10). The DEGs were further characterized into different groups 630 common genes between BT vs H4, BT vs C4 describing their putative functional significance. Whereas C4 vs H4 share very few genes with other conditions at week 4 (Figure 3.9).

During week 12, the 319 common DEGs genes between BT vs H12, BT vs C12 describe the effect and variation of expression. Whereas C14 vs H4 still share very few genes with other conditions a week 12 (Figure 3.10).

3.1.7 Volcano Plots

Heatmaps are useful for examining the expression levels of a large number of genes, but the volcano graphic provides a more global perspective. On the y-axis, log-transformed adjusted p-values are shown, and on the x-axis, log2 fold change values are plotted.

Another common comparison of interest between four weeks (BT4, H4, and C4) and twelve-week, the samples(BT12, H12, and C12) is the adjusted P-value versus log fold-change. These Figure are referred to as volcano plots distinguished by the 2 different stages (week 4 and 12), as it resembles an exploding volcano, with clusters of data points close to the origin and a fanning effect moving away from this central location (Figure 3.10). BT vs H4 display the statistical significance of the difference relative to the magnitude of difference for every single gene in the comparison, usually through the negative base-10 log and base-2 log fold-change, respectively. Statistically differentially expressed genes were shown.

The comparison of BT vs C4 detected 25 differential genes (11 up-regulated and 14 down-regulated) (Figure 3.11). Similarly, BT vs H4 comparison in the detected 23 genes (8 up-regulated and 15 down-regulated) (Figure 3.12). Whereas, C4 vs H4 expressed 5 differential genes (3 up-regulated and 2 down-regulated) (Figure 3.13). While for the twelve-week transcriptome profiling identified different types of differentially expressed genes as compared to the fourth week.

We observed BT vs C12 detected 20 genes (9 up-regulated and 11 down-regulated) (Figure 3.14). Similarly, BT vs H12 comparison in the detected 13 genes (6 up-regulated and 7 down-regulated) (Figure 3.15). Whereas, C12 vs H12 expressed 9 differential genes (5 up-regulated and 4 down-regulated) (Figure 3.16). C4 vs H4 and C12 vs H12 expressed very few differential genes as compare to others.

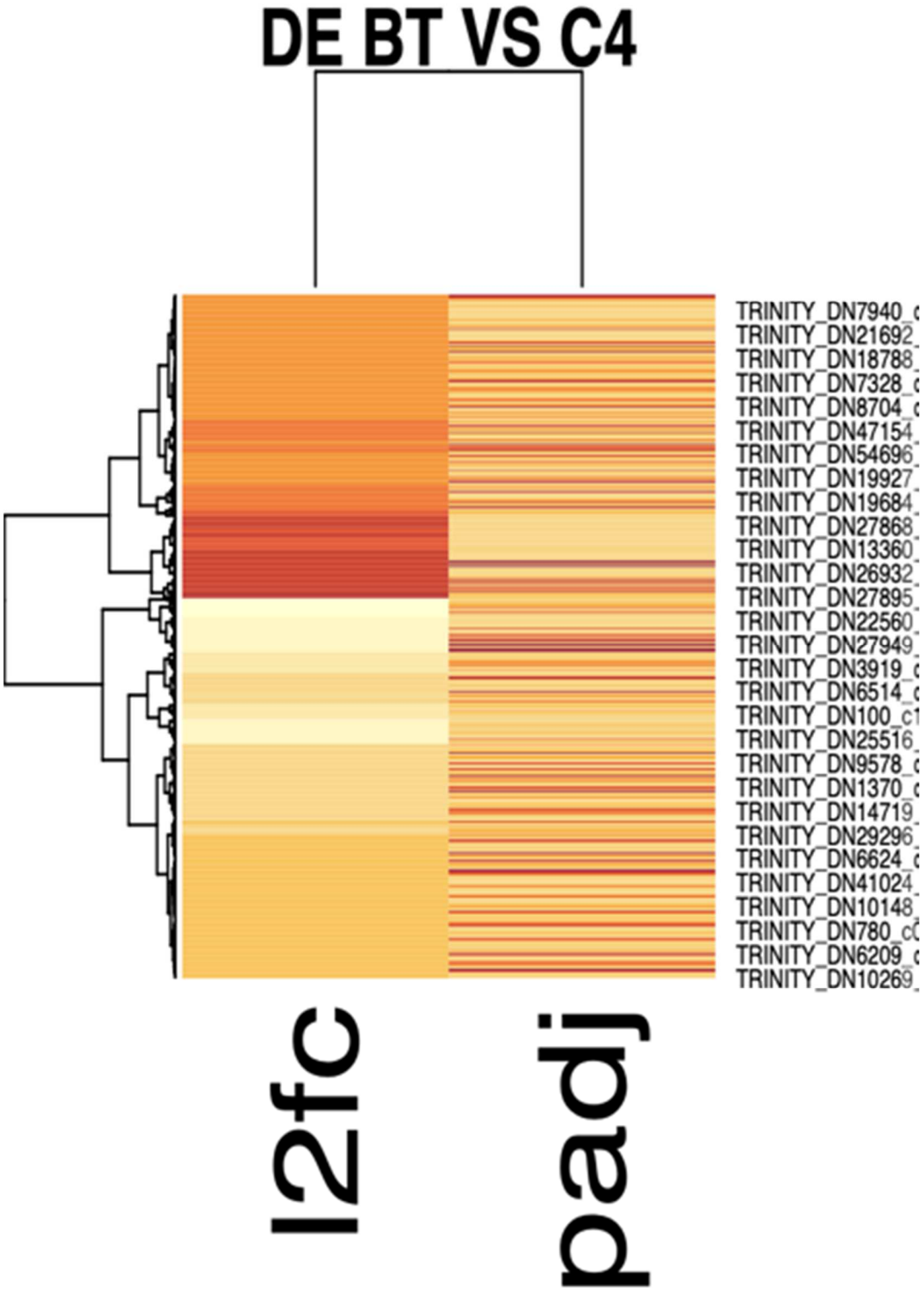

Figure 3. 1: Heatmap comparison of BT Vs C4 at the 4th week.

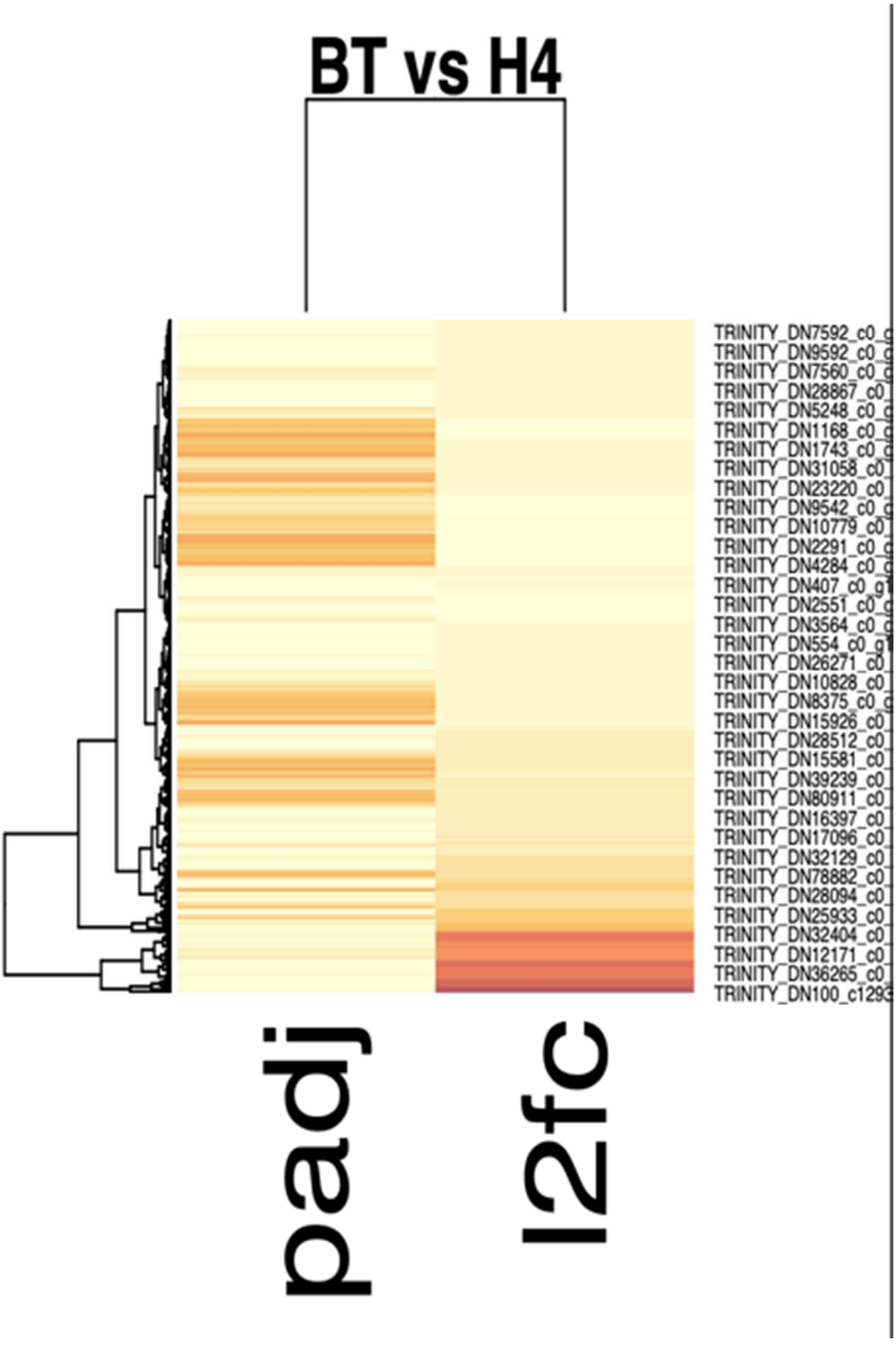

Figure 3. 2: Heatmap comparison of Bt Vs H4 at the 4th week .

