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Ana Sofia da Silva Duarte

Dissertação

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Resumo

A doença da tinta tem dizimado milhares de hectares de soutos na Europa desde o século XX, o que conduziu a um decréscimo na produtividade de madeira e castanha. O agente causador desta doença é um oomiceta, *Phytophthora cinnamomi* Rands, que possui formas de sobrevivência e disseminação, permitindo-lhe persistir nos solos. Quando as condições de humidade e temperatura são favoráveis, são produzidos zoósporos que se movimentam através da água e são conduzidos até às raízes não-lenhificadas. A infecção propaga-se até ao câmbio e floema que evolui para necrose das raízes. Na parte aérea da planta, os primeiros sintomas manifestam-se já numa fase mais avançada da doença. A presença de folhas de menores dimensões, amarelecidas e dessecadas, frutos de pequenas dimensões e a presença de um exsudado escuro são sintomas característicos da doença. O oomiceta está distribuído mundialmente e abrange mais de 950 hospedeiros, sendo, por isso, difícil controlar a sua dispersão. O castanheiro europeu, *Castanea sativa* Mill. é sensível à doença, no entanto, as espécies asiáticas, *Castanea crenata* e *Castanea mollissima* conseguem sobreviver em solos infectados. Com objectivo em reduzir as perdas, foram implementados programas de melhoramento do castanheiro. Através de sucessivos cruzamentos controlados entre as espécies asiáticas resistentes e a espécie europeia é teoricamente possível introduzir genes de resistência à doença. A obtenção de híbridos por este método e a sua utilização como porta-enxertos contribuiu para o melhoramento da compatibilidade em ensaios de enxertia com variedades nacionais, mantendo as características da qualidade da madeira e fruto do castanheiro europeu. No entanto, os genes que conferem resistência à doença da tinta e os seus mecanismos moleculares associados não foram ainda caracterizados. Sabe-se que as plantas desenvolveram mecanismos de defesa distintos em resposta a patogéneos. O primeiro baseia-se na detecção de elicitadores secretados pelo patogéneo que promovem alterações conformacionais e transdução de sinal conduzindo a uma resposta hipersensível, fortalecimento da parede celular em tecidos não infectados e morte celular no local da infecção. O segundo mecanismo baseia-se no reconhecimento de efectores através de genes de resistência denominados por genes R, que também levam à activação da transdução de sinal via MAPK e uma resposta hipersensível. Desta forma é possível restringir o crescimento de patogéneos biotróficos. No entanto, *Phytophthora cinnamomi* é hemibiotrófica, ou seja, tem capacidade de alternar o modo de obtenção de nutrientes para fases de necrotrofia e assim garantir a sua sobrevivência a partir de tecidos vegetais em necrose. Sabe-se que a biossíntese de ácido salicílico, assim como as interacções de outras hormonas como o ácido jasmónico, auxinas, ácido abcísico e etileno estão relacionadas

com a modulação da resposta de defesa a patogéneos hemibiotróficos. A activação dos dois mecanismos induz uma resistência sistémica que é conhecida por Resistência Sistémica Adquirida. Em subsequentes infecções verifica-se a redução dos sintomas da doença, devido à expressão de genes de defesa e acumulação de proteínas relacionadas com a patogénese, proteínas PR que dependem da acumulação de ácido salicílico. As interacções entre os três mecanismos de sinalização, a regulação e obtenção de resistência a *Phytophthora cinnamomi* ainda não foram caracterizados.

Foi recentemente iniciada uma abordagem transcriptómica, com objectivo em compreender a expressão diferencial de genes em *C.sativa* e *C.crenata* após inoculação com *Phytophthora cinnamomi*, tendo-se obtido a sequenciação de quatro bibliotecas de cDNA através da plataforma 454. Através da comparação das bibliotecas de *C.crenata* inoculado e não inoculado foram identificados 283 genes diferencialmente expressos. Este trabalho tem como principal objectivo a selecção de genes de resistência nessas sequências, e ulterior validação, através da sua quantificação por PCR digital contribuindo assim para a caracterização das interacções moleculares existentes no sistema Castanheiro-*Phytophthora cinnamomi*. Foram desenhados primers para vinte genes diferencialmente expressos, que por análise de homologia funcional, revelaram estar envolvidos no reconhecimento e regulação da resposta do hospedeiro, regulação da resposta à seca e stress hídrico, fortalecimento da parede celular, síntese de enzimas e metabolitos com propriedades anti-fúngicas. Para o estudo foram seleccionadas e produzidas por micropropagação, cinquenta e quatro plantas provenientes de sete genótipos por apresentarem diferentes níveis de resistência. Para representar os extremos de sensibilidade e resistência foi seleccionado um genótipo de *C.sativa* e outro de *C.crenata* respectivamente e ainda quatro híbridos com níveis intermédios de resistência, provenientes de cruzamentos entre *C.sativa* e *C.crenata,* e um híbrido resistente proveniente do cruzamento entre *C.sativa* x *C.mollissima.* Três réplicas biológicas, ou seja, plantas do mesmo genótipo que apresentavam desenvolvimento radicular e condições fisiológicas semelhantes 80 dias após a fase de aclimatação foram inoculadas com *Phytophthora cinnamomi*. As raízes inoculadas foram recolhidas às 24 ou 48 horas após a inoculação. Foram ainda recolhidas doze raízes não inoculadas provenientes dos genótipos *C.sativa* e *C.crenata*, que foram utilizadas como controlo. Procedeu-se à extracção de RNA e após o tratamento com DNase, verificou-se a existência de degradação em algumas amostras. Constatou-se que o desenvolvimento das raízes poderá não ter sido adequado à extracção de grandes quantidades de RNA com boa qualidade e por não ser possível obter novas plantas micropropagadas e aclimatadas, procedeu-se à concentração das amostras e síntese de cDNA.

A especificidade dos primers foi avaliada através da análise das curvas de dissociação obtidas por PCR em tempo real. Dezassete pares de primers revelaram ser específicos para os genes seleccionados. Para a quantificação numa primeira abordagem, seleccionaram-se oito genes envolvidos em diferentes níveis de regulação da defesa. *WRKY transcription factor 31* envolvido na transdução de sinal em resposta aos níveis de auxinas. *RING finger protein 5* que em presença de elicitadores regula a resposta hipersensível e morte celular programada. *Ethylene-responsive transcription factor ABR1* regula negativamente a via do ácido abscísico, que quando acumulado conduz ao decréscimo do potencial hídrico, da condutância estomática e da taxa fotossintética. *Myb-related protein Myb4* contribui para a produção de lenhina e fitoalexinas que impedem a colonização do patogéneo. *C2 domain-containing protein* envolvida na transdução de sinal após a percepção do agente patogéneo e mecanismos de morte celular programada. *Cysteine-rich repeat secretory protein 38* (Gnk) apresenta homologia a uma proteína secretada pelas sementes de Gingko e a indução está relacionada com a acumulação de ácido salicílico e espécies reactivas de oxigénio. As proteínas citoplasmáticas com domínios NBS-LRR (*LRR receptor-like serine/threonine-protein kinase)* participam na detecção e reconhecimento do patogéneo, tendo grande afinidade para efectores do patogéneo e representam uma família importante de genes de resistência. A enzima *Pectinesterase 2* catalisa uma reacção de de-esterificação da pectina e possibilita que iões de cálcio se intercalem fortalecendo as paredes celulares e impedindo a colonização.

A expressão foi analisada por PCR digital, com o sistema QuantStudio™ 3D Digital PCR (Life Technologies, Carlsbad, CA). Esta é uma técnica recente e inovadora, que se baseia na partição da amostra em 20.000 reacções individuais contidas num chip, possibilitando uma quantificação absoluta e por isso, não necessita de genes de referência. O software permite fazer a análise das três réplicas biológicas em conjunto e assim comparar os valores dos rácios de *fold change* obtidos por esta técnica com os resultados obtidos pela plataforma 454. A precisão dos resultados de quantificação de cada genótipo foi comprometida pela qualidade das amostras individuais, tendo-se verificado um maior número de cópias nos genótipos para os quais as amostras de RNA correspondentes têm rácios de A_{260}/A_{280} e A_{260}/A_{230} superiores a 1,5 e 1 respectivamente. A análise separada das amostras com RNA de melhor qualidade revelou que os genes *RING finger protein 5, Ethylene-responsive transcription factor ABR1* e *Cysteine-rich repeat secretory protein 38* (Gnk) apresentam níveis de expressão mais elevados nos genótipos mais resistentes. Estes genes poderão ter um papel importante no mecanismo de resistência, sugerindo o envolvimento das vias de sinalização do etileno e ácido salicílico deste modo, novos ensaios deverão ser desenvolvidos futuramente.

Palavras-chave: Castanheiro, *Phytophthora cinnamomi,* genes de resistência, quantificação, PCR digital

ABSTRACT

Ink disease is responsible for severe losses and poses a serious threat for European chestnut (*Castanea sativa*) populations. *Phytophthora cinnamomi* is a widely distributed oomycete that persists on soil or infected plants and penetrates non-lignified root tissues causing the decline and death of chestnut trees. Asian species revealed to be resistant in the presence of pathogen. As it is difficult to control effectively and limit the spread of the disease, the establishment of breeding programs with these species allowed the introgression of resistance genes and development of hybrids with different levels of resistance and improved grafting compatibility. Genes and mechanisms underlying resistance remain to be disclosed. The comparison of inoculated and non-inoculated *C.crenata* cDNA libraries obtained by 454 sequencing revealed the presence of 283 differentially expressed genes. To provide the first insights into Chestnut-*Phytophthora* interactions, this study involved the production of fifty-four chestnut plants by micropropagation techniques: *C.sativa*, *C.crenata*, one *C.sativa* x *C.mollissima* and four *C.sativa* x *C.crenata* hybrids with different levels of resistance to *P.cinnamomi*. RNA was extracted from inoculated roots collected at 24 and 48 hours post-infection and non-inoculated control plants. Primers were designed from twenty DEG sequences selected as strong candidates for resistance by homology and functional annotation analysis. Transcript abundance levels of eight candidate genes coding for *C2 domain-containing protein, WRKY transcription factor 31, Cysteine-rich repeat secretory protein 38 (Gnk), Ethylene-responsive transcription factor ABR1, Myb-related protein Myb4, LRR receptor-like serine/threonine-protein kinase, RING finger protein 5 and Pectinesterase 2* was assessed by digital PCR. Gene expression accuracy was compromised by RNA quality and it was not possible to obtain conclusive results regarding their role in resistance mechanisms. However, in resistant genotypes, when only the best quality were selected, *RING finger protein 5, Ethylene-responsive transcription factor ABR1 and Cysteine-rich repeat secretory protein 38 (Gnk)* revealed higher levels of expression, suggesting that ethylene and salicylic acid pathways may be involved in resistance. Transcript abundance levels for these genes should be investigated in more detail in the future.

Keywords: Chesnut, *Phytophthora cinnamomi,* resistance genes, transcript abundance, digital PCR

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Abbreviations

ABA - Abcisic acid
ATPase - Adenosine Triphosphatase
Avr - Avirulence
BAP - 6-benzilaminopurine
bHLH - Basic helix-loop-helix
C.crenata – Castanea crenata Siebold & Zuccarini
C.dentata - *Castanea dentata* Borkhausen
C.mollissima - Castanea mollissima Blume
C.parasitica - Cryphonectria parasitica Barr
C.sativa - *Castanea* sativa Miller
C.seguinii - Castanea seguinii Dode
Castanea spp - Castanea species
CTAB - cetyl trimethyl ammonium bromide
DEGs - Differentially Expressed Genes
ERF - *Ethylene-responsive factor*
ETI - Effector Triggered Immunity
ESTs - Expressed sequence tags
hpi - hours post-infection
IBA - Indol butyric acid
LRR - Leucine rich repeat
MAPK - Mitogen-activated protein kinase
MS - Murashige & Skoog culture medium
NBS - Nucleotide binding site
NFQ - Non-fluorescent Quencher-
P.cinnamomi - *Phytophthora cinnamomi* Rands
PAMP-Pathogen - Associated Molecular Patterns
Phytophthora spp. - *Phytophthora* species
PR proteins - Pathogenesis-related proteins
PTI - Pathogen-Associated Molecular Patterns Triggered Immunity
qPCR- quantitative real-time PCR
QTL - Quantitative trait loci
R genes - Resistance genes
ROS - Reactive oxygen species
SAR - Systemic Acquired Resistance

SC - *C sativa* x *C crenata hybrids*

SM - *C sativa* x *C mollissima* hybrids

1. INTRODUCTION

1.1. Chestnut (*Castanea* sp.)

1.1.1. Classification and distribution

Castanea genus belongs to the *Fagaceae* family, is found in north temperate climates and comprises 13 species: five Asian, seven American and one European species, *Castanea sativa* Mill. also known as European chestnut or sweet chestnut. The most important crop species are European chestnut, the Chinese chestnut (*C.mollissima* Blume) and Seguin chestnut (*C.seguinii* Dode), both of which can be found in China; Japanese chesnut (*C.crenata* Sieb.& Zucc.) appears in the Japanese islands and Korea and *C.dentata* Borkhausen the American chestnut is a nearly extinct species (Zohary & Maria, 1987; (GP, 1988); Dane et al., 2003). European and North American species appeared as a result of intercontinental dispersion and divergence of *Castanea* species originating in eastern Asia. Nowadays they are widespread throughout four continents with distinct soil and climate features: Europe, America, Asia and Oceania (Lang et al. 2006). Main species' distinctive features are summarized in table 1.

Table 1: Comparative description between main chestnut species
Adapted from (Gil Nelson, Christopher J. Earle, 2014)

Species	Maximum height (m)	Shoots	Buds	Leaves	Fruit
C.sativa	35	Thick angular, reddish	Thick, ovoid, hairy	Big dentate, dark green (10 to 20cm x 5 to 8 cm)	Lustrous brown, rounded on 1 side and flattened the other 2-3cm
C.mollissima	15	Thin, brown or grey slightly hairy brownish-red	Small, near ovoid, hairy	Long oval (15 to 20cm x 5 to 7 cm)	Lustrous reddish-brown, flattened on 1 or 2 sides (2 to 3 cm)
C.crenata	20	Thin round brownish-red and brightly	Small ovoid, pointed, no hairy, phyllotaxy 1	Smaller and narrower (9 to 15 cm x3 to 3,5 cm)	Small lustrous reddish brown nuts 2 to 3cm
C.dentata	35	Thin slightly angular, greenish-yellow, yellowish-red no hairy	Small, near ovoid, punted	Big narrow (15 to 20cm x 4,5 to 5,5 cm)	Small lustrous brown,flattened on one side (1,8-2,5cm)

1.1.2. Economic and ecological value

Castanea spp. has a significant economical value due to its edible fruits, good quality timber, tannins and also edible mushroom production. Moreover, chestnut has an ecological role in the ecosystem, providing shelter and food supply to small vertebrates, contributing for the forest diversity and soil fixation. Particularly, sweet chestnut appreciated in Mediterranean culinary traditions, has high organoleptic qualities and nutritional value with high starch, water content and low levels of fat. Italy, Turkey, Portugal and Spain are the main chestnut fruit producers for consumption in Europe (Borges et al., 2008).

1.2. Threatening pests and diseases

Since the 20th century, European and American chestnut populations have decreased in area mainly due to ink and blight diseases. Recently gall wasp emerged as a potentially devastating pest threatening *Castanea* spp. populations worldwide.

1.2.1. Chestnut blight (*Cryphonectria parasitica Barr*)

Cryphonectria parasitica Barr is an ascomycete, the causal agent of chestnut blight and affects particularly European and American chestnut trees. The fungus is wind-dispersed and when it comes in contact with wounds, colonizes bark tissue and develops cankers which expand around the stem and cambium causing wilting and death of the parts beyond the cankers. In America, it was first reported in 1904 in New York City and was responsible for the decimation of 3,5 billion *C. dentata* trees. *C.parasitica* is also present in Europe, and unlike *C.dentata, C.sativa* populations were able to recover from the disease due to the natural occurrence of hypovirulent strains. Coinoculation with hypovirulent strains as biological control has been adopted to reduce its impact as this method attenuates fungal pathogenesis (Anagnostakis 1987, Nuss, 1992). Asian trees are considered resistant to blight fungus as they develop few symptoms when infected. A breeding program based on crossing American with Chinese chestnut followed by successive backcrosses with *C.dentata* has been developed for *C.dentata* restoration in United States of America. As a complement to these efforts, Barakat et al.(2009;2012) have identified candidate genes for resistance to blight disease by comparison of Chinese and American chestnut's transcriptomes.

1.2.2. Gall wasp (*Dryocosmus kuriphilus*)

Dryocosmus kuriphilus Yasumatsu also known as gall wasp (*Cynipidae* family), is native to China, and attacks *C.crenata, C. dentata, C.mollissima* and *C.sativa* populations. In the twentieth century it was established as a pest in Japan, Korea and North America, and has been reported in Italy in 2002 (Zhang et al., 2009; Borowiec et al., 2014) Contingency plans are being adopted to control its dispersion. Gall wasp disrupts growth by laying eggs in early summer inside buds where the *larvae* remains during winter. In spring, larvae feed on the buds, and induce gall formation. Severe infestations can result in decline and death of the tree. No effective pesticides have been found, however, the parasitoid *Torymus sinensis* has been reported as a biological control to reduce infestations by attacking gall wasp eggs in Japan (Moriya et al., 1989).

1.2.3. Ink disease (*Phytophthora* spp.)

The main causal agent of ink disease, *Phytophthora cinnamomi* Rands, formerly integrated in *Fungi* kingdom due to their similar mode of nutrient acquisition and mycelial structures, is nowadays considered as belonging to *Oomycetes* (Petri, 1917; Hawksworth et al.,1995). Significant structural and biochemical differences such as the occurrence of beta-glucans and cellulose rather than chitin in the cell walls and aseptate hyphae with diploid nuclei, led to the inclusion of these species in *Chromista* Kingdom, Phylum *Oomycota* (Zentmeyer, 1980)

P.cinnamomi is present in soil and infected plant material and forms several spore stages involved in survival, infection and disease development. Under unfavourable conditions, survival structures that persist in soils for several years are produced. When in presence of low humidity conditions, chlamydospores differentiate within the roots and are released into the soil. Also, under low temperatures, oospores are produced. When in presence of a compacted poorly aerated and water saturated soil, chlamydospores and oospores differentiate into sporangia that produce motile zoospores favouring pathogen dissemination and infection. Zoospores are chemotactically attracted by root exudates and penetrate non-lignified root tissue or the base of stem or trunks producing effector molecules allowing it to establish within root cells. From the roots, hyphae colonizes intercellular regions, infection extends to the cortex and into phloem and xylem moving laterally along the root (Hardham 2005, Hardham & Cahill, 2010, Horta et al., 2010). Chlorotic and reduced in size leaves, thinning of the crown and the presence of immature fruits after leaf-fall, are common symptoms of the disease. After debarking, the necrotic regions are visible and can be clearly seen in young trees with smooth bark. This disease was named after the black exudates produced by the necrotic roots that stain the soils during the spring and fall. Over time the water and nutrient uptake inhibition lead to death in susceptible species (Hardham, 2005; Zentmeyer, 1980; Vannini & Vettraino, 2001).

This pathogen has a worldwide distribution and poses a serious threat to a wide range of plant genera: approximately 950 hosts as described by Zentmeyer (1980) including chestnut, walnut, oak, eucalyptus peach, avocado, among others. In Europe numerous cases have been described in different areas, with an elevate prevalence in Portugal, Spain, France Italy and the United Kingdom (A. R. Hardham, 2005). As a consequence of the growing international trade in living plants, the pathogen has spread overseas, being introduced in Europe from USA through Azores islands (Anagnostakis SL, 2001; Crandall, 1950). The first records date from 1838 in the north of Portugal (Fernandes CT, 1955). Devastating epidemics occurred between 1900 and 1950 (Moreau C, 1952; Peace, 1962). Also, *C.dentata* populations in the Southeastern USA were affected by ink disease and later were decimated by chestnut blight. Nowadays, ink disease is re-emerging in the United States, and is a serious threat to *C.dentata* reintroduction.

The impact in the ecosystems and the severe economic losses in agriculture and horticulture prompt the implementation of measures to control pathogen spread. Detection of the infection at an early stage is a complex task. Phenylamides and phosphonic acid are chemical compounds that have proved to be effective in inhibiting growth and limiting the spread, however they are not able to eradicate the pathogen successfully. Phosphite treatments are also reported to confer protection in threatened communities. Soil drainage, use of sterilized soil, containment, quarantine of new plants before transplanting can prevent the introduction of the disease (Pegg et al., 1985; Oros & Tamás, 1991; Hardy et al., 2001 Dunstan et al., 2010; Eshraghi et al., 2014). Developing resistant varieties and rootstocks is the most economical and environmentally safe method to control the disease.

1.3. Varieties and rootstock improvement

Chestnut production for commercial use must be approved according to certain selection criteria: level of resistance to *Phytophthora spp*. is one of them. There is a global interest from researchers and producers to develop new genomic tools to understand the mechanisms underlying the resistance to this oomycete. Different chestnut species display different levels of resistance to this pathogen. Ink disease is particularly threatening to sweet chestnut, *C. sativa* Mill contrasting with the resistant Chinese chestnut, *C. mollissima* and the Japanese *C.crenata* (Day, 1939).

Since the introduction of these Asian species in France in 1952, clones were selected as rootstocks to control the progression of the pathogen and produce high quality fruit cultivars (Vannini & Vettraino, 2001). However, genetic incompatibilities observed between both species led to the adoption of other strategies. Different breeding programs were then established, based on interspecific hybridization with Asian species aiming to produce ink-resistant plants with higher compatibility with C*.sativa varieties* (Elorrieta, 1949; Vieira Natividade, 1947; Gomes Guerreiro, 1948). In Europe, none of these programs were continued over time as the American program implemented for resistance to blight disease for American chestnut (http://www.acf.org) that has been developed with great success for American chestnut restoring and reintroduction.

Breeding for resistance to *P.cinnamomi*, by crossing the susceptible species with the resistant ones with further backcrosses of *C.sativa* with the most resistant hybrid genotypes of each generation, it is theoretically possible to establish the introgression of ink disease resistance-related genes in the next generation, maintaining the genetic background of *C.sativa.* This approach may lead also to the improvement of grafting compatibility with *C.sativa* varieties and thus preserving the most appreciated qualities of sweet chestnut and satisfy commercial demands. Following that approach a set of interspecific controlled crosses was implemented in 2006, between *C. sativa* x C. crenata *and C sativa x C mollissima* generating until now 52 SC hybrids (*C sativa* x *C crenata*) and 21 SM hybrids (*C sativa* x *C mollissima*) (Costa et al., 2011). The progenies were inoculated with *Phytophthora cinnamomi* to determine their level of resistance and to produce a segregant family with a ranking of phenotypes regarding the trait under study (from the most susceptible to the most resistant) for QTL mapping. Seven genotypes were selected as the most resistant (Santos et al., 2014). The mechanisms that may explain the higher resistance of certain genotypes remains to be disclosed.

1.4. *Phytophthora*-Chestnut interactions

Plants have developed two defense pathways to pathogenic microorganisms: basal defense and adaptative immune system. The first, PAMP-triggered immunity (PTI) depends on the perception of elicitors, also referred as PAMPs (pathogen-associated molecular patterns) which include glycoproteins, carbohydrates, polymers and lipids produced by the pathogen. The association with a pathogen molecule promotes conformational alterations that trigger downstream signalling (Zipfel & Felix, 2005; Jones & Dangl., 2006). Signal transduction leads to a hypersensitive response which is characterized by the producion of active oxygen species, phytoalexins and plant cell wall reinforcement, deposition of callose, synthesis of defense enzymes and accumulation of pathogenesis-related PR proteins. Infected cells are sacrificed, preventing energy obtention by biotrophic pathogens to restrict their growth. However, this system may be ineffective against *P. cinnamomi*, as it has effector proteins that actively suppress host defense and is hemibiotroph, capable of switching to a necrotrophic phase and use dead tissues as a nutrient source. Phytohormone balance plays an important role in the differentiation of biotrophic and necrotrophic pathogens defense mechanisms. Salicylic acid is important for the activation of defense responses to bio and hemibiotrophic pathogens (Vlot et al., 2009) and jasmonic acid and ethylene act as signals that promote defense against necrotrophs (Glazebrook, 2005; Jones & Dangl., 2006). The two systems are normally antagonistic, however synergistic interactions may occur, regulation can vary in some pathosystems (Van der Ent, et al. 2009). Auxins and abcisic acid negatively regulate salicylic acid pathways (Pieterse et al., 2012). In *Arabidopsis thaliana,* the auxin response pathway has been reported to play an important role in resistance during *P.cinnamomi*'s necrotrophic phase (Eshraghi et al., 2014)

Plants have evolved another system known as effector triggered immunity (ETI). It is based on specific recognition between proteins that act as a receptor resulting from resistance genes (R genes) and bind to the corresponding Avr proteins (avirulence) produced by the pathogen, activating signal transduction cascade and enhancing the accumulation of ROS. MAPK signalling cascades, ROS accumulation and hormone crosstalk induce multiple stress transcription factors that modulate response (Jones & Dangl., 2006; Dodds & Rathjen, 2010). Activation of PTI and ETI triggers systemic resistance in distal tissues from the site of the infection: systemic acquired resistance (SAR). SAR confers enhanced defensive capacity and is associated with increased expression of PR proteins and other defense genes (Vleeshouwers et al., 2000; Z. Q. Fu & Dong, 2013).

Host resistance to *P.cinnamomi* is an active area of research as signalling pathways and their regulation remains to be unravelled (Kamoun et al., 2015). *Phytophthora species* have evolved to evade, overcome and modulate host defensive responses. In 2014, Oßwald et al., suggested a model for woody-plant and *Phytophthora* interactions such as the release of elicitins and downregulation of plant defense genes to facilitate root penetration. In susceptible plants the level of abscisic acid (ABA) increases in roots, decreasing the leaf water potential. To cope with stress several mechanisms are activated including stomata closure to avoid water loss through transpiration, consequently decreasing carbon dioxide uptake in leaves reducing phtotosynthesis rate, resulting in chlorosis and wilting of leaves (Maurel et al., 2001; 2004).

There is little information regarding chestnut's genome and its response to *Phytophthora spp*., and it wasn't until recently that a transcriptomics approach was initiated.

Serrazina et al., (2015) compared the response of *C. sativa* and *C. crenata* to *P. cinnamomi* challenge using Roche 454 GSFLX platform. ESTs (Expressed Sequence Tags) generation is a tool that provides transcript specific information for the identification characterisation of genes. Total RNA was isolated from inoculated and non-inoculated *Castanea sativa* and *Castanea crenata* roots at 3 time points (2, 4 and 7 days after challenge). Four 454 pyrosequencing cDNA libraries were generated and are available online at Fagaceae Genomics website (http://fagaceae.org/). Sequencing generated 771,030 reads, and the assembly results displayed 15,683 contigs for *C. sativa* and 16,828 contigs for *C. crenata*. Differential gene expression analysis reveals that after inoculation, *C.crenata* upregulates twice more genes than *C.sativa* and also, *C.sativa* downregulates more than twice the genes than *C.crenata.* The results obtained also suggest that *C.crenata* may initiate the response earlier with an increased level of gene expression. Using a P value<5E-3, inoculated and non-inoculated *C.crenata* cDNA libraries comparison reveals the presence of 283 Differentially Expressed Genes (DEGs). Of these, 229 and 54 are respectively up and downregulated. Resistance is related with the ability to prevent pathogen spreading, however the genetic basis and pathways involved are not yet disclosed. A significant number of differentially expressed genes were identified, as candidates to ink disease resistance and have an important role in regulating plant immune response, anti-fungal enzymes and anti-fungal metabolite synthesis, cell wall strengthening by lignification and stress adaptation and recovery.

1.5. Candidate genes for resistance

Resistance response is a result of a complex interaction of many genes and may be associated with a strong up-regulation of key defense-related genes. Among all genes previously identified by Serrazina et al.,(2015) twenty differentially expressed genes were investigated and described with more detail as potential candidate genes for resistance.

Pathogen detection in plants relies solely on genes encoded in the genome. Nucleotide binding site-leucine rich repeat (*NBS-LRR*) gene family is a largest class of well known disease resistance genes (R genes). These cytoplasmatic R proteins are highly specific for Avr proteins or effector molecules and polymorphic: *NBS* have highly conserved motifs, however *LRR*s are adaptable structural domains. This gene family is a surface-localized plant pattern recognition receptor involved in PAMP-triggered immunity and development of systemic acquired resistance. The soybean *Rps* gene family that encodes for a *NBS-LRR* proteins has been reported to confer resistance to *Phytophthora sojae*. Also, in *Solanum* *pimpinellifolium, Ph-3* gene is involved in *Phytophthora infestans* resistance (Shan et al., 2004;DeYoung & Innes, 2006; Smart et al., 2007).

Calcium rapid and transient bursts act as a key second messenger in cell signaling and have an important role in response to biotic or abiotic stimuli triggering adaptive responses. Upon pathogen encounter, in response to elicitor *C2-domain* containing proteins are induced and translocated from the cytosol to plasma membrane binding to the membrane phospholipids in a calcium-dependent manner transducing the signal into the cells and recruiting several proteins to activate defense signalling pathways (Kim et al., 2003; Medvedev, 2005; Kim et al., 2003; Lecourieux et al., 2006). Also, endoplasmic reticulum Calcium-Transporting ATPases, regulate calcium efflux to maintain low calcium levels in cytoplasm. Intracellular Ca^{2+} increases as a consequence of pathogen perception signaling cascade, and this event activates Ca^{2+} cell death pathway (Ma & Berkowitz, 2007; Levine et al., 1996; Lecourieux et al., 2006). Downregulation of calcium-transporting ATPase may cause the accumulation of intracellular calcium inducing programmed cell death to prevent pathogen colonization.

Ring finger 5 is a member of Polycomb group proteins involved in chromatin conformation alterations. It is localized in the plasma membrane and upon pathogen elicitor detection, is upregulated and mediates hypersensitive response regulation by acting as E3 ubiquitin-protein ligase. Also, E3 ubiquitin-protein ligase may be involved in positive regulation of cell death and important for salicylic acid accumulation which is required for pathogenesis related gene expression (Buchwald et al., 2006; Zeng et al., 2006; Hondo et al., 2007).