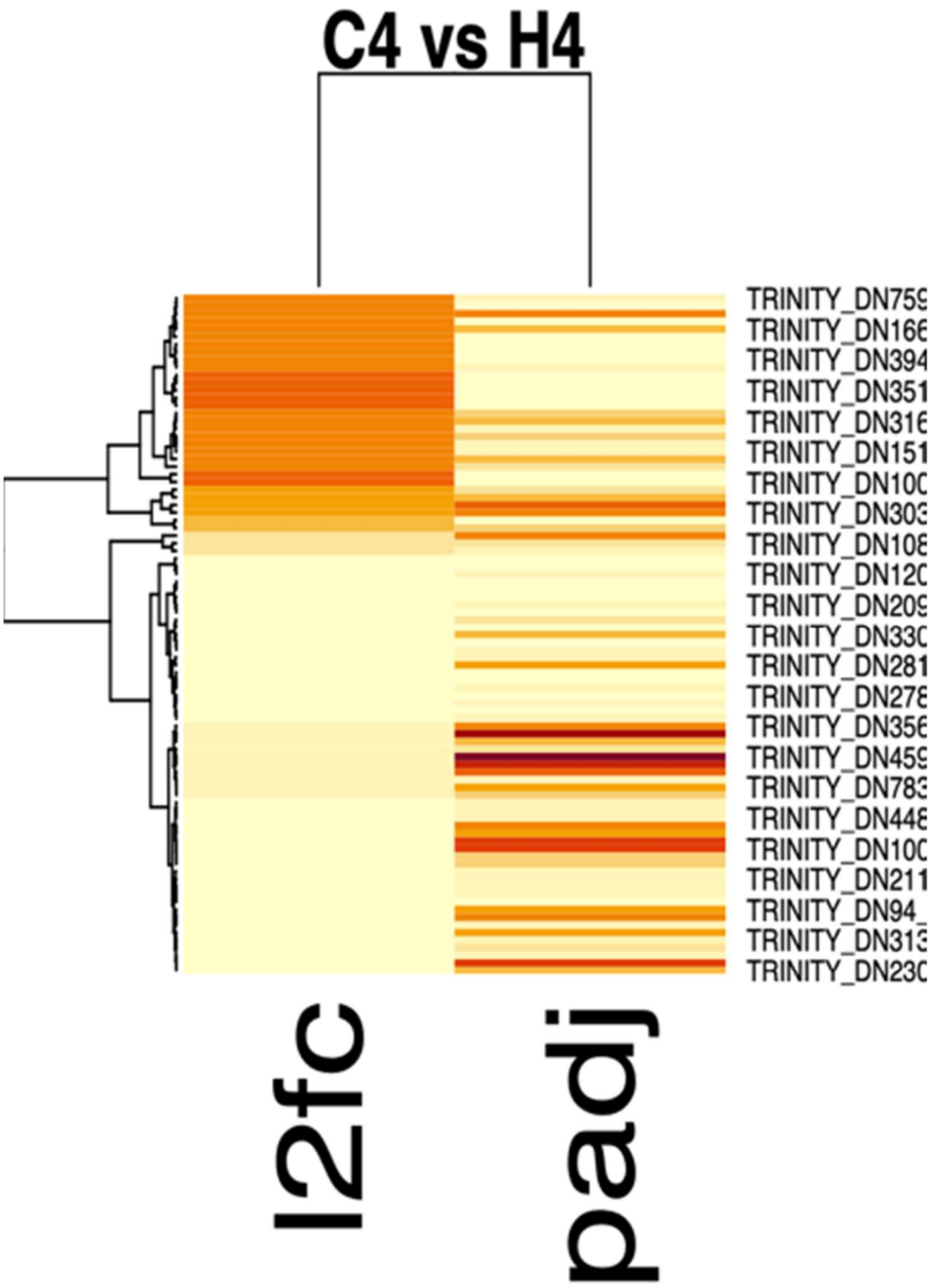

Figure 3. 3: Heatmap comparison of C4 Vs H4 at the 4th week .

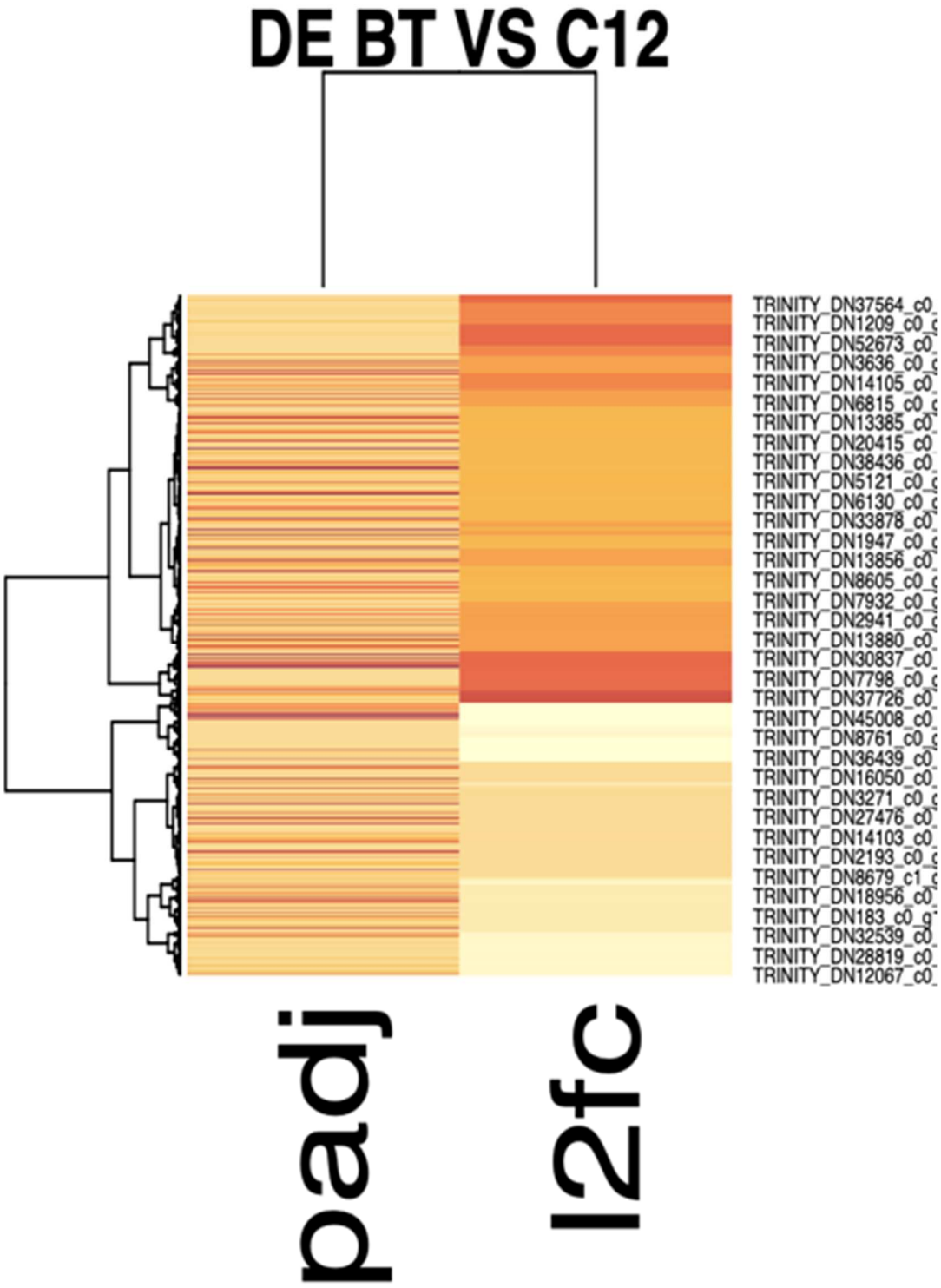

Figure 3.4: Heatmap comparison of BT Vs C12 at the 12th week.