Plants have a wide number of transcription factors to regulate gene expression in response to stresses. *WRKY transcription factors* are one of the largest families of transcriptional regulators in plants. They act in a complex network and have been reported to regulate response to fungus and hormones, such as auxin (Zhang et al., 2008; Eulgem et al., 2000). Particularly, *WRKY transcription factor 31* has been shown to be overexpressed in rice seedlings after treatment with a fungus, *Magnaporthe grisea*. It was observed blocked pathogen invasion indicating that it can be a transcriptional activator in defense response. Also WRKY31 might be important in crosstalk between auxin and defense signal transduction during the necrotrophic phase (Zhang et al., 2008). High auxin levels in potato young leaves demonstrated to be a natural defense against *Phytophthora infestans* spread and development *in vitro*, (Martinez-Noël et al., 2001). Also, auxin homeostasis revealed to be critical for survival in *Arabidopsis thaliana* in response to *Phytophthora cinnamomi* (Eshraghi et al., 2014).

Ethylene regulates plant responses to biotic stresses and abiotic stresses. *Ethylene-responsive transcription factors* modulate GCC box-mediated gene expression in response to extracellular signals (Fujimoto et al., 2000). Members from this transcription factor family, (e.g. *ERF027 and ABR1*) may be important in the pathogen-responsive gene expression. *Ethylene-responsive transcription factor ABR1* is a negative regulator of abcisic acid signalling pathway in *Arabidopsis thaliana* (Pandey et al., 2005). Ethylene and Jasmonic acid defense responses activate ERF and PR genes in response to necrotrophic pathogens (Shin, S. et al., 2014). NAC-domain transcription factors specifically bind to 5'-CATGTG-3' motif and are strongly induced by biotic, abiotic stresses, abscisic acid, lateral root formation and also regulate response after pathogen perception. Most NAC genes have yet to be characterized as they may play important roles in plant physiology and immunity (Nuruzzaman et al., 2013; Xie et al., 2000)

MYB transcription factor family genes are involved in the control of specific processes including primary and secondary metabolism and responses to biotic stresses (Dubos et al., 2010). *MYB4* has been shown to repress flavonol biosynthesis pathway allowing the production of defense compounds that restrict the pathogen spread: lignin and phytoalexins. (Mengiste et al., 2003). *MYB-related protein 306* activates flavonoid synthesis genes (Jackson et al, 1991). Flavonoids have antioxidant properties and active oxygen scavengers (Kandaswami and Middleton, 1994). MYB transcription factors could also participate in the precise coordination of ROS production and scavenging mechanisms during hypersensitive response for the regulation of cell death. Other enzymes are also produced: catalase, peroxidase, superoxide dismutase and glutathione-S-transferase are synthesized to protect cells from oxidative damage (García-Pineda et al., 2010). *Basic/helix-loop-helix (bHLH*) proteins are a superfamily of transcription factors recognize E-Box and are included in many processes including flavonoid biosynthesis and increased tolerance to osmotic stresses. In *Arabidopsis bHLH92* gene is also involved in lateral root formation and root elongation inhibition in response to osmotic stresses (Robinson et al., 2000; Jiang et al., 2009; (Castilhos et al., 2014). Roots cease to grow at 24 hours after infection with *P.cinnamomi* and in resistant species resumes 48 hours later from lateral roots (Tippett & Hill., 1984). As *P.cinnamomi* zoospores are attracted to the root elongation sites, lateral root formation can be important in survival for the first 24 hours after infection. Also transcription factors that control development processes may be involved. *DNA-binding Escarola* is a transcription factor that belongs to the AT-hook motif nuclear localized family and co-regulate transcription by modification of chromatin structure acting as negative regulator of cell elongation in hypocotyl and leaves (Street et al., 2008).

Allene oxide synthase is a key enzyme in jasmonic acid biosynthetic pathway in response to pathogens. *Solanum tuberosum* Allene oxide synthase 2 was reported to increase resistance to *Phytophthora infestans* (Pajerowska-Mukhtar et al., 2008 and 2009). Jasmonic acid is able to induce phytoalexin production and enhancing the expression of defense related genes, PR proteins (Mei et al., 2006). PR proteins accumulation prevents pathogen growth and spread and are associated with the development of Systemic Acquired Resistance. ß-1,3-glucanase (PR2), chitinase (PR3) and thaumatin-like (PR5) proteins belong to the PR family. Glucan 1,3 ß-glucosidase is strongly induced after infection and catalyzes the cleavage of 1,3-ß-D glucosidic linkages in ß-1,3-glucans present in *Phytophthora*'s cell wall (Zevenhuizen & Bartnicki-Garcia, 1970; Doxey et al., 2007). *Acidic endochitinase* and *Endochitinase PR4* catalyses the hydrolysis of N-acetyl-ß-D-glucosaminide 1,4-ß-linkages in chitin also present in the cell wall (Zentmeyer, 1980). *Endochitinase* and PR5 activity has been shown to be activated upon infection of *Persea americana* with *Phytophthora cinnamomi*, however could not be directly linked to the observed phenotypic resistance (Engelbrecht & Van den Berg, 2013)

Secondary metabolite biosynthesis, enzymes are able to modify cell walls, antifungal protein secretion can lead to resistance. Plants have a vast array of enzymes that synthesize defensive secondary metabolites (Bennett, et al., 1994; Jeandet et al., 2013). Terpenoid phytoalexins synthesis has been reported to be upregulated after *P.cinnamomi* inoculation in *Zea mays* roots. *δ-cadinene synthase* is a jasmonic acid-responsive sesquiterpene cyclase involved in secondary metabolite biosynthetic pathways (Allardyce et al., 2013; Taniguchi et al., 2014). *Neomenthol dehydrogenase* is responsible for the conversion menthone monoterpene to d-neomenthol enhancing resistance to the hemibiotrophic pathogen *Pseudomonas syringae* in tomato (Choi et al., 2008). *Pectinesterase* catalyses the de-esterification of pectin into pectate and methanol. This reaction may allow interchelation of calcium ions strengthening the cell walls to prevent further pathogen colonization (Wen et al., 2013). *Ginkbilobin-2 (Gnk2)* is an secreted antifungal protein found in Ginkgo seeds and also other plants. Contains Gnk2-homologous domains and cysteine-rich receptor kinases, and are induced by pathogen infection or salicylic acid and reactive oxygen species treatments. In *Arabidopsis thaliana* 60 genes with cysteine-rich secreted proteins and Gnk2-homologous domains have been identified, however their role is not yet clear (Miyakawa et al., 2009). Novel roles can be identified for these genes as their functions in *Castanea* spp remain unknown.

2. AIM OF STUDY

This work is integrated in the implemented and on course research programme at INIAV (Costa et al., 2011) aiming to address the comprehension of chestnut defense mechanisms related with ink disease resistance using mapping and transcriptomic approaches.

The main aim of this dissertation is to validate the previously *C.crenata* Differential Expressed Genes sequences, using hybrids with intermediate resistance phenotypes obtained from controlled crosses performed between *C.sativa*, *C crenata* and *C.mollissima*, following the next steps:

1- Production of 7 genotypes with 3 replicates each, by micropropagation: *Castanea sativa (CS12)*, *Castanea crenata (CC14),* 1 SM hybrid (*Castanea sativa* x *Castanea mollissima*) and 4 SC hybrids (*Castanea sativa* x *Castanea crenata*) with different phenotypes of resistance to *P. cinnamomi* ranging from the most susceptible to the most resistant: SC912, SC914, SC51, SC55 and SM904

2- Perform root inoculation assays with *P. cinnamomi* for collection of plant material in two time points after inoculation: T1 – 24h and T2 - 48h.

3- Select candidate resistance genes and primer design to analyze differential expression profiles between the genotypes under study.

4- Perform RNA extraction from the roots and cDNA synthesis.

5- Design Primer specificity determination by qPCR

6- Determine the levels of gene expression by digital PCR.

This study is an important contribution for the understanding of the molecular mechanisms underlying resistance of *Fagacea* family to *P cinnamomi*: The final aim of the research program on course is to identify genomic regions related with ink disease resistance in *Castanea* genus to develop, in the near future molecular markers linked with resistance for marker-assisted selection and breeding. At the end these new tools will enable the production of resistant/less susceptible chestnut rootstocks with a consequent positive impact in orchard’s productivity.

3. MATERIALS AND METHODS

3.1. Plant material

In 2006 and 2009, 52 SC hybrids were obtained from controlled crosses between *C. sativa* (var. Aveleira) x *C. crenata* and 21 SM hybrids from *C.sativa* (var. Aveleira) x *C. mollissima*. All hybrids were submitted to screening tests to evaluate their level of resistance/susceptibility to *P. cinnamomi* (Santos et al., 2014). Based on these results five genotypes with diverse levels of resistance/susceptibility were selected for gene validation: SC912, SC914, SC55, SC51, SM904 (ranging from the most susceptible to the most resistant). *C. crenata* (CC14) and *C. sativa* (CS12) genotypes, kindly provided by Beatriz Cuenca, Tragsa, Spain, were used respectively as controls of resistance and susceptibility.

3.2. Micropropagation of plants from the selected SC and SM hybrids

To obtain multiple copies of each selected genotype, micropropagation was implemented through *in vitro* meristems establishment. Plants were cultured on Murashige and Skoog multiplication medium (Murashige & Skoog, 1962) with half strength nitrates (modification number 3B) supplemented with 0,1mg/L BAP (6-benzilaminopurine), 60 mg/L ascorbic acid, 3% sucrose and 0,8% agar, pH at 5,8. The explants were maintained in a culture chamber S600 ARALAB™, with temperatures ranging from 23 ºC at day and 20 ºC at night, under a photoperiod of 16h of daylight and 8 hours of darkness. After three weeks in multiplication medium, explants were transferred to MS medium containing 0,3% of active charcoal without phytoregulators, where they were maintained for another two weeks. After the elapsed time, plantlets were ready for root induction. The explants were washed with tepid water and an antifungal solution, benlate at 0,4 g/L. The basal region was cut and dipped in IBA (indol butyric acid) at 1g/L for 2 minutes. Plants were then transferred to a container with 2 cm of hydrated perlite and vermiculite (1:1) for root development where they remained for 3 weeks. After this period, rooted plants were transferred to substrate containing peat, perlite and vermiculite (3:1:1), and maintained in an environmental controlled chamber Fitoclima 4600 ARALAB™ under the conditions previously described. Relative humidity was maintained at 80% initially, and gradually adjusted to 65% in order to obtain acclimatized plants.

3.3. Root inoculation test with *P. cinnamomi*

Plants exhibiting similar root development and physiological conditions, 80 days after acclimatization were submitted to the root inoculation test, using a hypervirulent strain, PH107 (Santos et al., 2014). The experiment comprised six plants inoculated, per selected genotype. Six CS12 and six CC14 plants were not inoculated and were used as control.

The inoculum was prepared 21 days before the inoculation day. *P. cinnamomi* was grown in Potato Dextrose Agar medium for 6 days at 20 ºC in darkness. For soil infestation, mycelia were grown on a mixture containing sterilized millet seeds (*Ponicium milleaceum*) moistened with V8 broth (20% (v/v) with 3g/L of $CaCO_3$), and incubated for two weeks in darkness at 24 ºC.

Inoculum was placed into the substrate of each pot at a concentration of 5% (v/v), minimizing root disturbance, and flooded for 1 hour to stimulate zoospore release, promoting the disease development and homogenizing root infection. These experiments were performed under environmental controlled conditions, 16/8h photoperiod, with temperatures ranging from 20 to 23 ºC and 65% relative humidity. Three biological replicates were collected at 24 and 48 hours post inoculation. The whole plant was removed from the pot and gently washed to remove the substrate and avoid root wounding. Roots were separated from the aerial part, frozen in liquid nitrogen and stored at -80ºC until RNA isolation.

3.4. RNA extraction and cDNA synthesis

Total RNA extraction was performed according to a CTAB extraction protocol as described by Le Provost et al., 2007. Samples were eluted in 10µL of DEPC-treated ddH_2O. RNA integrity and purity were evaluated by gel electrophoresis under denaturating conditions: 1,2% (w/v) agarose gel in 0,5X Tris-Borate-EDTA buffer (TBE) stained with SYBR® Safe (Invitrogen, Carlsbad, CA, USA) and visualized with a UV transilluminator (Bio Rad). Quantification and quality was measured using Nanodrop ND-100 Spectophotometer (Nanodrop Technologies Inc. Montchanin, DE, USA).

Genomic DNA was removed by treatment with DNase (Turbo DNase-free kit Ambion, Inc., USA) according to manufacturer's instructions. Samples were concentrated using a Speed Vacuum Concentrator (Centrivap, Labconco corporation, Kansas City, MO, USA). 500 ng RNA were reverse transcribed to cDNA with Improm-II Reverse Transcription System kit (Promega, Madison, WI) and using Oligo(dT) primer and Random Primers in the same reaction. Reactions were incubated at 25ºC for 5 minutes, 42ºC for 1 hour followed by 15 min at 72ºC for reverse transcriptase inactivation. cDNA was visualized by gel electrophoresis in 1% (w/v) agarose stained with SYBR Safe (Invitrogen®, Carlsbad, CA, USA) and visualized with a UV transilluminator (Bio Rad).

3.5. Candidate genes selection

P. cinnamomi resistance candidate genes were selected from ESTs with homology to genes available at a private database (http://transcriptomics.biocant.pt/Castanea/) provided by BIOCANT (Cantanhede, Portugal) that comprises the four transcriptomes obtained by Serrazina et al., (2015). DEGs were identified as genes showing significant higher/lower expression levels in inoculated against non-inoculated plant material. The most significant candidate genes of resistance considered are present among *C.crenata* inoculated and non-inoculated DEGs. The comparison of *C.crenata* inoculated and non-inoculated cDNA libraries using a P value<5E-3, reveals the presence of two hundred and eighty three Differentially Expressed Genes (DEGs). Two hundred and twenty nine, and fifty four of these are respectively up and downregulated. The number of reads was used as an approximate estimation of gene expression level following the equation 1 (Serrazina et al., 2015):

$$ratio = \log_2\left(\frac{Cc\,inoculated\,reads}{Cc\,non-inoculated\,reads}\right)$$

Equation 1

From these, twenty DEGs putatively related to stress response, with a ratio above 2 and bellow -2, were selected as EST-based candidate resistance genes.

3.6. Primer design and specificity tests

Primers and probe sequences were designed for twenty gene sequences using Primer 3 software version 0.4.0 (available at http://bioinfo.ut.ee/primer3-0.4.0/) and synthesized by STAB Vida Lda, Portugal. Blastn and Blastx tools were used to find homologous sequences in related-species genomes and to identify and avoid conserved domain motifs within the sequence, respectively. Primers were designed with the following options: size between 18-20bp, to amplify a product of 100-150bp with melting temperatures ranging from 58-60ºC, 30 to 60% G/C content, no more than two G/C in the last five 3' end nucleotides and no more than three G's runs within the sequences (Table 2). Probes were labelled with FAM or VIC dye on the 5' end and NFQ (Non-fluorescent Quencher) on the 3', size between 18-30bp with melting temperatures ranging 68-70ºC.

Table 2: Primer and probe sequences of defense-related genes following infection with *Phytophthora cinnamomi*

RecName	**Interpro Accession number**	**Ratio[a]**	**Homology**	**Primer Forward (F), Reverse (R) and probe (P) sequences[b]**	**Product size (bp)**
Acidic endochitinase	IPR001223	2,44	*Nicotiana tabacum* (tobacco)	F 5’ GTGCCAAGGTACCCTCATTT R 5’ ACTATGGCATCAAATCTGTCAAG P 5’ ACCCCAGTAGATAGCGATTCCACCAG	130
Allene oxide synthase	IPR001128	3,34	*Zea Mays* (Maize)	F 5’ TGGCTTTGGTCAACGAAGT R 5’ AATGTTGGTGGAGCTTTTCC P 5’ CAACGTCCCAGCCTCGATCG	101
Calcium-transporting ATPase, endoplasmic reticulum-type	IPR006068	-6,83	*Oryza sativa* (Rice)	F 5’ GAAGGAGTCAAAGATGGATAGCA R 5’ AGAATGGACCTCCCAATCTG P 5’ TGGCATGACAAAATTTCAGGCTCAA	109
C2 domain-containing protein At1g63220	IPR000008	2,48	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ CCATGTGGAAGAGGAAACCT R 5’ GGAAAACTGAAATCAATTGAAG P 5’ **VIC-**ACGGTGGATGGAAACAGTCTGCA	129
Cysteine-rich repeat secretory protein 38 (Gnk)	IPR002902	2,88	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ CACCACGACAAAGAGCAAGT R 5’ CCACCAATGACCCATATGAA P 5’ **FAM**-ACCAAAGCCCAGGAGAGG	138
Endochitinase PR4	IPR000726	2	*Ginkgo biloba* (Maidenhair tree)	F 5’ GGTTTCCACCATCACATTCA R 5’ TGGCTTGTGGTATTGGATGA P 5’ TGGCTCGAATCGTTGCTCCG	104
Ethylene-responsive transcription factor ABR1	IPR001471	6,53	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ GATGTGGAGTCTCCCTGTGA R 5’ TCTGCTCCTGCTTTTGCTT P 5’ **VIC**-CGCCCCCTCTTTTGGCCA	149
Ethylene-responsive transcription factor ERF027	IPR001471	3,46	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ CCAGTGCCAGCCTCTACAT R 5’ TGGAGTTGTGACTGGCTTTC P 5’ ACGCTGCCACAGCCGCTG	101
Glucan 1,3-beta-glucosidase	IPR001547	2,67	*Oryza sativa* (Rice)	F 5’ GCACCCAATGGATTCTTCTT R 5’ CACTTGAAACTCGCCCTGTA P 5’ TGCTGGTGACTGCAGATTTTGCAG	141
LRR receptor-like serine/threonine-protein kinase	IPR000719	2,32	Zea Mays (Maize)	F 5’ CAATTCTCGAAAGTTGAACGA R 5’ GCTTAGGACTCACCCAATGC P 5’ **VIC**-TCACCGGCCAATCTGCAATTG	134
Myb-related protein Myb4; Transcription factor RLTR1	IPR001005	2,95	*Oryza sativa* (Rice)	F 5’ TACAGCCCAATTTCCATTCA R 5’ CCAGCTCCAATGAAAAGGTT P 5’ **FAM**-TGGAACCAGACTATAGCGATGGCTCA	114
Myb-related protein 306	IPR001005	2,84	*Antirrhinum majus* (Garden snapdragon)	F 5’ AAGCTCGATCTGGTGTTGTG R 5’ TGCTCGAGAACTGGAAGAAA P 5’ CTGGAACCAGTTGCCCCAAGG	147
NAC domain-containing protein	IPR003441	2,66	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ CCTGAATTCGCTGTTGAA R 5’ TCCCCGAGTTTGTTCTGAGT P 5’ CCCACAGCTTTGCCACGTGG	148
Pectinesterase 2	IPR000070	2,98	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ TGACATCAACGGCAAGAGAT R 5’ AATGTCAAGTGCAGCCAAAC P 5’ **VIC**-CCAGGCCCGACACGTCTCAA	125
Putative DNA-binding protein ESCAROLA	IPR005175	-2,49	*Oryza sativa* (Rice)	F 5’ ACCAACTGCAGCTGTTCCTA R 5’ CCCTCCAGGCAAAGATACAT P 5’ CATCTTCCACGTCATTCCGTGGA	139
RING finger protein 5	IPR001841	2,97	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ GGATTCCGTCAGCGTACAG R 5’ AGCAGCTCATGTTCCGATAG P 5’ **FAM-**TGAAGAGGCTGCTTTTGCTTATCGC	135

Transcription factor bHLH92;	IPR001092	-4,95	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ AGAATTGCAAAGGCGAAACT R 5’ ATGGAATCAACCCCAGATGT P 5’ TGGCTGCGGTGGAAGGAGAA	120
WRKY transcription factor 31	IPR003657	1,71	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ GGTCTCTTCATCGGAAGGAA R 5’ ACAAGCCGCTCCTCACTAAT P 5’ **VIC**-ACGGTCAGGATCGCCCGGTA	117
(+)-delta-cadinene synthase isozyme XC14	IPR001906	3,91	*Gossypium arboreum* (Tree cotton)	F 5’ GCTCAGCTGTTGTGAAAGATG R 5’ AGAGGACAAGGATATTCATTTACG P 5’ CTCGTTTCTCACCTCATCTTCCAGCTC	109
(+)-neomenthol dehydrogenase	IPR002198	4,95	*Arabidopsis thaliana* (Mouse-ear cress)	F 5’ ACAATCCTTGTGTAGGCATTGA R 5’ GCCTAACTGAAGAAAGAGTGGAT P 5’ CCAGCCTTTGGTTTCCAAGGA	145

[a] Values for ratio were calculated using Equation 1: ratio=$\log_2$(*C.crenata* inoculated reads/ *C.crenata* non-inoculated reads.

[b] Probes with FAM or VIC label were selected for dPCR

Primer pairs’ specificity and successful amplification of target genes was first tested by conventional PCR on agarose gel electrophoresis and melting curve analysis by qPCR. qPCR reaction was carried out using Biorad iQ SYBR Green Supermix and analyzed by an iCycler iQ Real Time detection system following recommended manufacturer’s instructions. 4 cDNA pools (CC14, CS12, all SC hybrids and SM904 alone) were prepared. A final concentration of 300 nM of each primer and 100ng of cDNA template from each cDNA pools was used in 20µL reactions. All reactions were performed in duplicate in the same 96-well PCR plate. Thermal cycling was initiated at 95ºC for 3 minutes, followed by 40 cycles of 15 seconds at 95ºC and 30 seconds at 60ºC.

3.7. Gene expression determination by dPCR

Transcript abundance of eight *Castanea crenata* resistance-related genes was investigated using QuantStudio™ 3D Digital PCR System (Life Technologies, Carlsbad, CA). All reactions were performed using QuantStudio ™ 3D Digital PCR Master Mix following manufacter’s instructions. Two genes were evaluated per reaction. Each 14,5 µL reaction consisted of 50ng cDNA, 0,9 µM of each primer and 0,25µM of each FAM™ and VIC® labeled probes. Each reaction was loaded onto a QuantStudio ™ 3D Digital PCR chip, and cycled under standard conditions for 40 cycles. Transcript abundance was analysed by fluorescence measurements using QuantStudio ™ 3D Digital PCR Instrument (Life Technologies, Carlsbad, CA). Data was processed with QuantStudio ™ 3D Analysis Suite™ software (absolute quantification mode) available online at (https://apps.lifetechnologies.com/quantstudio3d/).

4. RESULTS AND DISCUSSION

4.1. RNA extractions and cDNA synthesis

Fifty-four successfully acclimatized plants were obtained by *in vitro* culture within 6 months. Plants were inoculated with *P. cinnamomi* and twelve remained non-inoculated as controls (Figure 1). Sensible species exhibited dark brown lesions in the roots indicating that the pathogen had colonized the roots.

Figure 1: Plant material and root inoculation with *P.cinnamomi*. A- *In vitro* culture of *Castanea* spp. B-Three biological replicates, 80 days after acclimatization (CC14 genotype) before inoculation C- Root inoculation technique with PH107 strain (adapted from Santos et al., 2014) D- Root collection at 24 and 48h post-infection.

Whole plants were immediately frozen in liquid nitrogen and then roots were separated from the above part to avoid other stresses. RNA was extracted according to the protocol described by Provost et al., (2007). The same protocol was used also by Serrazina et al.,(2015) with good results using chestnut roots from plants with 5 years of age. Intact 28S and 18S ribosomal RNA subunits were observed for all samples on gel electrophoresis indicating minimal degradation, as shown in Figure S1. Additionally, quantity and quality was estimated using spectrophotometric analysis. Concentrations ranged from 100 to 1800ng/µL and the ratio of absorbance at 260 and 280 nm values ranged between 1.8 and 2.2 indicating the presence of genomic DNA in some samples. Also A_{260}/A_{230} ratio was higher than 1.8, suggesting low levels of polysaccharide and phenol contamination (Table S1).

DNase treatment was used to remove contaminating DNA and to avoid genomic DNA amplification. On average, 70% of total RNA was lost during this procedure. A_{260}/A_{280} and A_{260}/A_{230} ratios were also affected. As the sample volume was low residual chemical contaminations and the presence of DNA that absorbs at 260 nm resulted in an overestimation of ratios, particularly A_{260}/A_{280}, and as a consequence the values dropped significantly. A_{260}/A_{230} ratio values of some samples dropped below 1 which can be a consequence of buffer salts in the samples.

As it was not possible to obtain new acclimatized plants in due time to repeat RNA extraction procedure, the studies proceeded with these samples. CentriVap vaccum concentrator was used for concentrating the samples and to uniformize the values.

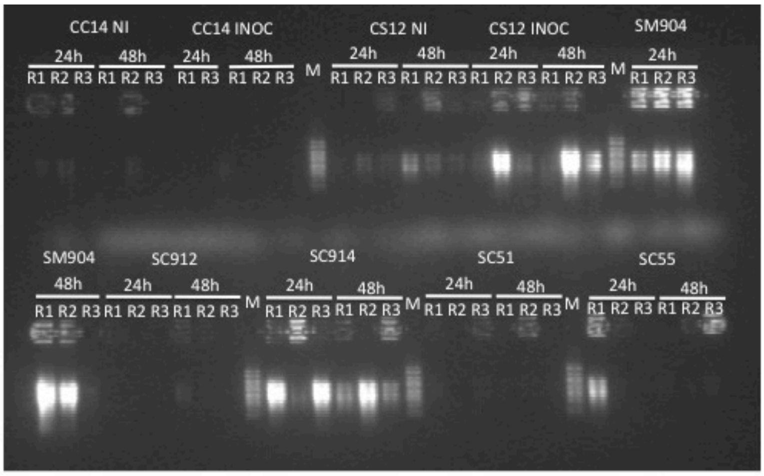


Figure 2: Denaturing gel electrophoresis of total RNA isolated for all samples prior to cDNA synthesis. M- RiboRuler High Range RNA Ladder (Fermentas) that ranges from 200 to 6000 bp

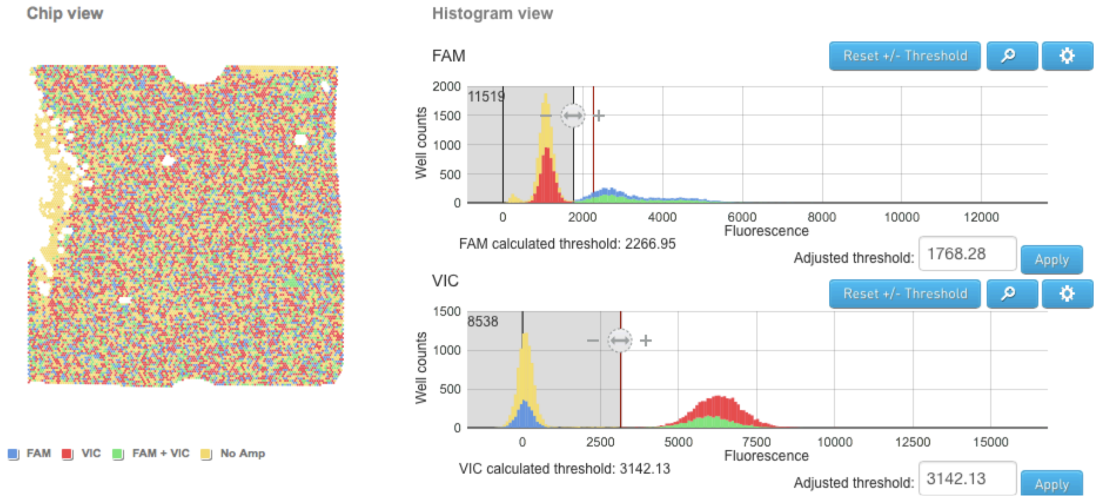
After these procedures, some degradation can be observed (Figure 2). SM904, SC914, CS12 samples show the presence of integrate RNA. RNA concentration was measured by Nanodrop (Table S1), and 500ng was used for cDNA synthesis.

4.2. Primer specificity tests with Real-time PCR

Primer pairs' specificity and successful amplification of target genes was first tested by displaying the PCR products on agarose gel electrophoresis and Real Time PCR melting curve analysis with SYBR Green. 4 cDNA pools were prepared using all samples: CC14, CS12, all SC hybrids and all SM904 samples alone. As SM904 is a hybrid clone obtained from *C.mollissima* and *C.sativa* crosses, and the primers were designed to amplify *C.crenata* genes, some variation could occur in the results, and for this reason this sample was tested apart from all SC hybrids. The presence of a single PCR product with the expected size (100-150 bp), confirmed that seventeen primers were specific. Only "*Myb-related protein 306*", "*Neomenthol dehydrogenase*" and "*Delta-cadinene synthase*" were discarded for the occurrence of primer-dimer association at lower temperatures and/or nonspecific amplification above or below the expected product. From these, eight genes were chosen for validation: *C2 domain-containing protein, WRKY transcription factor 31, Cysteine-rich repeat secretory protein 38 (Gnk), Ethylene-responsive transcription factor ABR1, Myb-related* *protein Myb4, LRR receptor-like serine/threonine-protein kinase, RING finger protein 5 and Pectinesterase* 2.

4.3. Gene expression profiling

The differential gene expression levels acquired with 454 sequencing technologies were compared with absolute quantification by digital PCR. QuantStudio™ 3D Digital PCR System (Life Technologies, Carlsbad, CA) is a chip-based technology that partitions the sample into 20.000 individual reactions and allows highly precise and accurate, absolute quantification of one or two genes in the same chip without the use of a reference gene. A total of 218 chips were prepared and analysed by QuantStudio™ 3D AnalysisSuite™ Software (Figure 3).



FAM calculated threshold: 2266.95

Adjusted threshold: 1768.28

VIC calculated threshold: 3142.13

Adjusted threshold: 3142.13

Figure 3: QuantStudio™ 3D AnalysisSuite™ Software Absolute Quantification mode. Chip view distribution of non-amplified wells (Yellow) and amplified wells with FAM™(Blue) or VIC® (Red) and FAM™+VIC® (Green). The histogram view allows the distinction of each population individually.