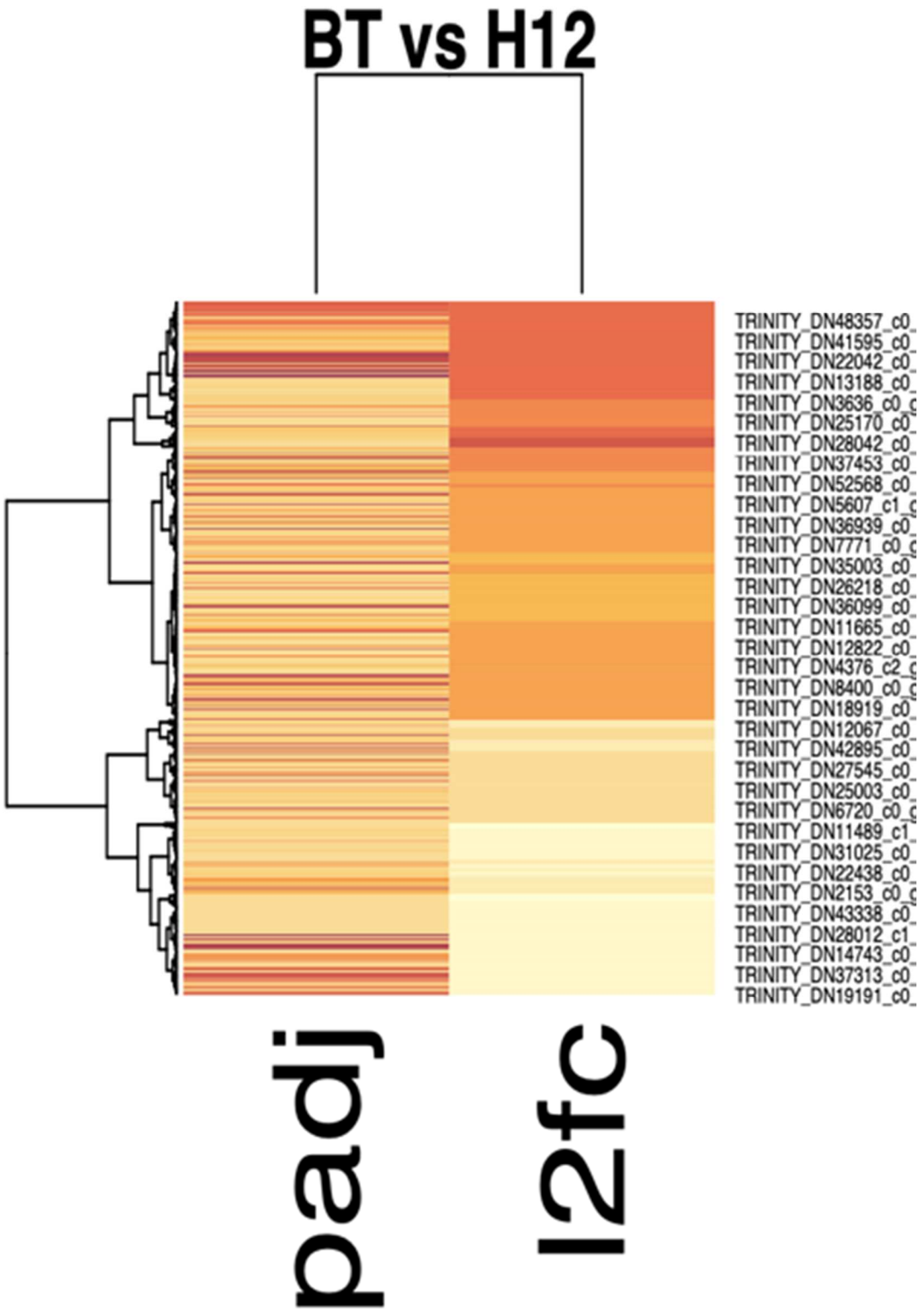

Figure 3.5: Heatmap comparison of BT Vs H12 at the 12th week.

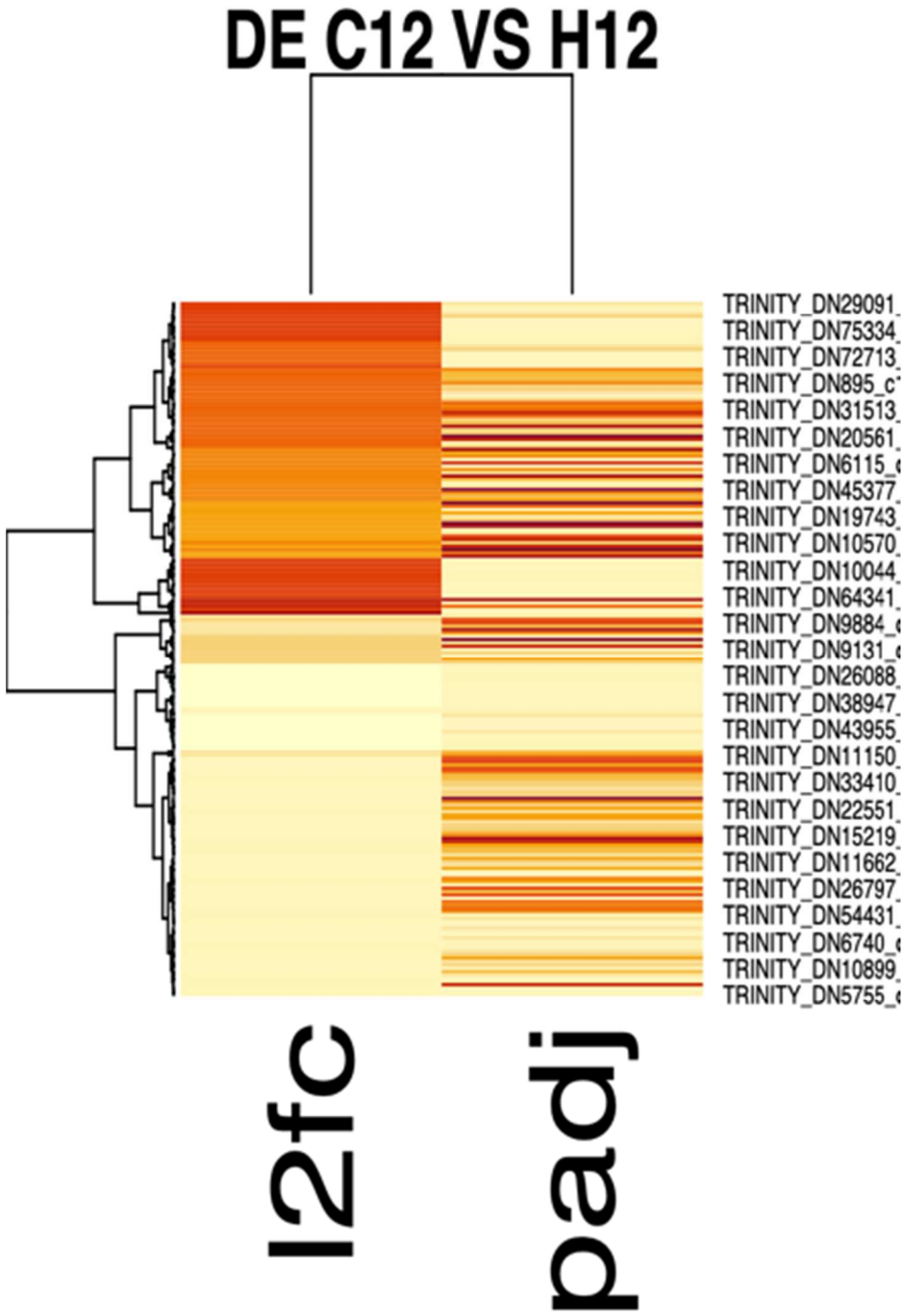

Figure 3.6. Heatmap comparison of C12 Vs H12 at the 12th week.

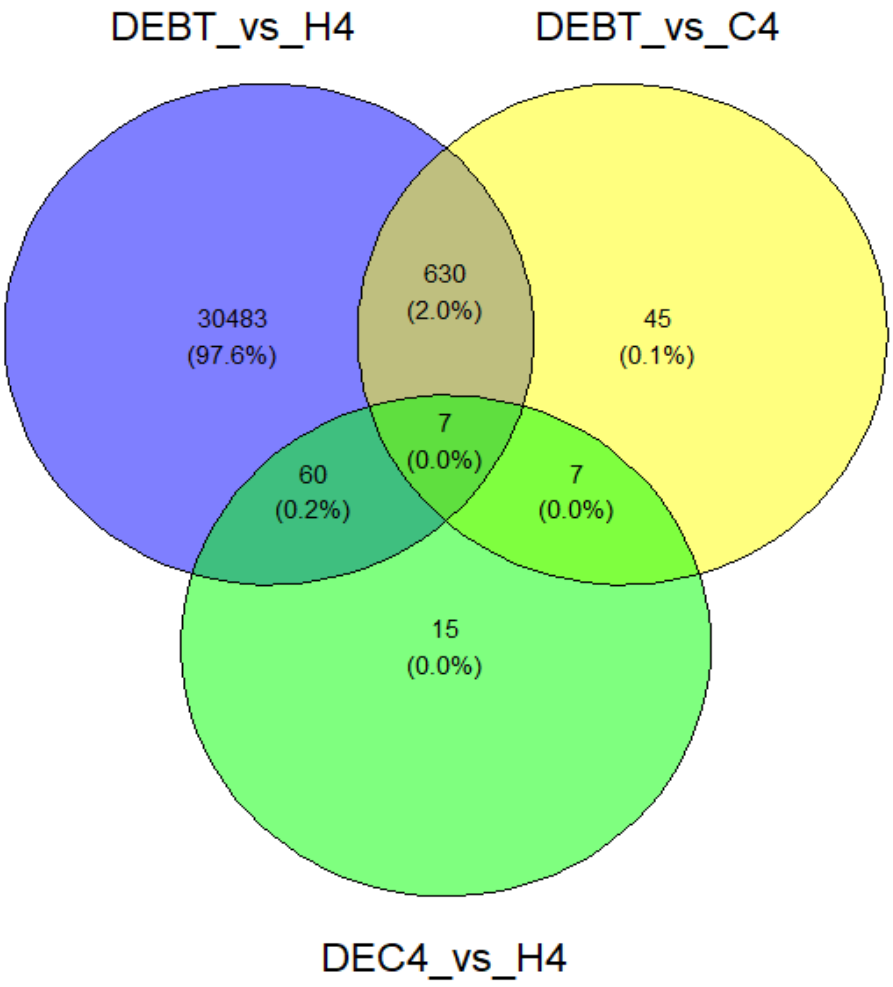

Figure 3.7: Venn Diagram Comparison of treatments at 4th week. Venn Diagram of the comparison of three states of the plant representing the number of genes that were present common in all three conditions at the 4th week. Venn diagram shows the unique and common DEGs in all three conditions at the 4th week.