To analyze the results, some PCR reactions must be negative (no amplification depicted as yellow in figure 3), while others will be considered as positive for containing either one or more copies. The software performs analysis for each chip individually, the distribution of molecules throughout the partitions fits a Poisson distribution, the confidence interval is calculated for Poisson error distribution and the output is the number of copies/µL. The software then calculates precision which enables to distinguish between multiple sample measurements with a certain confidence. Lower precision values correspond to narrower confidence intervals. To achieve good precision levels, replicate chips data can be combined. Data reflects values from one large combined chip and not individual chips. Biological replicates were assembled in groups. When low or no copies were identified in one or more sample replicates, the software excluded samples from the combined chip, and in those cases, samples are represented with "NA" (not available). Some chips were also omitted from results due to leaks/evaporation and condensation and are represented with "NA".

The following charts show all samples numbered (1 to 9) from the most susceptible to the most resistant: *C. sativa* CSNI (CS12 non-inoculated) and CSI (CS12 inoculated); SC hybrids (SC912, SC914, SC55, SC51, SM904); *C. crenata* CCNI (CC14 non-inoculated) and CCI (CC14 inoculated). Non-inoculated samples CSNI (24hpi and 48hpi) and CCNI (24hpi and 48hpi) differ in the day that the samples were collected. No significant differences were expected, although some individual variance may be present.

Fold change ratios were calculated according to equation 2 and compared with the 454 ratio results obtained by Serrazina et al., (2015)(Table 2).

$$ratio = \log_2\left(\frac{B}{A}\right)$$

where "A" and "B" represent:
-the number of transcripts at 24hpi and 48hpi respectively, when comparing two time points
-total number of transcripts (24hpi and 48hpi) of non inoculated and inoculated samples respectively, when comparing inoculated with non-inoculated samples

Equation 2

It is also important to note that these results correspond to 2, 4 and 7 days after inoculation, and here we used two different time points, aiming to understand the early expression after inoculation. Expression variations can be observed at later stages of infection and not so evident for the chosen time points which may contribute to value discrepancies after comparison.

4.3.1. *Ethylene-responsive transcription factor ABR1*

Figure 4 shows that transcript the levels of *Ethylene-responsive transcription factor ABR1* are very low and similar in resistant and susceptible genotypes. Only the intermediate phenotype SC914 has a high number of copies at 48hpi: 1736,5 which is 87 times higher than CC14 levels. After inoculation CC14 transcript levels increased (ratio=3,75); most significant changes occurred at 48hpi. Values are inferior to what was predicted by 454 technologies (ratio=6,53). SC51 and SC912 show opposite behavior: SC51 increases at 48hpi with a 0,87 ratio, and SC912 decreases with a -0,86 ratio. *C.sativa* increased the number of copies after inoculation with 153 copies μL^{-1} (ratio=2,84).

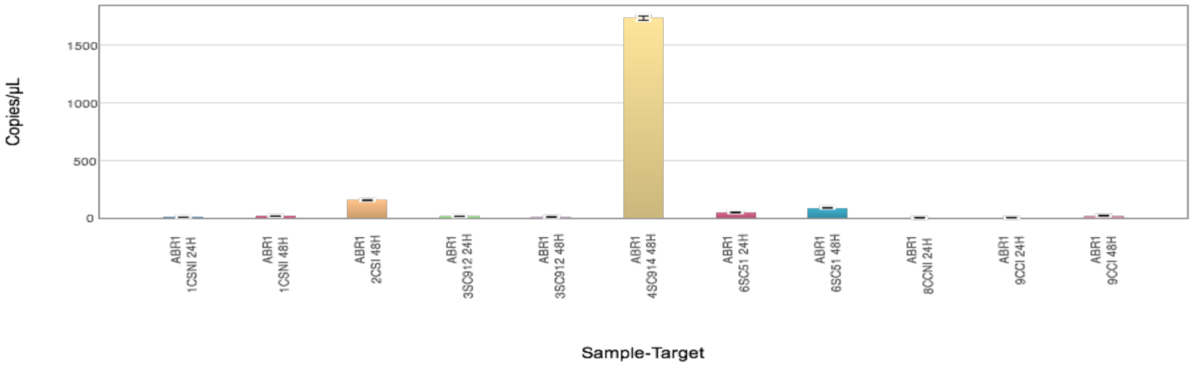


Figure 4: Absolute quantification results of "*Ethylene-responsive transcription factor ABR1*" transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.2. RING finger protein 5

Regarding *RING finger protein 5* transcript abundance, (Figure 5) *a*s *C.crenata* upregulates this gene after infection, with a 2,97 ratio, according to 454 sequencing results, higher expression was expected for CCI at 24hpi and 48hpi. Instead, the number of copies decreased after inoculation. *C.sativa* transcript levels are higher than those obtained for *C.crenata*, and is upregulated after inoculation (ratio=4,32). SC914 has the highest copy number value: 673 and 849 copies µL^{-1} at 24hpi and 48hpi respectively (ratio=0,33). The number of copies for SC912 was reduced at 48hpi (ratio=-0,86). Resistant genotypes, SC55 and SC51 have lower expression levels when compared with CSI and SC914, and higher than *C.crenata.*

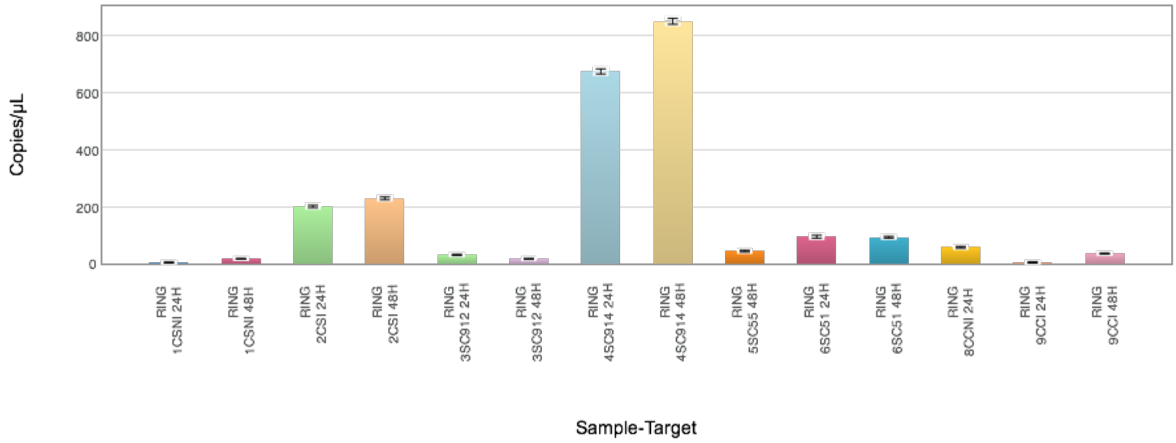


Figure 5: Absolute quantification results of "*RING finger protein 5*" transcripts by QuantStudio™ 3D PCR™ (Absolute Quantification mode). Error bars represent 95% confidence intervals.

4.3.3. *WRKY transcription factor 31*

WRKY levels increase after inoculation with *P.cinnamomi* (Figure 6), in sample CCI at 48hpi, with a 4,23 ratio, significantly higher than the levels expected by 454 technologies (ratio= 1,71). However expression at 24hpi is inferior to non-inoculated values. At 24hpi, CSI *WRKY* abundance increases with a 3,9 ratio, similar to what is observed in CC 48hpi. At 48hpi the expression is significantly reduced to the levels observed in non-inoculated plants. *WRKY* levels also decreased at 48hpi for SC912. SM904 has the highest copy number for this target, followed by SC914. No significant differences were observed between SC914 samples at 24hpi and 48hpi (ratio=0.43) while SM904 increased the number of copies at 48hpi, from 279 to 726 copies/µL (ratio=1,38). Low transcript levels are present among SC51 and SC55 samples; number of copies/µL also increased at 48hpi for SC51 (ratio=2,65).

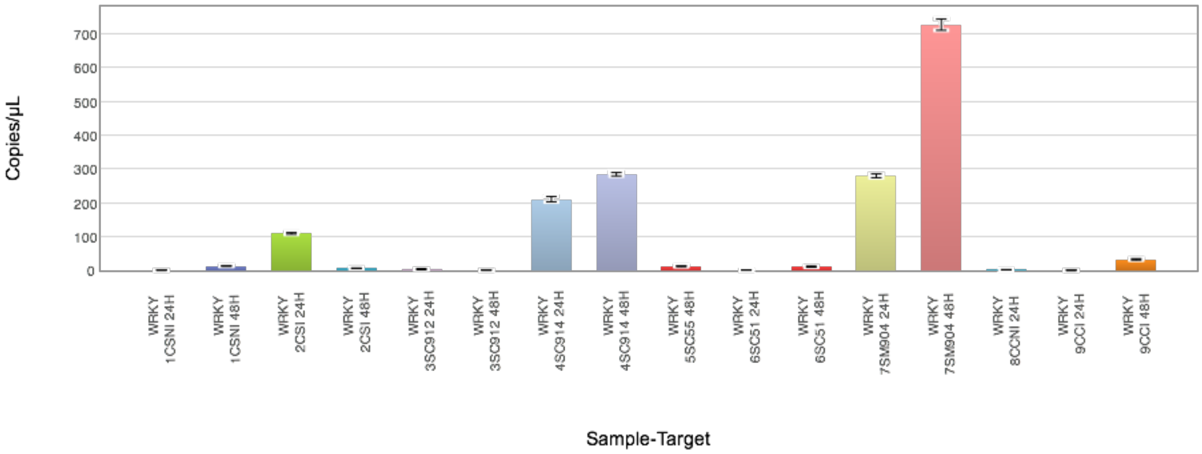


Figure 6: Absolute quantification results of “*WRKY transcription factor 31*” transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.4. *Myb-related protein Myb4*

Low *Myb4* expression levels were observed for most of the samples (Figure 7). CSNI-CSI comparison shows no significant differences after and before inoculation (ratio=0,05). In opposition to what was expected by 454 sequencing results, CCI at 24hpi and at 48hpi has fewer copies than non-inoculated samples, suggesting that this gene is dowregulated after inoculation (ratio=-0,79). SC914 has the highest number of copies followed by SM904. Myb4 expression levels decreases at 48 hpi for SC912 and SC914 with -1,63 and -2,83 ratios respectively, in opposition to what occurs for SC51 and SM904 that increase the number of copies at 48hpi (3,88 and 0,93 ratios respectively).

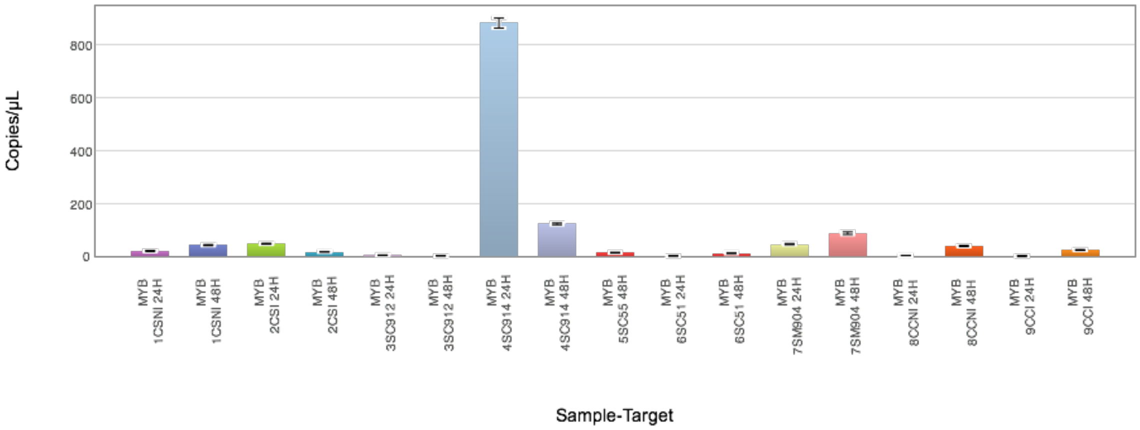


Figure 7: Absolute quantification results of “*Myb-related protein Myb4*” transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.5. Cysteine-rich repeat secretory protein 38 (Gnk)

Cysteine-rich repeat secretory protein 38 (Gnk) abundance (Figure 8) was higher in intermediate (SC914), and resistant genotypes (SC51 and SM904). CSI at 24hpi also exhibited high levels, however lower when compared to these genotypes. *C.sativa* significantly increased the expression at 24hpi, however low levels are present at 48hpi. CCI at 24 hpi showed no differences when compared to non-inoculated samples, and was significantly upregulated at 48hpi (ratio=5,3). Similar results can be observed for other samples: expression increases at 48hpi in SC914 and SC51 (with ratios= 0,64 and 7,49 respectively). SC55 at 48hpi and CCI at 48hpi have similar copy number values. SC912 has the lowest number of copies.

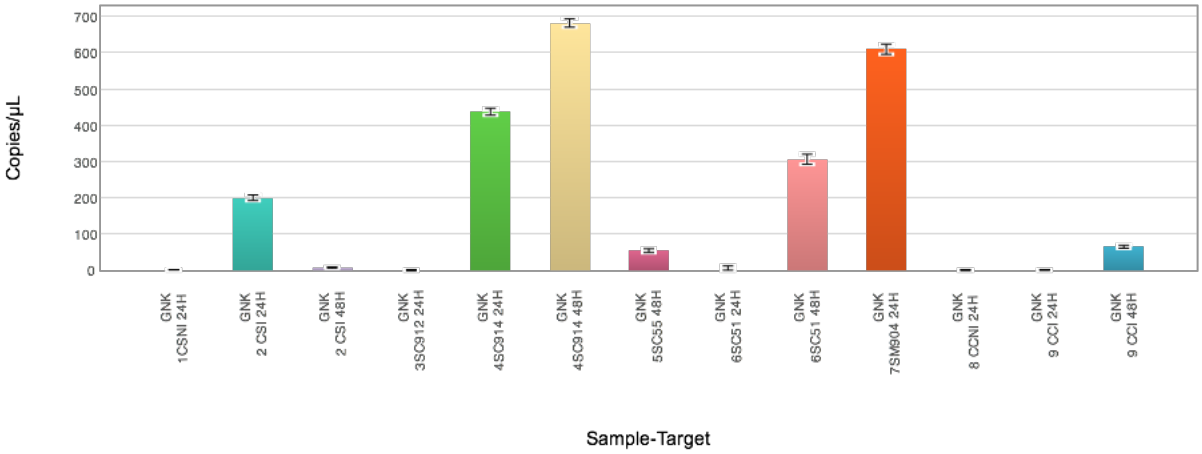


Figure 8: Absolute quantification results of “*Cysteine-rich repeat secretory protein 38 (Gnk)*” transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.6. *LRR receptor-like serine/threonine-protein kinase*

CCI *LRR* transcript levels obtained at 48hpi when compared to non-inoculated plants, increased with a 2,09 ratio, similar to what was expected by 454 results, a 2,32 ratio (Figure 9). CSI showed increased and stable expression of *LRR* after inoculation with *P.cinnamomi,* with a 2,8 ratio, a higher ratio and similar copy number levels when comparing with *C.crenata* results. SC912 has the lowest number of copies, which increase at 48hpi (ratio= 2,25). SC914 registered the highest number of copies at 24hpi, and levels decreased at 48hpi (ratio=-0,72). The number of copies for SM904 are similar to SC914, and also decreased at 48hpi (ratio=-0,15). No significant variance is shown for SC55; SC51 increases the expression at 48hpi (ratio=0,41). The number of copies for both genotypes is inferior to when compared to inoculated *C.sativa* and *C.crenata* values.

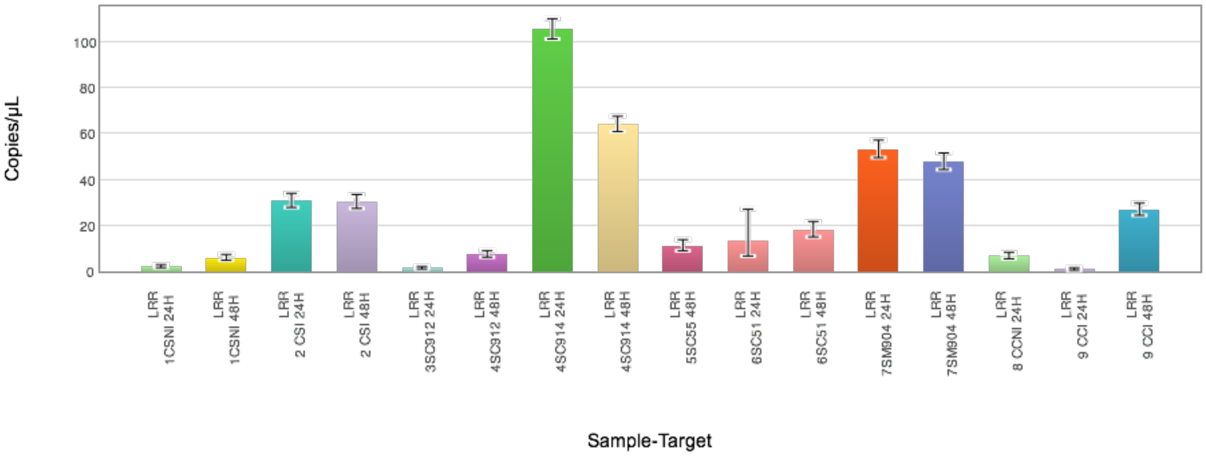


Figure 9: Absolute quantification results of "*LRR receptor-like serine/threonine-protein kinase*" transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.7. Pectinesterase 2

C.crenata's Pectinesterase 2 transcript levels were significantly upregulated upon inoculation (ratio=3,5), similar to what was expected from 454 data (ratio=2,98). Copy number levels increased for *C.sativa* after inoculation (ratio=0,82) and a decrease in the values at 48hpi is observed (ratio=-2,58). SC912 has a low copy number value, and also registered a decrease at 48hpi (ratio=-2,67). SC914 has the highest number of copies/µL with 539 and 1034 at 24hpi and 48hpi (ratio=0,94), followed by SM904 with 277 and 490 at 24hpi and 48hpi respectively and a 0,69. SC55 is upregulated at 48hpi in comparison to 24hpi levels with a 5,38 ratio. SC51 shows no significant differences when comparing 24hpi and 48hpi values (ratio=0,29).

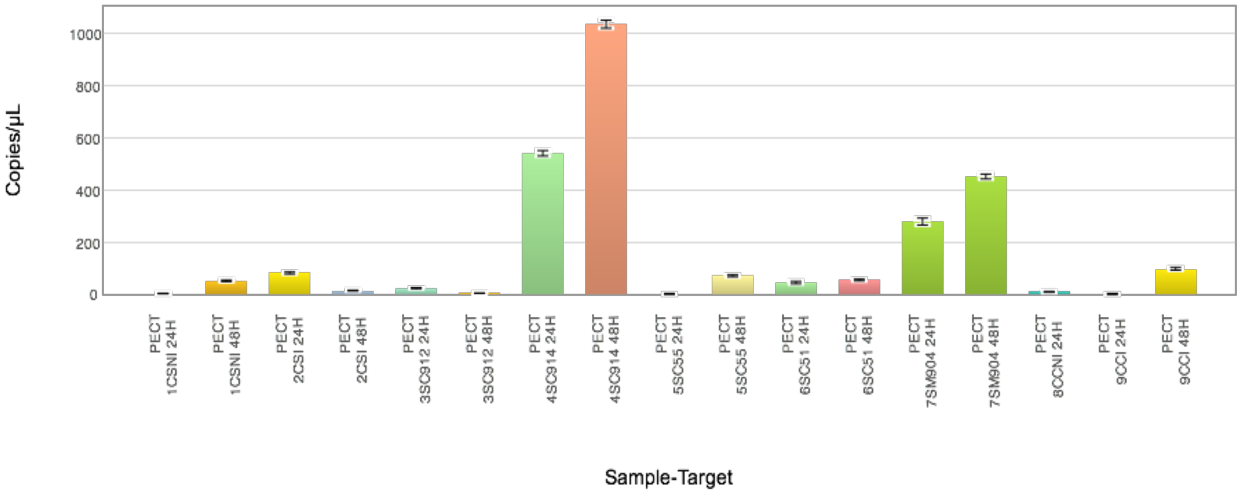


Figure 10: Absolute quantification results of "*Pectinesterase 2"* transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.3.8. *C2 domain-containing protein*

The gene encoding for *C2 domain-containing* protein was constitutively expressed for all samples (Figure 11). After inoculation, CSI transcript level observed at 24hpi and 48hpi was significantly upregulated with a 3,37 ratio. Transcript abundance decreases for *C.crenata* after inoculation (ratio=-0,31) with a marked decrease at 24hpi (-3,04), however the non-inoculated sample exhibited similar transcript levels at 48hpi. Results differ from those obtained with 454 sequencing: *C.crenata* upregulated transcript levels with a 2,48 ratio. SC912 and SC51 transcript levels decreased at 48hpi with -2,39 and -2,11 ratios respectively, opposed to SC914, SC55 and SM904 which upregulate the number of copies at 48hpi (0,42; 3,17 and 1,57 ratios respectively). Resistant SM904 has inferior number of copies when compared to SC914.

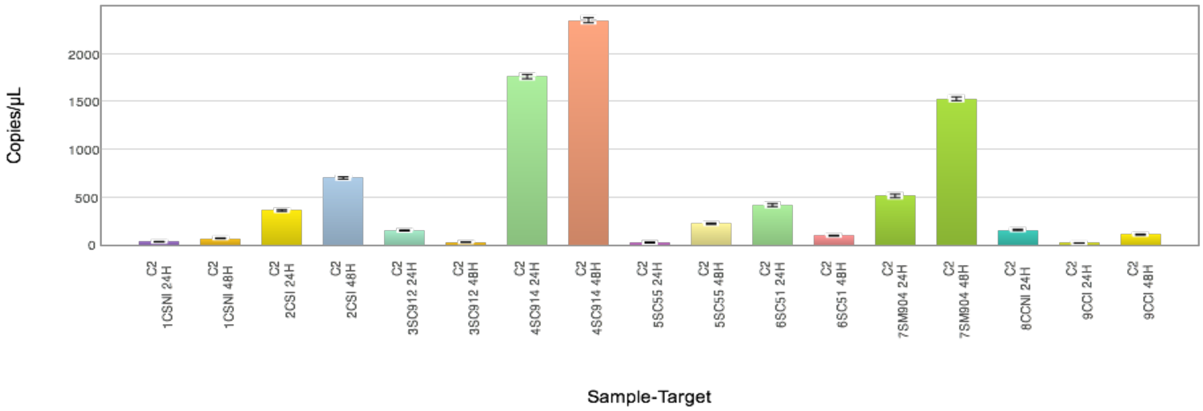


Figure 11: Absolute quantification results of "*C2 domain-containing protein*" transcripts by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

4.4. Analysis of best quality RNA samples

Samples with better RNA quality with A260/280 and A260/230 ratios superior to 1,5 and 1 respectively (Supplementary table 1) were chosen for a separate analysis, when good quality chips were available. It is possible to verify that in comparison to CSI, SC55 has the highest number of copies/µL, 5206 and 1763 for ABR1 transcription factor and RING finger protein 5 respectively, followed by SC914 with a maximum at 48hpi of 1363 and 924 copies respectively. RING finger protein levels for *C.sativa* at 48hpi are similar to SC914 at 24hpi with 637 and 532 copies µL^{-1} respectively. As SC914 levels increase at 48hpi with 924 copies/µL (Figure 12).

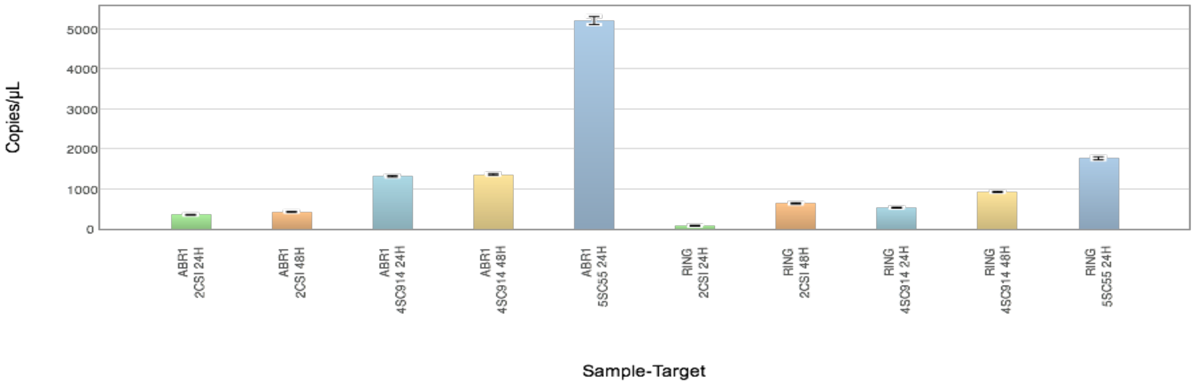


Figure 12: Absolute quantification results of "*Ethylene-responsive transcription factor ABR1*" and "*RING finger protein 5*" transcription factors abundance for best quality samples by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

Regarding Myb-related protein Myb4 transcript abundance has low variance among resistant and susceptible genotypes. WRKY transcription factor 31 expression is higher at 48hpi in resistant SM904 with 726 copies. At 24hpi SC55 and SM904 have similar number of copies/µL (342 and 377 respectively). Other samples have similar number of transcripts, 352 and 398 copies/µL for *C.sativa* and SC914 respectively (Figure 13).

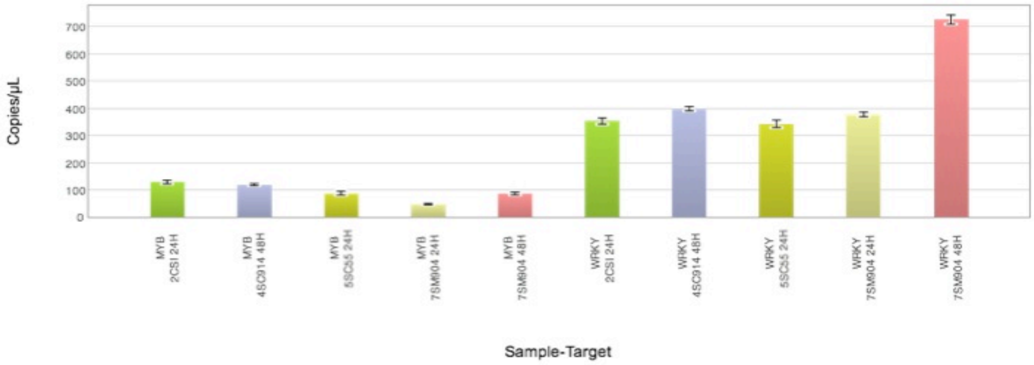


Figure 13: Absolute quantification results of "*Myb-related protein Myb4*" and "*WRKY transcription factor 31*" abundance for best quality samples by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

Cysteine-rich repeat secretory protein 38 is also highly expressed in SM904 with 3796 copies in comparison to other genotypes that range from 553 to 919 copies. CSI has the lowest GNK trancript abundance with 397 copies/µL. LRR number of copies is superior in SC55 with 380 copies/µL. SM904 and CSI have similar number of copies/µL: SM904 with 53 and 82 at 24hpi and 48hpi and CSI with 52 at 24hpi (Figure 14).

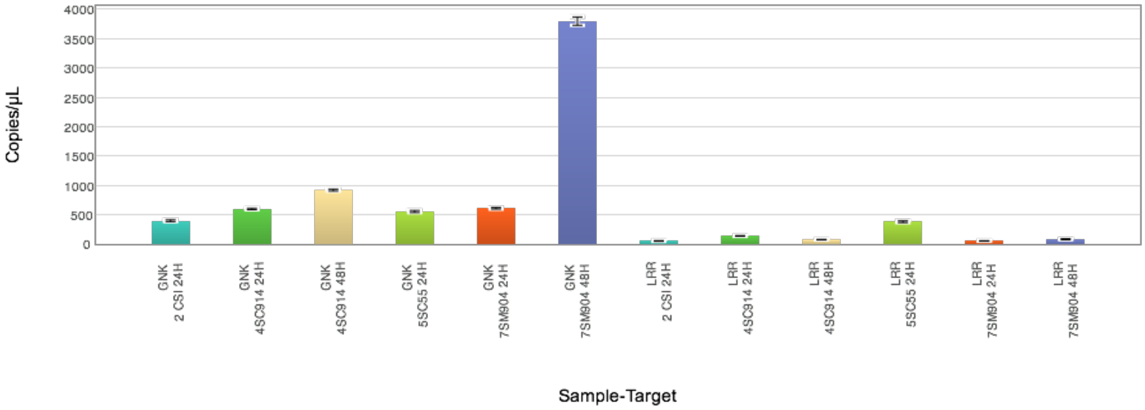


Figure 14: Absolute quantification results of "*Cysteine-rich repeat secretory protein 38"* and "*LRR receptor-like serine/threonine-protein kinase"* transcript abundance for best quality samples by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

SM904 C2-domain containing protein transcript abundance at 24hpi is lower when compared to CSI 24hpi: SM904 has 511 and CSI 890 copies. However it is highly expressed at 48hpi with 1967 copies. SC914 has the highest number of copies/µL with 2630 and 2257 copies/µL at 24 and 48hpi respectively. Pectinesterase 2 abundance is similar to C2-domain: SC914 at 24 and 48hpi have the highest number of copies/µL (814 and 1427 respectively). At 24hpi SM904 has 277 copies/µL slightly above CSI (233 copies/µL) and 719 copies/µL at 48hpi (Figure 15). When compared to inoculated *C.sativa*, copy number are higher in resistant genotypes, only SM904 at 24hpi is similar to *C.sativa.*

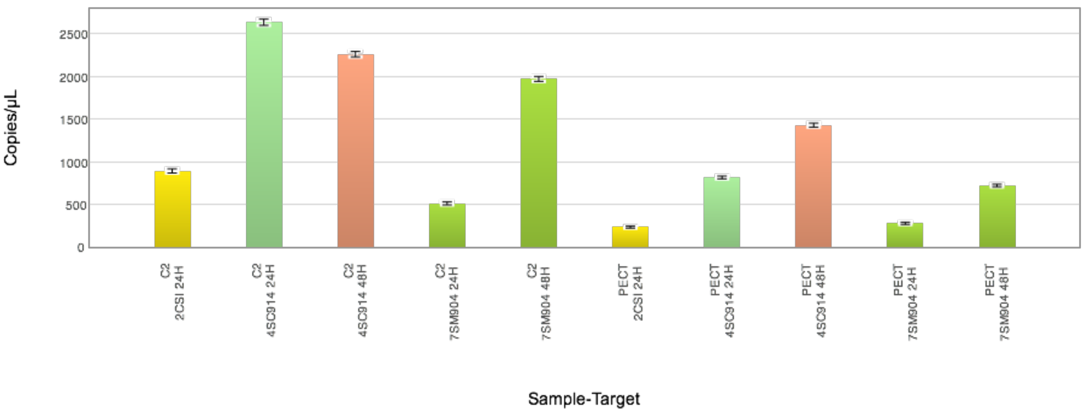


Figure 15: Absolute quantification results of "*C2 domain-containing protein"* and "Pectinesterase 2" transcript abundance for best quality samples by QuantStudio™ 3D PCR™ Absolute Quantification mode. Error bars represent 95% confidence intervals.