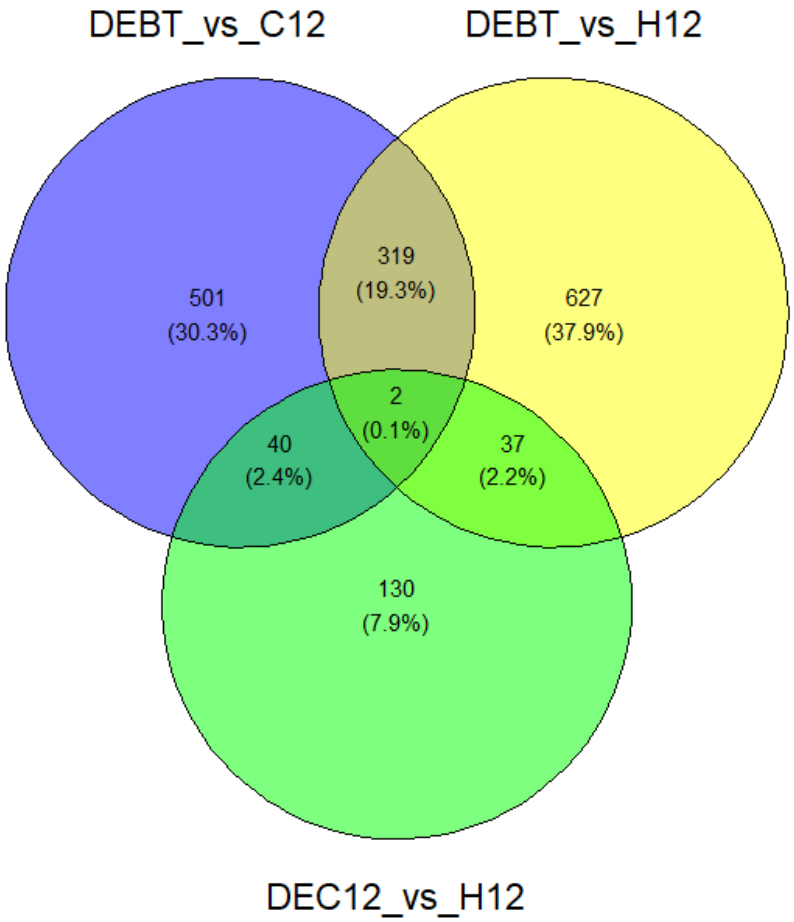

Figure 3.8: Venn Diagram Comparison of treatments at 12th week. Venn Diagram of the comparison of three states of the plant representing the number of genes that were present common in all three conditions at the 12th week. **Venn diagram shows the unique and common DEGs in all three conditions at the 12th week**

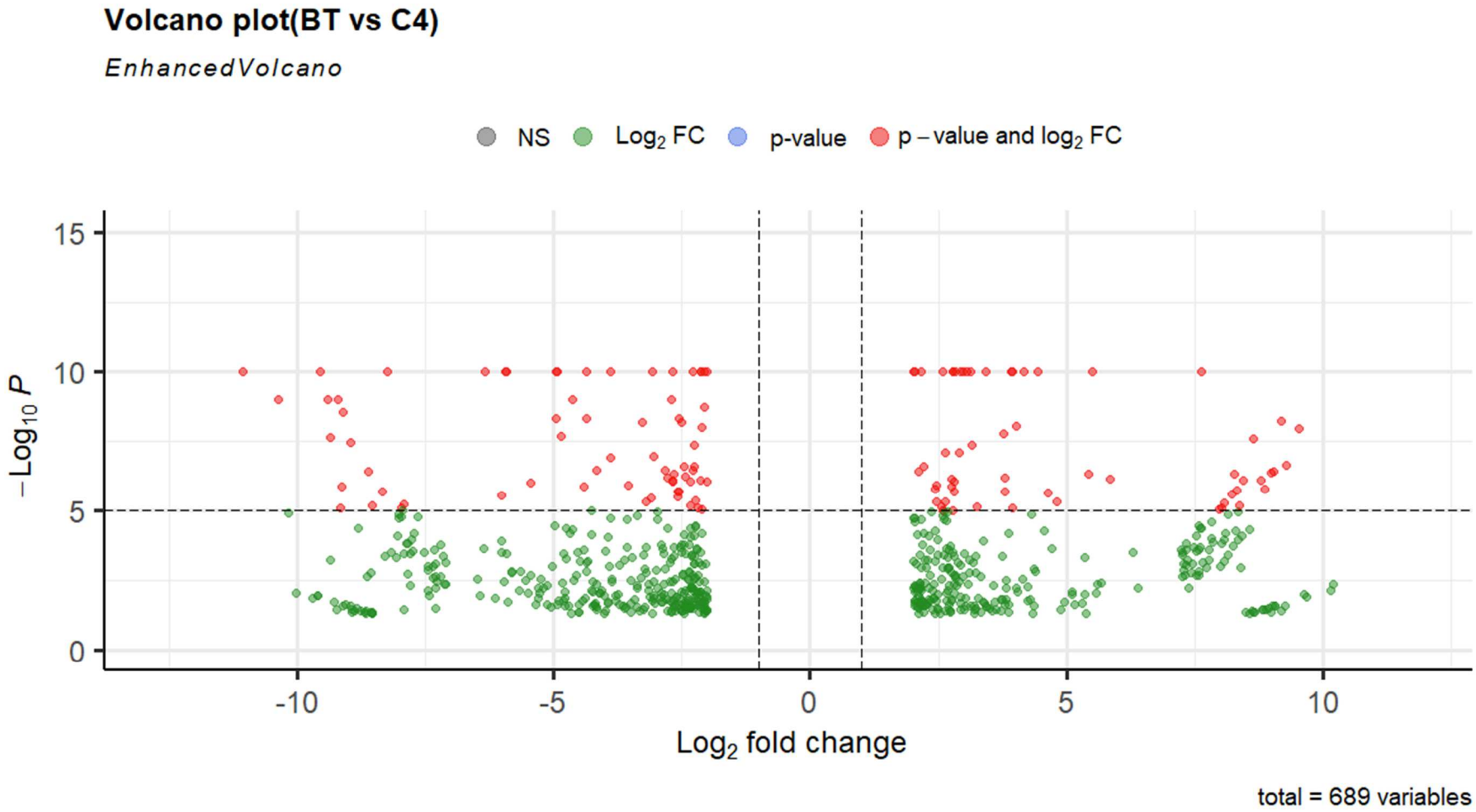

Figure 3.9: Volcano plot BT at the 4th week. Volcano plot BT vs C4 vs C4 with default log fold-change thresholds of −2 and 2 and an adjusted P-value threshold of 0.05 at the 4th week

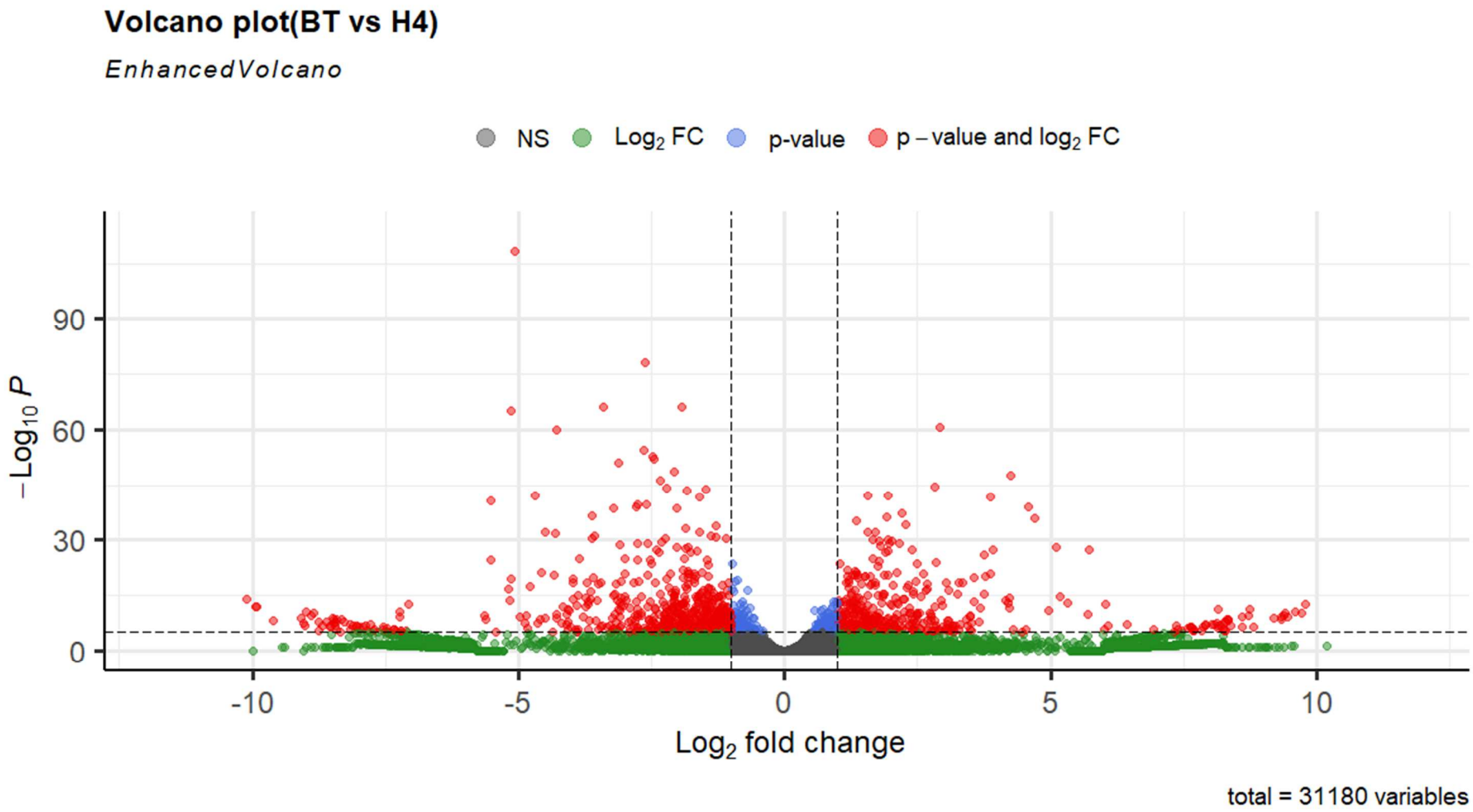

Figure 3.10: Volcano plot BT vs H4 at 4th week. Volcano plot BT vs H4 with default log fold-change thresholds of -2 and 2 and an adjusted P-value threshold of 0.05 at the 4th week.

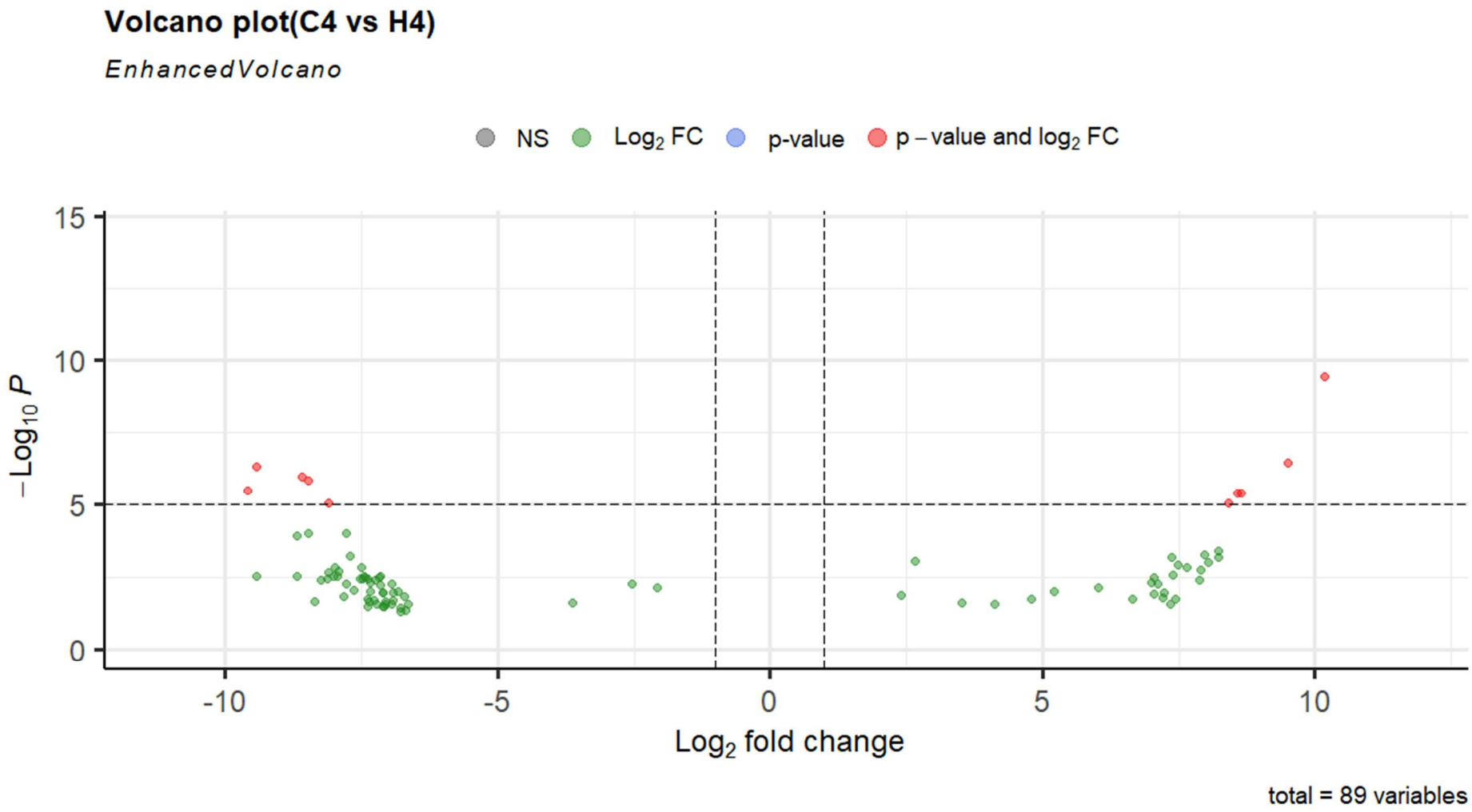

Figure 3.11: Volcano plot C4 vs H4 at 4th week. Volcano plot C4 vs H4 with default log fold-change thresholds of -2 and 2 and an adjusted P-value threshold of 0.05 at the 4th week.

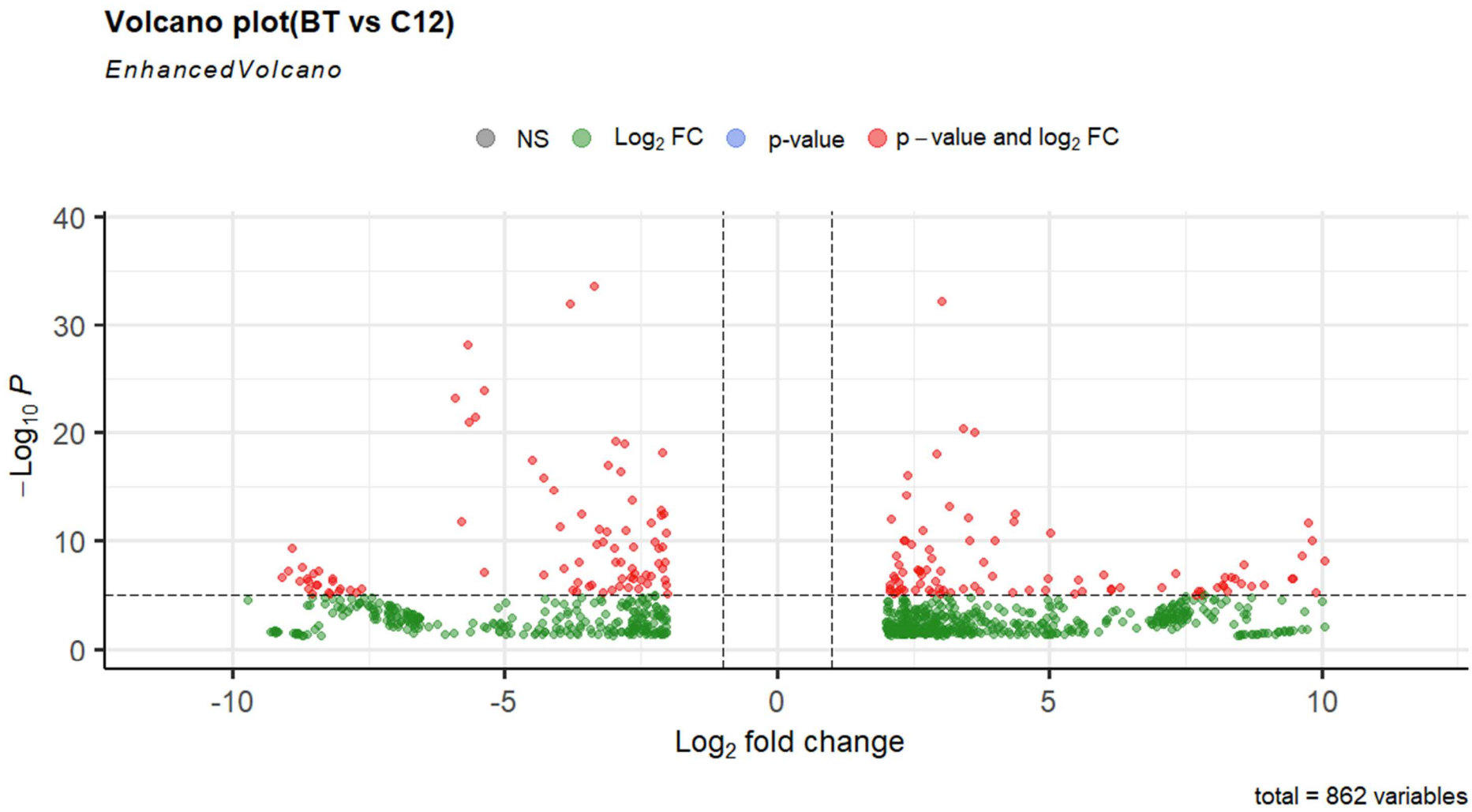

Figure 3.12: Volcano plot BT vs C12 at 12th week. Volcano plot BT vs C12 with default log fold-change thresholds of −2 and 2 and an adjusted P-value threshold of 0.05 at the 12 the week.

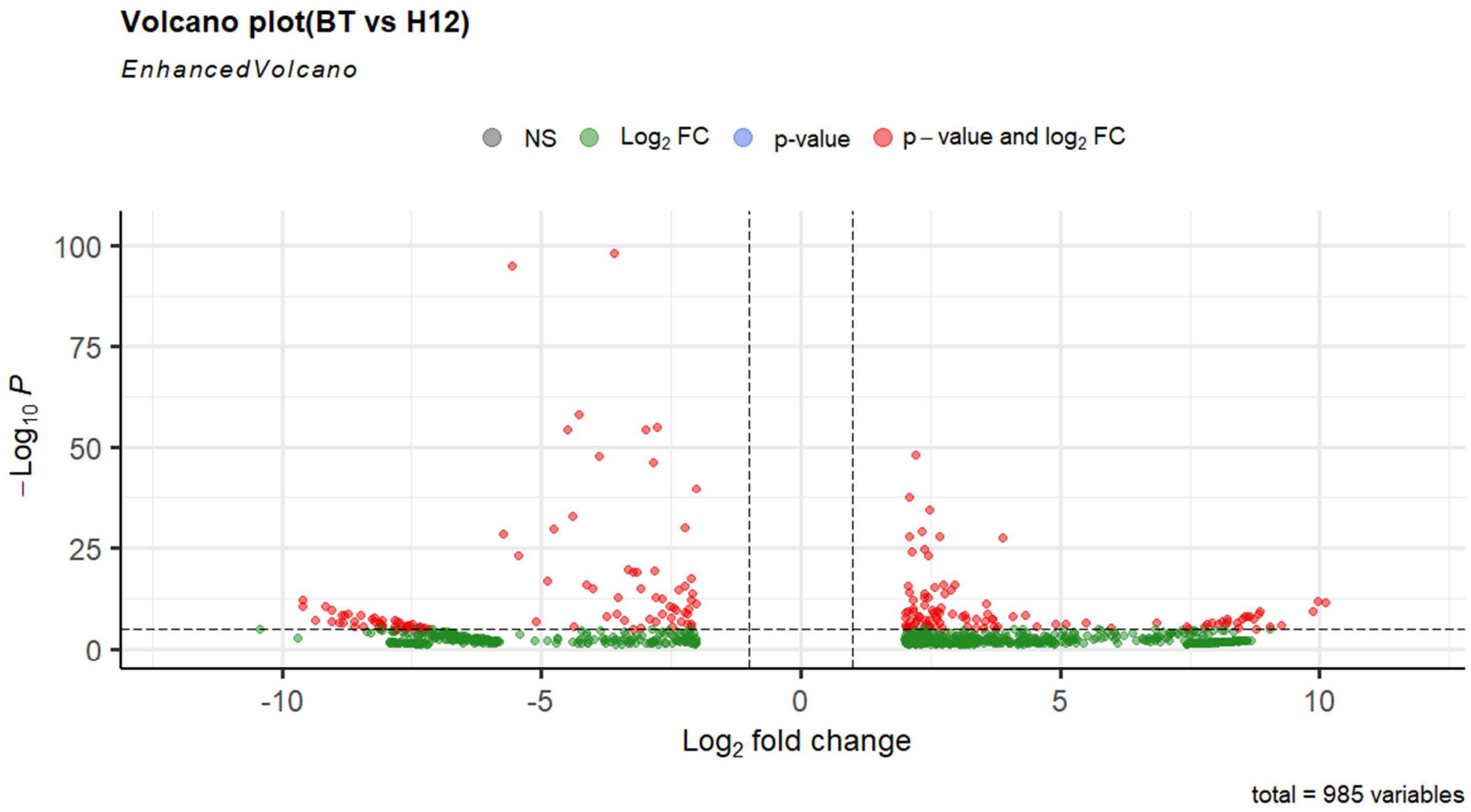

Figure 3.13: Volcano plot BT vs H12 at 12th week. Volcano plot BT vs H12 with default log fold-change thresholds of −2 and 2 and an adjusted P-value threshold of 0.05 at the 12 the week.