Digital PCR results differ from the values predicted by 454 sequencing method. Sample-to-sample variations have occurred: when comparing the number of positive amplification for all the genotypes tested, CS12, SC914 and SM904 have the highest number of copies. These samples previously showed the presence of intact RNA, and that fact may explain the highest number of copies obtained for those samples. As digital PCR allows the detection of small fold change differences, these results prove that for a sensible and reliable quantification, RNA quality and purity is critical. To compensate sample inter variation from RNA quality, data normalization can be carried out by testing an invariant endogenous control for all samples using QuantStudio™ 3D PCR™ Relative Quantification mode. Pairing absolute quantification with reference normalizations could give more accurate results in this case, however, no reference genes have been described for *Castanea spp*.

As *RING finger protein 5, Ethylene-responsive transcription factor ABR1 and Cysteine-rich repeat secretory protein 38 (Gnk)* indicate that expression increases when the resistance level increases, from SC914 to SM904, when compared to inoculated *C.sativa*. Transcript abundance levels for these genes should be investigated in more detail in the future.

5. CONCLUSIONS

Resistance to *P.cinnamomi* is an appealing area of research as a wide range of species is affected and we still lack knowledge on the mechanisms and genes involved. The interactions between *Castanea* genus and *P.cinnamomi* can be an interesting topic of research due to the natural occurrence of variation in susceptibility/resistance observed in host species and also challenging as the genome sequence is not yet available. Gene expression profile studies are essential for the comprehension of biological processes and to identify gene function. RNA sequencing is a technique that allows gene expression profiling or transcript abundance and does not require prior knowledge of the transcriptome under consideration (Robles et al. 2012). To quantify gene expression, qPCR is the most commonly used technique. All results are analysed in comparison to reference genes that have a stable expression, independent of the experimental conditions, developmental stages and tissues. Alternatively digital PCR offers a new approach that enables the absolute and accurate quantification of nucleic acids without the use of reference genes. This is the first approach aimed at unravelling chestnut's molecular mechanisms of resistance, by validating eight candidate genes, for seven genotypes, with different levels of resistance to *P.cinnamomi.* To understand the phenotypic resistance observed in the previous study

(Santos et al., 2014) and to validate data obtained by 454 sequencing (Serrazina et al., 2015), the transcript abundance levels were monitored by digital PCR. This is the first attempt in using Quantstudio 3D digital PCR for quantifying gene expression in plants. The results obtained by the two methods were not similar. The accuracy of gene expression determination is strongly influenced by RNA quality. Root's development revealed to be critical for the success of these experiments and for the efficiency of downstream applications. Nevertheless new primer and probe sequences have been developed for eighteen candidate resistant genes and are available for new quantification assays in the future.

These preliminary results show that three genes involved in different stages of disease response were overexpressed in resistant genotypes. In the first 48 hours following the infection, salicylic acid and ethylene phytohormones crosstalk can play an important role in resistance to *P.cinnamomi*. After pathogen recognition, RING-type E3 ubiquitin ligases may coordinate host defense responses signalling by targeting regulatory proteins for ubiquitination and lead to salicylic acid accumulation. Endogenous salicylic acid accumulation is required for SAR which is associated with faster activation of salicylic acid induced-genes and PR proteins production. Gnk2 may be secreted as a consequence of SAR induction and may contribute to limit pathogen growth. Ethylene signalling induces transcription factors such as ABR1 involved in the dowregulation of ABA maintaining water balances and preventing stomata closure and decreases in photosynthesis rates and ensure survival. It is possible that *C.sativa* fails to activate salicylic acid pathways in early stages of infection allowing pathogen colonization and compromising survival. It would be interesting to evaluate if exogenous applications of salicylic and ethylene can confer resistance to *P.cinnamomi* in *C.sativa* plants comparable to what is observed for *C.crenata* and *C.mollissima*.

The understanding of molecular mechanisms involved in resistant hosts will enable the identification of defense marker genes for marker-assisted breeding and selection of resistant rootstocks. Further studies are needed to unravel the complex interactions between chestnut and *P.cinnamomi*.

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SUPPLEMENTARY MATERIAL

Supplementary Tables

Table S 1: RNA quality results after each treatment assessed by Nanodrop

Genotypes	Time point	After RNA extraction				After DNase treatment			% RNA loss	After concentration		
		ng/µL	Total Yield (ng)	260/280	260/230	ng/µL	260/280	260/230		ng/µL	260/280	260/230
CS12 inoculated	24h R1	688,4	6884	1,88	1,83	139,4	1,56	0,92	79,75	273,2	1,37	0,9
	24h R2	989,5	9895	1,92	1,85	510,3	1,77	1,14	48,43	2014	1,89	1,64
	24h R3	754,8	7548	1,88	1,71	201	1,3	0,89	73,37	675	1,54	0,93
	48h R1	1177,6	11776	1,88	1,68	214,2	1,53	0,85	81,81	446,8	1,52	0,86
	48h R2	1427,2	14272	1,92	1,85	499,2	1,9	1,65	65,02	1782,4	1,77	1,11
	48h R3	1412,8	14128	1,77	1,41	536,7	1,59	1,02	62,01	1153,4	1,57	0,89
CS12 non-inoculated	24h R1	768,9	7689	1,92	1,81	124,1	1,47	0,79	83,86	230,3	1,42	0,78
	24h R2	905,7	9057	1,93	1,85	96,1	1,48	0,78	89,39	328,1	1,51	0,82
	24h R3	675,7	6757	1,87	1,7	114,6	1,43	0,75	83,04	346,1	1,41	0,64
	48h R1	940,8	9408	1,87	1,67	222,9	1,52	0,88	76,31	1039,1	1,47	1
	48h R2	807,2	8072	1,92	1,78	103,1	1,38	0,7	87,23	103,1	1,38	1,05
	48h R3	1008,8	10088	1,94	1,89	109,6	1,44	0,76	89,14	332,4	1,39	0,71
CC14 non-inoculated	24h R1	412,2	4122	1,94	2	49,8	1,38	0,68	87,92	152,1	1,42	0,59
	24h R2	281,1	2811	1,94	2,09	72,6	1,55	0,93	74,17	285,9	1,56	0,89
	24h R3	612,8	6128	1,98	2,2	30,6	1,54	0,63	95,01	72,3	1,45	0,58
	48h R1	1016,7	10167	1,98	2.,26	201,9	1,38	0,73	80,14	425,8	1,36	0,5
	48h R2	408,3	4083	1,95	1,97	54,3	1,3	0,58	86,7	836,9	1,48	0,6
	48h R3	720,9	7209	2	2,23	29	1,43	0,54	95,98	79,3	1,39	0,55
CC14 inoculated	24h R1	470	4700	1,95	1,95	39	1,48	0,63	91,7	112	1,46	0,65
	24h R2	405,7	4057	1,95	2,09	74,1	1,54	0,86	81,74	74,1	1,54	0,86
	24h R3	1012,1	10121	1,97	2,14	68,1	1,59	0,84	93,27	371,7	1,58	0,88
	48h R1	572,3	5723	1,96	2,05	47,5	1,42	0,64	91,7	157	1,46	0,7
	48h R2	788,2	7882	1,98	2,13	50,3	1,43	0,57	93,62	186,9	1,42	0,59
	48h R3	712,5	7125	1,91	2,09	58,3	1,47	0,69	91,82	559,5	1,54	0,86
SC912 inoculated	24h R1	155,4	1554	1,88	1,65	190,9	1,39	0,65	None	147,3	1,34	0,62
	24h R2	202,3	2023	1,82	1,45	187,5	1,3	0,81	7,32	453,9	1,54	0,79
	24h R3	123,2	1232	1,92	1,66	77	1,36	0,58	37,5	148,9	1,47	0,65
	48h R1	101	1010	1,8	1,32	142,3	1,39	0,74	None	136,7	1,29	0,49
	48h R2	290,3	2903	1,91	1,82	70,1	1,32	0,68	75,85	678,6	1,42	0,93
	48h R3	95,3	953	1,77	1,25	107,4	1,41	0,56	None	185,5	1,31	0,48
SC51 inoculated	24h R1	581,1	5811	2,02	2	43,3	1,38	0,59	92,55	218	1,38	0,62
	24h R2	1140,8	11408	2,03	2,18	64,2	1,68	0,82	94,37	556,3	1,63	0,78
	24h R3	241,6	2416	1,86	1,45	72,3	1,42	0,72	70,07	343,6	1,44	0,54
	48h R1	240,3	2403	1,94	1,73	51,6	1,28	0,6	78,53	286,5	1,58	0,78
	48h R2	119,9	1199	1,9	1,64	121,8	1,4	0,57	None	551,9	1,6	0,84
	48h R3	146,5	1465	1,78	1,35	116	1,4	0,56	20,82	238,9	1,38	0,59

SC55 Inoculated	24h R1	493,7	4937	1,92	1,9	535,6	1,7	0,84	None	1437,7	1,8	1,19
	24h R2	782,3	7823	2,03	2,11	87,2	1,4	0,56	88,85	160,6	1,48	0,64
	24h R3	1872,8	18728	2	2,13	245,6	1,46	0,57	86,89	351,9	1,35	0,55
	48h R1	121,7	1217	1,69	1,14	56,3	1,48	0,63	53,74	322,2	1,37	0,88
	48h R2	466,2	4662	1,89	1,67	890,2	1,13	1,32	None	238,1	1,37	0,58
	48h R3	390,5	3905	1,96	2,01	253,1	1,19	0,93	35,19	128,9	1,32	0,65
SM904 inoculated	24h R1	331,1	3311	1,87	1,87	156	1,81	1,36	52,88	1117,3	1,7	0,8
	24h R2	362,6	3626	1,9	1,94	218,6	1,82	1,42	39,71	1193,5	1,78	1,18
	24h R3	510,8	5108	1,91	2,11	271,3	1,79	1,39	46,89	1435,3	1,74	1,16
	48h R1	1519,9	15199	1,97	2,25	691,8	2	2,09	54,48	1843,8	1,82	1,16
	48h R2	675,2	6752	1,9	2,1	110,8	1,65	1,01	83,59	1612,5	1,49	1,43
	48h R3	766,7	7667	1,95	2,05	315,2	1,92	1,79	58,89	1194,3	1,55	1,3
SC914 inoculated	24h R1	852,9	8529	1,93	2,06	352,3	1,93	1,72	58,69	1297,6	1,88	1,45
	24h R2	178,5	1785	1,89	1,86	79,8	1,79	1,17	55,29	216,9	1,72	0,99
	24h R3	1134,8	11348	1,93	1,93	549,1	1,89	1,49	51,61	1029,9	1,94	1,74
	48h R1	761,5	7615	1,93	2,03	313,1	1,91	1,69	58,88	745,7	1,82	1,24
	48h R2	1520,3	15203	1,98	1,99	640,9	1,99	1,9	57,84	1176,2	1,97	1,73
	48h R3	413,2	4132	1,94	1,96	186,8	1,8	1,42	54,79	392	1,6	0,76

Table S 2: Transcript abundance results for all samples using QuantStudio™ 3D AnalysisSuite™ Software (Absolute Quantification mode) with a confidence interval of 95%.

Gene	**Sample**	**Copies/µL**	**Confidence Interval copies/µL**	**Precision**	**Chips**
Ethylene-responsive transcription factor ABR1	1CSNI 24H	5.862	5.057 -- 6.796	15.921%	2
	1CSNI 48H	80.618	78.714 -- 82.568	2.419%	3
	2CSI 24H	NA	NA	NA	3
	2CSI 48H	NA	NA	NA	2
	3SC912 24H	14.405	13.123 -- 15.813	9.77%	3
	3SC912 48H	7.908	6.864 -- 9.11	15.202%	3
	4SC914 24H	NA	NA	NA	3
	4SC914 48H	1736.5	1717.8 -- 1755.3	1.087%	3
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	NA	NA	NA	3
	6SC51 24H	47.776	44.209 -- 51.632	8.07%	2
	6SC51 48H	87.733	84.891 -- 90.67	3.348%	3
	7SM904 24H	NA	NA	NA	0
	7SM904 48H	NA	NA	NA	0
	8CCNI 24H	1.647	1.245 -- 2.18	32.313%	3
	8CCNI 48H	NA	NA	NA	3
	9CCI 24H	2.315	1.899 -- 2.821	21.895%	3
	9CCI 48H	19.907	18.642 -- 21.258	6.787%	3
RING finger protein 5	1CSNI 24H	4.094	3.431 -- 4.885	19.331%	2
	1CSNI 48H	115.03	112.73 -- 117.37	2.036%	3
	2CSI 24H	200.6	196.19 -- 205.1	2.244%	3
	2CSI 48H	NA	NA	NA	2
	3SC912 24H	30.717	28.811 -- 32.749	6.615%	3
	3SC912 48H	16.837	15.278 -- 18.554	10.201%	3
	4SC914 24H	673.51	664.59 -- 682.54	1.342%	3