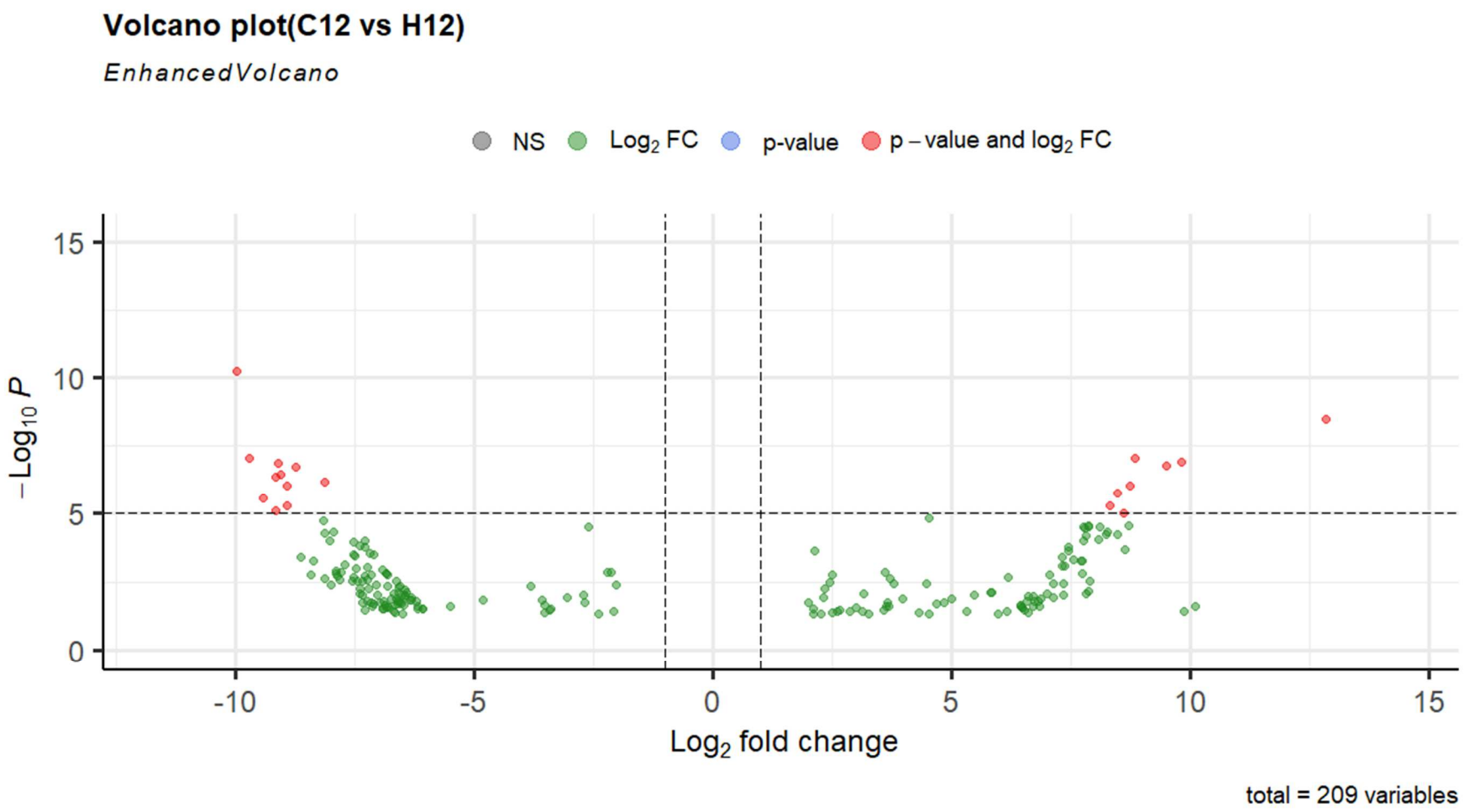

Figure 3.14: Volcano plot C12 vs H12 at 12th week. Volcano plot C12 vs H12 at 12th week with default log fold-change thresholds of −2 and 2 and an adjusted P-value threshold of 0.05 at the 12 the week

CHAPTER IV

ANALYSIS AND CONCLUSION

Infections of citrus with citrus greening disease (CGD) is a major challenge to produce this crop. Although extensive study has been undertaken on the transcriptome profile of HLB-affected citrus plants, the first steps in pathogenesis are still not well comprehended. (Wei, Mira, Yu, & Gmitter, 2021)This study evaluated two time points, four and twelve weeks of three distinct M. Paniculata treatments: (1) non-grafted and non-infected plant, grafted and non-infected plant (C), and grafted and infected plant (H). Determine the responsiveness of Orange Jasmine (M. Paniculata) to CLas infection following graft infection. These high-quality transcriptome assemblies may represent a significant step forward in the comprehension of this incredibly complicated disease, and they may complement the recently developed resources for other citrus diseases.

In this study, we had used Illumina technology to develop transcriptome assemblies. This, de novo assembly had 81,400 trinity genes arising from nine samples. However, in some of the transcriptomes there is no functional annotation available for comparison. After filtering for log to fold change (l2fc) of greater and equal to 2 and less and equal to -2 and and the adjusted P value (Padj) of P minor to 0.05. The genes of interest reduced to 6679, from which 4625 belong to the four-week treatment from samples: BT4, C4 and H4, and 2054 to the twelve-week treatment from sample BT12, C12 and H12.

Two Ven diagrams (Figures 3.7 and 3.8) were constructed to determine the relationship of three data set, one Venn Diagram per time point. The first Venn diagram compare the samples BT, C4 and H4 (Figure 3.7) and showed that from those 4625 genes, 3048 belong only to the comparison of the samples BT vs H4, 45 belong to the comparison of BT and C4 and 15 to the comparison of C4 vs H4. However, it also provides the information that 630 are present in the comparisons of BT vs H4 and BT vs C4, 60 genes are present in the comparison of BT vs H4 and C4 vs H4, and 7 genes are present on the comparison of BT vs C4 and C4 vs H4. Lastly this first diagram at four weeks reveals that only 7 genes are present on three comparisons BT vs H4,BT vs C4 and C4 vs H4.

On the second Venn diagram constructed (Figure 3.8) which evaluate the gene overlapping at the twelve-week time point, demonstrate that from the 2054 genes present on all treatment at this time point; 501 solely belong to the interaction of BT vs C12, 627 belong to the comparison of BT vs H12 and 130 from the comparison of C12 vs H12. The comparison BT vs C12 and BT vs H12 share 319 genes, the comparison of BT vs C12 and C12 vs H12 shared 40 genes in common, and the comparison of BT vs H12 and C12 vs H12 share 37 genes in common, meanwhile 2 genes were found to be in all three comparisons BT vs H12, BT vs C12 and C12 vs H12.

A volcano plot is a variation of scatterplot that depicts the relationship between statistical significance (P value) and magnitude of change (fold change). It permits rapid visual detection of genes with statistically significant and large substantial fold changes. These may be the genes with the greatest biological significance. Six volcano plots were constructed for this study that analyses the samples among them within the same timepoint: BT vs C4 (Figure 3.9) with 689 variables, BT vs H4 (Figure 3.10) with 31180 variables, C4 vs H4 (Figure 3.11) with 89

variables, BT vs C12 (Figure 3.12) with 862 variables, BT vs H12 (Figure 3.13) with 985 variables and C12 vs H12 (Figure 3.14) with 209 variables. Because the samples BT which represents the non-grafted non-infected treatment when is compared with samples C, that represent the grafted non-infected treatment gave us very useful information to identify genes up and down regulated (Using heatmaps) that are related to the healing process of the plant due to the mechanical damage suffered by the graft. Therefore, could be eliminated from the comparison of the samples C with samples H (grafted and infected) to discard genes that were present in this comparison due to both been inoculated and aid us to identify potential candidates' genes that may have relation to CGD infection resistance that *M. paniculata* has demonstrated.

From the volcano plot analysis of C4 vs H4 (Figure 3.10) and taking into considerations of the l2fc of greater and equal to 2 and less and equal to -2 and the adjusted P value (Padj) of $P < 0.05$ we were able to identify five candidate genes (Table 6.) from which four doesn't have annotated description in NCBI library, leaving just one gene (Contig TRINITY_DN33844_c0_g1; NCBI ID KDO62333.1) with an annotated description on the same library. On the other hand, during the analysis of the volcano plot of C12 vs H12 (Figure 3.14), we identify eight candidates genes (Table 6.), from which three doesn't have annotated description in NCBI library, leaving five genes (Contig TRINITY_DN1820_c5_g1; NCBI ID KDO66008.1, Contig TRINITY_DN37458_c0_g1; NCBI ID KAF1857764.1, Contig TRINITY_DN33844_c0_g1; NCBI ID XP_024039421.1, Contig TRINITY_DN100_c13466_g1; NCBI ID ESR66196.1 and Contig TRINITY_DN42068_c0_g1; NCBI ID GAY39689.1) with annotated description. It also interested that the up and down regulation almost even in both time points for the comparison of C4 vs H4, three of the

candidates were down regulated, this include the which information is available, and two were up regulated. From the comparison of C12 Vs H12, four genes were down regulated, from which two of the description is available (Contig TRINITY_DN100_c13466_g1; NCBI ID ESR66196.1 and Contig TRINITY_DN42068_c0_g1) and four were up regulated from which three of them description is available (Contig TRINITY_DN1820_c5_g1; NCBI ID KDO66008.1, Contig TRINITY_DN37458_c0_g1; NCBI ID KAF1857764.1, Contig TRINITY_DN33844_c0_g1).

For the gene KDO62333.1 the description provides refer to as a hypothetical protein CISIN_1g024706mg [Citrus sinensis]. After reviewing literature it was found that this gene aims in the regulation of heat shock proteins (HSPs) in citrus(Shafqat et al., 2020). It is described that this protein has been found to be produce and secreted during period of abiotic and biotic stress of the citrus.(Shafqat et al., 2020)

For the gene KDO66008.1 the description provides refer to as hypothetical protein CISIN_1g025482mg [Citrus sinensis]. And its analysis relates it to the proteins in the exosome complex component RRP41 homolog. Which is required for normal development of female gametophytes(Chekanova et al., 2007). Is made reference that the lack of this protein creates Endosperm mutants developed to varied degrees, but never advanced beyond the cellularization stage.(Chekanova et al., 2007). Is important to recall that cell cycle 14 marks the beginning of cellularization, the process that generates a distinct cell membrane for each nucleus. This procedure involves the synchronous ingression of membrane surrounding each nucleus to form an epithelial cell sheet.

For the contig KAF1857764.1 The description provided refer to hypothetical protein Lal_00041143 [Lupinus albus]. According to the literature review this protein belongs to a group of auxin modulators, a group of hormones that control the plan grow and some morphologic characteristics. This protein in conjunction to several other, allow the plant to maximize the phosphorus uptake efficient particularly in soils with low amount of this element (Xu et al., 2020). Phosphorus is essential to ATP, the "energy unit" of plants. Phosphorus is present in the structure of ATP, which is produced during photosynthesis and functions from the commencement of seedling growth through the production of grain and maturity. Phosphorus is therefore necessary for the general health and vitality of all plants. (Meng et al., 2021).

For the contigs: XP_024039421.1, ESR66196.1 and GAY39689.1 The contig XP_024039421.1 the description provided refer to as a LOW QUALITY PROTEIN: uncharacterized protein LOC112098046 [Citrus clementina]. For the contig ESR66196.1 the description provided refer to as a hypothetical protein CICLE_v10007851mg [Citrus clementina] domain found in CTC-interacting domain proteins CID5, CID6, CID7 and similar proteins; cd14371 and Finally, for contig GAY39689.1 the description provided refer to as a hypothetical protein CUMW_046370 [Citrus unshiu]. For these three genes, there was no additional information provided in the literature in regard to their function or possible contribution in citrus.

Further experimentation is required for the contigs that were identified and have functional description has relationship to grow process and plant macronutrients absorption, which possible can lead to explain *M. Paniculata* resistant to CGD.

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APPENDIX

APPENDIX

Sample	**BTA**		**BTB**		**BTC**	
Parameters of Filtering						
% of Uncalled bases(N)	5		5		5	
Mean quality value threshold	20		20		20	
Minimum size of reads	0		0		0	
Filtering mode for quality value	Filter		Filter		Filter	
Filtering Statistics for given Paired						
Quality format	illumina 1.8+	illumina 1.8+	ill+L17:M38umina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+
Total number of reads analyzed	20317096	20317096	22445677	22445677	20367546	20367546
Total Bases (ATGC) in unfiltered reads (bp)	3047154883	3047326269	3366400451	3366590200	3054717821	3054890164
Total Bases (ATGC) in filtered reads (bp)	3031228364	3031387607	3346425632	3346601325	3042021364	3042182114
Minimum size (bp) of unfiltered reads	150	150	150	150	150	150
Maximum size (bp) of unfiltered reads	150	150	150	150	150	150
Mean size (bp) of unfiltered reads	150	150	150	150	150	150
Average Quality value for unfiltered reads	39.16413008	38.16151129	39.15504324	38.17527201	39.6951939	38.83671133
Average Quality value for filtered reads	39.18689598	38.26174526	39.17542378	38.29001252	39.71212719	38.91863198
Total number of reads below minimum size	0	0	0	0	0	0
Total unfiltered reads containing at least one uncall	313046	208546	344927	229802	316411	211011
Total filtered reads containing at least one uncalled	307362	204838	338479	225582	311080	207427
Reads filtered out with more than given % of N	3261	1443	3482	1529	3291	1513
Reads filtered out for quality	6266	97031	6286	123570	4191	78086
Average %GC content in unfiltered reads	45.98567312	46.02125008	45.81518873	45.86445556	45.58229967	45.61397595
Average %GC content in filtered reads	45.98245301	45.99911938	45.81355892	45.84364701	45.58157483	45.59669453
Total unfiltered reads with >Q30 mean quality value	19155095	18622063	21165195	20570288	19995605	19567527
Total filtered reads with >Q30 mean quality value	19074172	18617340	21057550	20565144	19926289	19562382
Total number of reads kept	20210649	20210649	22312218	22312218	20282631	20282631
Minimum size (bp) of filtered reads	150	150	150	150	150	150
Maximum size (bp) of filtered reads	150	150	150	150	150	150
Mean size (bp) of filtered reads	150	150	150	150	150	150
The total number of sequences removed	**106447**		**133459**		**84915**	

Table 1. Quality Control of Data BT. Filtering parameters and Statistical filtering of Untreated Plants (BT) in triplicates.

Sample	**C4A**		**C4B**		**C4C**	
Parameters of Filtering						
% of Uncalled bases(N)	5		5		5	
Mean quality value threshold	20		20		20	
Minimum size of reads	0		0		0	
Filtering mode for quality value	Filter		Filter		Filter	
Filtering Statistics for given Paired						
Quality format	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+
Total number of reads analyzed	25149080	25149080	24568569	24568569	24637733	24637733
Total Bases (ATGC) in unfiltered reads (bp)	3771857441	3772069548	3684789719	3684996451	3695163938	3695371952
Total Bases (ATGC) in filtered reads (bp)	3754428918	3754625217	3673163421	3673356303	3677026154	3677218626
Minimum size (bp) of unfiltered reads	150	150	150	150	150	150
Maximum size (bp) of unfiltered reads	150	150	150	150	150	150
Mean size (bp) of unfiltered reads	150	150	150	150	150	150
Average Quality value for unfiltered reads	39.65092523	38.6193685	39.8327085	38.9422591	39.3562452	38.3505103
Average Quality value for filtered reads	39.6704094	38.70973763	39.8486126	39.0035434	39.3773613	38.4450442
Total number of reads below minimum size	0	0	0	0	0	0
Total unfiltered reads containing at least one uncall	384529	255414	378448	252825	379162	252799
Total filtered reads containing at least one uncalled	377593	250922	372284	248766	372374	248350
Reads filtered out with more than given % of N	4089	1827	3923	1741	3972	1759
Reads filtered out for quality	4081	108116	3014	70695	6316	110796
Average %GC content in unfiltered reads	46.26551699	46.3745824	45.5866696	45.6914006	45.5750753	45.9462347
Average %GC content in filtered reads	46.26512486	46.35536523	45.585926	45.6740174	45.5726314	45.9263192
Total unfiltered reads with >Q30 mean quality value	24544253	23838438	24242109	23672518	23490376	22838762
Total filtered reads with >Q30 mean quality value	24449810	23832450	24181130	23666815	23396145	22832839
Total number of reads kept	25032550	25032550	24490741	24490741	24516488	24516488
Minimum size (bp) of filtered reads	150	150	150	150	150	150
Maximum size (bp) of filtered reads	150	150	150	150	150	150
Mean size (bp) of filtered reads	150	150	150	150	150	150
The total number of sequences removed	116530		77828		121245	

Table 2. Quality Control of Data C4. Filtering parameters and Statistical filtering of Plants with mechanical damage (Grafting) (C) in triplicates at fourth week.

Sample	**C12A**		**C12B**		**C12C**	
Parameters of Filtering						
% of Uncalled bases(N)	5		5		5	
Mean quality value threshold	20		20		20	
Minimum size of reads	0		0		0	
Filtering mode for quality value	Filter		Filter		Filter	
Filtering Statistics for given Paired						
Quality format	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+
Total number of reads analyzed	19353632	19353632	27185844	27185844	30116065	30116065
Total Bases (ATGC) in unfiltered reads (bp)	2.9E+09	2.9E+09	4.08E+09	4.08E+09	4.52E+09	4.52E+09
Total Bases (ATGC) in filtered reads (bp)	2.88E+09	2.88E+09	4.06E+09	4.06E+09	4.5E+09	4.5E+09
Minimum size (bp) of unfiltered reads	150	150	150	150	150	150
Maximum size (bp) of unfiltered reads	150	150	150	150	150	150
Mean size (bp) of unfiltered reads	150	150	150	150	150	150
Average Quality value for unfiltered reads	39.10897	37.94433	39.26815	38.32698	39.77652	38.8174
Average Quality value for filtered reads	39.1313	38.08629	39.28608	38.41111	39.79356	38.8997
Total number of reads below minimum size	0	0	0	0	0	0
Total unfiltered reads containing at least one uncall	296283	197853	418744	279408	462795	308436
Total filtered reads containing at least one uncalled	290078	193811	411453	274573	454909	303220
Reads filtered out with more than given % of N	3092	1294	4408	1960	4825	2052
Reads filtered out for quality	5346	133858	6607	108670	4166	117154
Average %GC content in unfiltered reads	45.75156	45.96747	45.78645	45.86441	45.5375	45.76272
Average %GC content in filtered reads	45.75068	45.94646	45.78412	45.84544	45.53708	45.74582
Total unfiltered reads with >Q30 mean quality value	18122103	17488645	25711611	25066923	29585603	28836620
Total filtered reads with >Q30 mean quality value	18004319	17484293	25618404	25060894	29481523	28829434
Total number of reads kept	**19211199**	**19211199**	**27065990**	**27065990**	**29989547**	**29989547**
Minimum size (bp) of filtered reads	150	150	150	150	150	150
Maximum size (bp) of filtered reads	150	150	150	150	150	150
Mean size (bp) of filtered reads	150	150	150	150	150	150
The total number of sequences removed	142433		119854		126518	

Table 3. Quality Control of Data C12. Filtering parameters and Statistical filtering of Plants with mechanical damage (Grafting) (C) in triplicates at twelfth week.