	4SC914 48H	849.12	838.72 -- 859.64	1.239%	3
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	43.878	41.176 -- 46.757	6.562%	3
	6SC51 24H	94.788	89.659 -- 100.21	5.72%	2
	6SC51 48H	92.706	89.78 -- 95.727	3.259%	3
	7SM904 24H	NA	NA	NA	0
	7SM904 48H	NA	NA	NA	0
	8CCNI 24H	57.297	54.61 -- 60.116	4.92%	3
	8CCNI 48H	NA	NA	NA	3
	9CCI 24H	4.326	3.743 -- 5.001	15.591%	3
	9CCI 48H	35.265	33.562 -- 37.055	5.074%	3
C2 domain-containing protein	1CSNI 24H	32.576	29.695 -- 35.737	9.703%	2
	1CSNI 48H	68.787	65.314 -- 72.446	5.318%	3
	2CSI 24H	359.28	351 -- 367.75	2.359%	3
	2CSI 48H	699.92	687.99 -- 712.05	1.734%	2
	3SC912 24H	149.94	144.75 -- 155.32	3.587%	3
	3SC912 48H	28.542	25.982 -- 31.355	9.853%	2
	4SC914 24H	1758.5	1736.9 -- 1780.3	1.243%	3
	4SC914 48H	2348.6	2321.4 -- 2376.1	1.171%	3
	5SC55 24H	24.486	22.073 -- 27.163	10.931%	2
	5SC55 48H	220.98	214.57 -- 227.58	2.986%	3
	6SC51 24H	415.25	400.04 -- 431.03	3.8%	1
	6SC51 48H	95.86	91.611 -- 100.31	4.638%	3
	7SM904 24H	511.31	493.22 -- 530.07	3.668%	1
	7SM904 48H	1527.4	1507.7 -- 1547.3	1.307%	3
	8CCNI 24H	156.75	150.25 -- 163.53	4.325%	3
	8CCNI 48H	NA	NA	NA	3
	9CCI 24H	19.007	17.293 -- 20.892	9.913%	3
	9CCI 48H	107.23	102 -- 112.72	5.127%	3
Pectinesterase 2	1CSNI 24H	2.122	1.257 -- 3.582	68.849%	2
	1CSNI 48H	51.144	48.166 -- 54.305	6.181%	3
	2CSI 24H	81.082	77.314 -- 85.035	4.875%	3
	2CSI 48H	13.546	12.08 -- 15.189	12.132%	2
	3SC912 24H	22.887	20.938 -- 25.017	9.307%	3
	3SC912 48H	3.589	2.756 -- 4.675	30.25%	2
	4SC914 24H	539.92	529.59 -- 550.46	1.952%	3
	4SC914 48H	1034.6	1019.2 -- 1050.2	1.507%	3
	5SC55 24H	1.682	1.367 -- 2.268	85.857%	2
	5SC55 48H	70.245	66.73 -- 73.945	5.267%	3
	6SC51 24H	44.208	39.614 -- 49.336	11.599%	1
	6SC51 48H	54.187	51.03 -- 57.539	6.187%	3
	7SM904 24H	277.82	264.9 -- 291.37	4.877%	1
	7SM904 48H	490.79	480.92 -- 500.86	2.052%	3
	8CCNI 24H	8.653	7.246 -- 10.334	19.416%	3
	8CCNI 48H	NA	NA	NA	3
	9CCI 24H	1.528	1.3 -- 1.93	76.086%	3
	9CCI 48H	96.641	91.688 -- 101.86	5.402%	3
Cysteine-rich repeat secretory protein 38 (Gnk)	1CSNI 24H	1.28	0.884 -- 1.854	44.832%	3
	1CSNI 48H	NA	NA	NA	2
	2 CSI 24H	199.87	192.23 -- 207.82	3.976%	2
	2 CSI 48H	7.507	6.152 -- 9.16	22.019%	2
	3SC912 24H	1.145	1.046-- 1.448	210.06%	3
	3SC912 48H	NA	NA	NA	3

	4SC914 24H	436.64	427.48 -- 445.99	2.143%	3
	4SC914 48H	681.6	670 -- 693.4	1.731%	3
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	53.81	48.811 -- 59.322	10.243%	1
	6SC51 24H	1.694	0.239 -- 12.028	609.93%	1
	6SC51 48H	305.71	292.07 -- 319.99	4.671%	1
	7SM904 24H	608.79	595.23 -- 622.66	2.278%	2
	7SM904 48H	NA	NA	NA	2
	8 CCNI 24H	1.35	1.146 -- 1.842	140.26%	2
	8 CCNI 48H	NA	NA	NA	2
	9 CCI 24H	1.638	1.332 -- 2.227	92.194%	2
	9 CCI 48H	64.802	60.827 -- 69.036	6.534%	2
LRR receptor-like serine/ threonine-protein kinase	1CSNI 24H	2.241	1.694 -- 2.965	32.313%	3
	1CSNI 48H	6.042	4.897 -- 7.455	23.384%	2
	2 CSI 24H	30.716	27.858 -- 33.866	10.256%	2
	2 CSI 48H	30.254	27.392 -- 33.415	10.448%	2
	3SC912 24H	1.591	1.131 -- 2.238	40.662%	3
	3SC912 48H	7.566	6.282 -- 9.113	20.446%	3
	4SC914 24H	105.21	100.92 -- 109.68	4.252%	3
	4SC914 48H	63.988	60.728 -- 67.423	5.368%	3
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	11.218	9.07 -- 13.875	23.688%	1
	6SC51 24H	13.557	6.78 -- 27.109	99.963%	1
	6SC51 48H	18.065	15.059 -- 21.671	19.959%	1
	7SM904 24H	53.103	49.428 -- 57.051	7.435%	2
	7SM904 48H	47.723	44.24 -- 51.48	7.872%	2
	8 CCNI 24H	6.808	5.579 -- 8.306	22.019%	2
	8 CCNI 48H	NA	NA	NA	2
	9 CCI 24H	1.993	1.588 -- 2.676	68.848%	2
	9 CCI 48H	26.943	24.434 -- 29.71	10.269%	2
Myb-related protein Myb4	1CSNI 24H	19.205	17.653 -- 20.893	8.792%	2
	1CSNI 48H	41.385	39.396 -- 43.474	5.048%	3
	2CSI 24H	46.58	44.814 -- 48.417	3.942%	3
	2CSI 48H	16.11	14.865 -- 17.459	8.374%	2
	3SC912 24H	3.344	2.828 -- 3.953	18.229%	3
	3SC912 48H	1.081	0.756 -- 1.546	43.024%	3
	4SC914 24H	839.01	828.68 -- 849.54	1.954%	1
	4SC914 48H	121.68	118.39 -- 125.06	2.778%	2
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	12.906	11.489 -- 14.498	12.334%	3
	6SC51 24H	1.742	1.421 -- 2.307	76.097%	2
	6SC51 48H	10.959	9.372 -- 12.814	16.932%	1
	7SM904 24H	45.209	43.128 -- 47.39	4.824%	3
	7SM904 48H	86.325	81.478 -- 91.46	5.949%	1
	8CCNI 24H	2.32	1.777 -- 3.029	30.567%	3
	8CCNI 48H	38.025	35.755 -- 40.44	6.349%	2
	9CCI 24H	1.228	1.123 -- 1.423	85.857%	3
	9CCI 48H	23.045	21.383 -- 24.837	7.775%	3
WRKY transcription factor 31	1CSNI 24H	1.021	0.71 -- 1.47	43.902%	2
	1CSNI 48H	13.371	12.268 -- 14.574	8.993%	3
	2CSI 24H	109.77	107 -- 112.61	2.587%	3
	2CSI 48H	6.536	5.762 -- 7.414	13.427%	2
	3SC912 24H	3.955	3.391 -- 4.613	16.648%	3

	3SC912 48H	1.117	0.785 -- 1.588	42.194%	3
	4SC914 24H	211.04	200.44 -- 222.06	3.921%	1
	4SC914 48H	284.46	279.22 -- 289.8	1.876%	2
	5SC55 24H	NA	NA	NA	3
	5SC55 48H	12.724	11.317 -- 14.305	12.427%	3
	6SC51 24H	1.299	0.847 -- 1.992	53.388%	2
	6SC51 48H	11.309	9.695 -- 13.192	16.648%	1
	7SM904 24H	279.71	274.19 -- 285.35	2.015%	3
	7SM904 48H	726	709.45 -- 742.94	2.334%	1
	8CCNI 24H	2.234	1.702 -- 2.931	31.232%	3
	8CCNI 48H	NA	NA	NA	2
	9CCI 24H	1.797	1.572 -- 2.11	39.278%	3
	9CCI 48H	32.838	30.838 -- 34.968	6.487%	3

Table S 3: Transcript abundance results for samples with better RNA quality using QuantStudio™ 3D AnalysisSuite™ Software (Absolute Quantification mode) with a confidence interval of 95%.

Gene	Sample	Copies/µL	Confidence Interva copies/µLl	Precision	Chips
Ethylene-responsive transcription factor ABR1	2CSI 24H	353.55	343.44 -- 363.95	2.943%	1
	2CSI 48H	429.4	418.09 -- 441.02	2.705%	1
	4SC914 24H	1315.8	1298.3 -- 1333.4	1.344%	2
	4SC914 48H	1363	1344.8 -- 1381.4	1.353%	2
	5SC55 24H	5206.2	5105.6 -- 5308.7	1.969%	1
RING finger protein 5	2CSI 24H	81.132	76.639 -- 85.889	5.863%	1
	2CSI 48H	637.49	623 -- 652.32	2.326%	1
	4SC914 24H	532.35	523.21 -- 541.65	1.747%	2
	4SC914 48H	924.18	910.77 -- 937.79	1.473%	2
	5SC55 24H	1763.5	1726.7 -- 1801.2	2.134%	1
Myb-related protein Myb4	2CSI 24H	128.49	122.12 -- 135.2	5.221%	1
	4SC914 48H	119.26	115.25 -- 123.4	10.23%	1
	5SC55 24h	87.735	81.204 -- 94.792	2.202%	1
	7SM904 24H	47.533	44.827 -- 50.403	3.476%	2
	7SM904 48H	86.325	81.478 -- 91.46	8.043%	1
WRKY transcription factor 31	2CSI 24H	352.71	341.5 -- 364.29	6.038%	2
	4SC914 48H	398.12	390.28 -- 406.13	5.949%	1
	5SC55 24h	342.82	329.3 -- 356.9	3.282%	1
	7SM904 24H	377.17	368.81 -- 385.71	19.161%	1
	7SM904 48H	726	709.45 -- 742.94	1.983%	1
C2 domain-containing protein	2CSI 24H	925,2	916.81 -- 943.02	5,602%	2
	4SC914 24H	2630.1	2593.7 -- 2667.1	1.405%	2
	4SC914 48H	2257.5	2225.2 -- 2290.2	1.449%	2
	7SM904 24H	511.31	493.22 -- 530.07	3.668%	1
	7SM904 48H	1967.4	1938.7 -- 1996.6	1.483%	2
Pectinesterase 2	2CSI 24H	233.3	221.96 -- 245.23	5.112%	1
	4SC914 24H	814.67	798.45 -- 831.23	2.032%	2
	4SC914 48H	1427.1	1403.9 -- 1450.6	1.648%	2
	7SM904 24H	277.82	264.9 -- 291.37	4.877%	1
	7SM904 48H	719.46	704.51 -- 734.72	2.122%	2
Cysteine-rich repeat secretory protein 38 (Gnk)	2 CSI 24H	397.52	382.14 -- 413.51	4.023%	1
	4SC914 24H	597.58	584.54 -- 610.92	2.232%	2
	4SC914 48H	919.78	902.81 -- 937.07	1.88%	2
	5SC55 24H	553.66	535.72 -- 572.19	3.348%	1
	7SM904 24H	608.79	595.23 -- 622.66	2.278%	2

	7SM904 48H	3796.5	3726.1 -- 3868.3	1.891%	1
LRR receptor-like serine/ threonine-protein kinase	2 CSI 24H	52.664	47.447 -- 58.455	10.996%	1
	4SC914 24H	137.77	131.88 -- 143.92	4.465%	2
	4SC914 48H	75.042	70.72 -- 79.627	6.111%	2
	5SC55 24H	380.54	366 -- 395.66	3.973%	1
	7SM904 24H	53.103	49.428 -- 57.051	7.435%	2
	7SM904 48H	82.503	76.123 -- 89.419	8.382%	1

Supplementary Figures

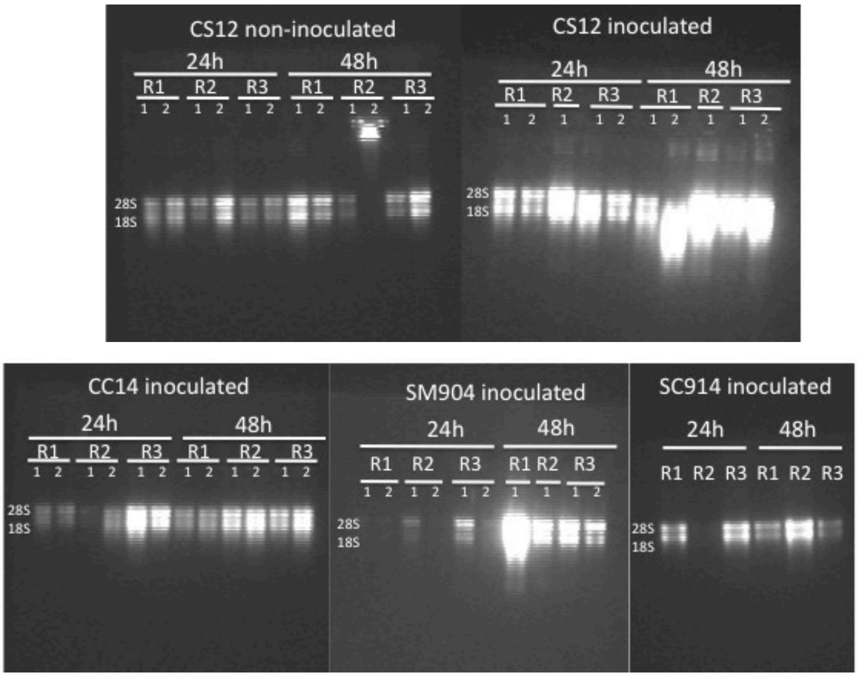

Figure S 1: Denaturing gel electrophoresis of total RNA extracted. Three biological replicates (R1, R2, R3) corresponding to two time points after inoculation 24h and 48h are depicted. Clear 28S and 18S bands are visible for most of the samples.

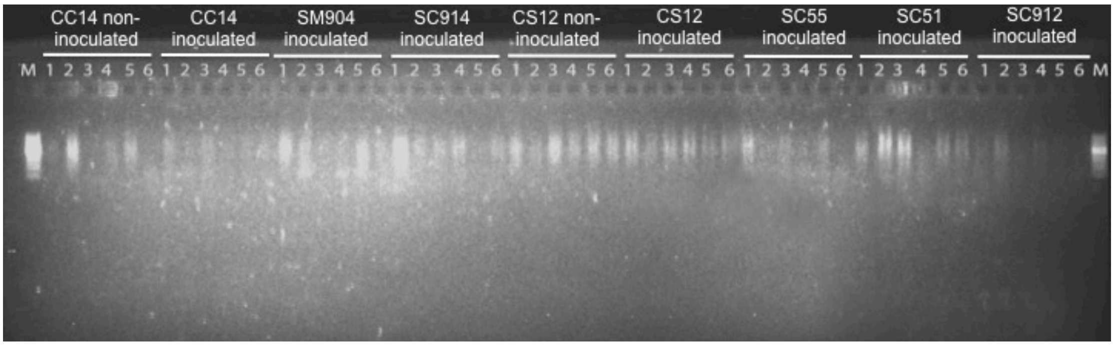

Figure S 2: Gel electrophoresis of cDNA synthesis using random and oligodT primers. A nearly uniform smear can be observed in most of the samples.

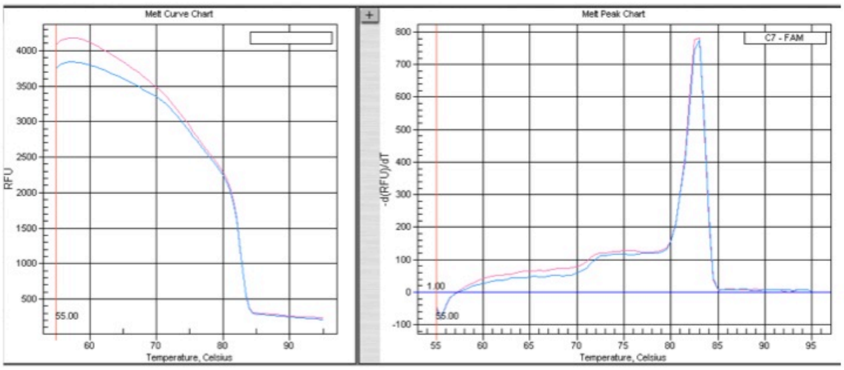

Figure S 3: Melting curves obtained for the gene coding for Cysteine-rich repeat secretory protein. Single peaks show primer specificity for the expected product. Image collected from iQ5 optical system software 1.0 (Bio-rad).