Sample	**H4D**		**H4E**		**H4F**	
Parameters of Filtering						
% of Uncalled bases(N)	5		5		5	
Mean quality value threshold	20		20		20	
Minimum size of reads	0		0		0	
Filtering mode for quality value	Filter		Filter		Filter	
Filtering Statistics for given Paired						
Quality format	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+
Total number of reads analyzed	20884076	20884076	53921600	53921600	20947025	20947025
Total Bases (ATGC) in unfiltered reads (bp)	3.13E+09	3.13E+09	8.09E+09	8.09E+09	3.14E+09	3.14E+09
Total Bases (ATGC) in filtered reads (bp)	3.12E+09	3.12E+09	8.07E+09	8.07E+09	3.13E+09	3.13E+09
Minimum size (bp) of unfiltered reads	150	150	150	150	150	150
Maximum size (bp) of unfiltered reads	150	150	150	150	150	150
Mean size (bp) of unfiltered reads	150	150	150	150	150	150
Average Quality value for unfiltered reads	39.74635	38.77195	39.68327	38.86165	38.16869	37.21151
Average Quality value for filtered reads	39.76625	38.84831	39.69739	38.91282	38.19084	37.29293
Total number of reads below minimum size	0	0	0	0	0	0
Total unfiltered reads containing at least one uncall	318961	212690	832656	553329	325494	216442
Total filtered reads containing at least one uncalled	313466	209042	819463	544770	319826	212704
Reads filtered out with more than given % of N	3383	1545	8764	3744	3426	1483
Reads filtered out for quality	3306	74459	7085	128784	10160	82194
Average %GC content in unfiltered reads	46.36104	46.15493	46.65054	46.39836	46.78331	46.88145
Average %GC content in filtered reads	46.35947	46.12671	46.64988	46.38152	46.77882	46.86185
Total unfiltered reads with >Q30 mean quality value	20602607	20060281	52878439	51811898	18242190	17782601
Total filtered reads with >Q30 mean quality value	20539789	20055346	52767112	51799157	18177512	17777797
Total number of reads kept	20803210	20803210	53776134	53776134	20851118	20851118
Minimum size (bp) of filtered reads	150	150	150	150	150	150
Maximum size (bp) of filtered reads	150	150	150	150	150	150
Mean size (bp) of filtered reads	150	150	150	150	150	150
The total number of sequences removed	80866		**145466**		**95907**	

Table 4. Quality Control of Data H4. Filtering parameters and Statistical filtering of Plants with Grafting and pathogen (H) in triplicates at fourth week.

Sample	**H12D**		**H12E**		**H12F**	
Parameters of Filtering						
% of Uncalled bases(N)	5		5		5	
Mean quality value threshold	20		20		20	
Minimum size of reads	0		0		0	
Filtering mode for quality value	Filter		Filter		Filter	
Filtering Statistics for given Paired						
Quality format	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+	illumina 1.8+
Total number of reads analyzed	23246725	23246725	27566856	27566856	75644889	75644889
Total Bases (ATGC) in unfiltered reads (bp)	3.49E+09	3.49E+09	4.13E+09	4.13E+09	1.13E+10	1.13E+10
Total Bases (ATGC) in filtered reads (bp)	3.47E+09	3.47E+09	4.12E+09	4.12E+09	1.13E+10	1.13E+10
Minimum size (bp) of unfiltered reads	150	150	150	150	150	150
Maximum size (bp) of unfiltered reads	150	150	150	150	150	150
Mean size (bp) of unfiltered reads	150	150	150	150	150	150
Average Quality value for unfiltered reads	39.68823	38.85826	39.73257	38.7659	39.84093	38.90294
Average Quality value for filtered reads	39.70472	38.92541	39.7509	38.84982	39.85714	38.9577
Total number of reads below minimum size	0	0	0	0	0	0
Total unfiltered reads containing at least one uncall	359057	239337	423948	282560	1165473	776976
Total filtered reads containing at least one uncalled	353102	235493	416718	277907	1146793	764660
Reads filtered out with more than given % of N	3805	1601	4341	1868	12258	5451
Reads filtered out for quality	3800	73096	4259	108924	8082	195079
Average %GC content in unfiltered reads	45.96511	46.0412	45.68411	45.90506	45.88946	45.86905
Average %GC content in filtered reads	45.96455	46.02548	45.68353	45.8877	45.88881	45.85149
Total unfiltered reads with >Q30 mean quality value	22739585	22247113	27052663	26360828	74693770	72902547
Total filtered reads with >Q30 mean quality value	22675991	22241295	26957356	26354216	74529111	72885529
Total number of reads kept	23165813	23165813	27449235	27449235	75428237	75428237
Minimum size (bp) of filtered reads	150	150	150	150	150	150
Maximum size (bp) of filtered reads	150	150	150	150	150	150
Mean size (bp) of filtered reads	150	150	150	150	150	150
The total number of sequences removed	**80912**		**117621**		**216652**	

Table 5. Quality Control of Data H12. Filtering parameters and Statistical filtering of Plants with Grafting and pathogen (H) in triplicates at twelfth week.

C4 VS H14

TRINITY ID (Contig)	NCBI ID	l2fc	padj	Description
TRINITY_DN196_c14_g1	*Unavailable*	10.1752	3.59E-10	*Unavailable*
TRINITY_DN100_c12137_g1	KDO62333.1	-9.3483	2.29E-08	hypothetical protein CISIN_1g024706mg [Citrus sinensis]
TRINITY_DN35125_c1_g1	*Unavailable*	8.4118	8.33E-06	*Unavailable*
TRINITY_DN30039_c0_g1	*Unavailable*	-8.5912	1.06E-06	*Unavailable*
TRINITY_DN2182_c1_g1	*Unavailable*	-0.2937	9.50E-01	*Unavailable*

C12 vs H12

TRINITY ID (Contig)	NCBI ID	l2fc	padj	Description
TRINITY_DN49177_c0_g1	*Unavailable*	12.8355	3.23E-09	*Unavailable*
TRINITY_DN1820_c5_g1	KDO66008.1	8.8579	8.92E-08	hypothetical protein CISIN_1g025482mg [Citrus sinensis]
TRINITY_DN37458_c0_g1	KAF1857764.1	8.7565	9.59E-07	hypothetical protein Lal_00041143 [Lupinus albus]
TRINITY_DN33844_c0_g1	XP_024039421.1	8.3223	5.28E-06	LOW QUALITY PROTEIN: uncharacterized protein LOC112098046 [Citrus clementina]
TRINITY_DN100_c13466_g1	ESR66196.1	-9.9687	5.89E-11	hypothetical protein CICLE_v10007851mg [Citrus clementina]
TRINITY_DN42197_c0_g3	*Unavailable*	-8.7170	1.85E-07	*Unavailable*
TRINITY_DN42068_c0_g1	GAY39689.1	-8.9050	9.59E-07	hypothetical protein CUMW_046370 [Citrus unshiu]
TRINITY_DN26088_c0_g1	*Unavailable*	-8.9008	5.28E-06	*Unavailable*

Table 6: Candidate Genes for *M. Paniculata* Resistance to Citrus Greening Disease. The table present the potential candidates genes identified that could have relationship with the resistance of *M. Paniculata* to Citrus Greening Disease

BIOGRAPHICAL SKETCH

Jeffrey Aquino Gomez was born in Aguadilla, Puerto Rico, where he attended elementary school, middle school, and high school. In June 2012, he received his Bachelor of Science in Biology from The Interamerican University of Puerto Rico- Aguadilla Campus, where he completed his undergraduate studies. In 2018, he graduated from Touro University with a Master of Arts in Industrial and Organizational Psychology with a concentration in Human Resource Management and Magna Cum Laude Honors. In January of 2019, he was accepted into a Master of Science program at The University of Texas, Rio Grande Valley, from which he graduated in August of 2022.

Mr. Aquino has held many positions in a range of organizations and departments throughout his career. From 2009 to 2014, he was employed by Walgreens Boots Alliance, Puerto Rico's Region, where he exercised several roles in Retails Operations. In 2014, he was promoted and transitioned to Field Human Resources for the District Puerto Rico South, where he oversaw the training and development of 30 stores. He served the South Texas Area and oversaw 83 stores in training, development, and performance management from South Houston (Fort Bend-Sugar Land) to Brownsville, Texas, after being selected as Organizational Learning Specialist and relocating to the Texas Region following a company-wide organizational restructuring in late 2015.

In 2016, he becomes the General Manager of Sunoco Company, a post he held until 2017, when he accepted the General Manager position at The Vitamin Shoppe in Corpus Christi, Texas. He relocated to the Rio Grande Valley in 2018 after receiving a promotion to a higher load store.

In 2018, he chose to leave the retail industry and begin a new profession as a Laboratory Specialist II in the Biology Department at South Texas College. From September 2018 to August 2021, when he was promoted to Biology Lecturer, he held this job. Mr. Aquino-Gomez obtained his permanent position and promotion to Biology Instructor in August 2022.

Jeffrey Aquino Gomez can be contacted through his email account aquino.gomez@gmail.com.