



**Silencing the *gip* gene of *Phytophthora cinnamomi* by iRNA
and studying the subcellular localization of GIP and NPP1
proteins**

ABDESSALEM CHAHED

*Dissertation submitted to Escola Superior Agrária de Bragança to
obtain the Degree of Master in Biotechnological Engineering*

Supervised by

Altino Choupina

Maria João De Sousa

Noureddine Chatti

Bragança

July 2016



*Dissertation made under the agreement of **Double Diploma** between the Escola Superior Agrária de Bragança | IPB and the High Institute of Biotechnology of Monastir | ISBM, Tunisia to obtain the Degree of Master in Biotechnological Engineering*

ACKNOWLEDGEMENTS

There are so many people who have helped me and have made my time at the Polytechnic Institute of Bragança so enjoyable and productive.

First and foremost, I would like to thank my supervisor Pr. Altino Choupina for his guidance, support, and infinite patience during this year. I would like also to express my sincere appreciation to my co-supervisor Pr. Maria João De Sousa for encouragement and sharing her valuable expertise and advice. Also thank, to Pr. Anabela Martins the coordinator of the Biotechnological Engineering, Masters Program for supporting me all through those difficult moments in Bragança.

I also want to acknowledge the effort of Rodrigo Costa for his excellent support in the laboratorial experiments. You have been really helpful and gave all necessary information for the successful completion of this thesis.

I am grateful to all the teaching and non-teaching staff of the Polytechnic Institute of Bragança. A special thanks to Pr Elsa Ramalhosa, Pr Antonio Peres and Pr Paula Rodrigues for their kindness and friendship.

I would also like to thank all researchers in the Mountain Research Center (CIMO) and all my friends who have been there for me all through my stay in Bragança specially Vitor, Taofiq and Sana.

Lastly, I would like to thank my parents Nejib and Sonia, my brothers Alaa and Samer for their never-ending love and support throughout my entire life.

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ABBREVIATIONS LIST

% (w/v): percentage expressed in weight by volume

BLAST: Basic Local Alignment Search Tool

bp: base pairs

CaCl₂: Calcium chloride

CaCO₃: Calcium carbonate

cDNA: Complementary DNA

CECT: Spanish Type Culture Collection

CIAP: Calf Intestinal Alkaline Phosphatase

CPMP: coat protein mediated protection

DNA: Deoxyribonucleic acid

dNTP: Deoxynucleotide solution mix

dsRNA: double stranded RNA

EDTA: Ethylenediaminetetraacetic acid

EGase: endoglucanases

EMBL-EBI: European Molecular Biology Laboratory-European Bioinformatics Institute

ER: endoplasmic reticulum

Exp5: exportin 5

g: gram

gfp: green fluorescent protein gene

GFP: green fluorescent protein

gip: glucanase inhibitor protein gene

GIP: glucanase inhibitor protein

HIV: human immunodeficiency virus

hpt: Hygromycin phosphotransferase gene

HPT: Hygromycin phosphotransferase protein

Hsp70: 70 kilodalton heat shock proteins

Kb: kilo base pairs

kD: kiloDalton

L: liter

LB: Luria-Bertani medium

LGC-1: low glutenin content-1

M: molar

Map kinases: mitogen activated protein kinases

miRNA: micro RNA

mRNA: messenger RNA

NaCl: Sodium chloride

NCBI: National Center for Biotechnology Information

NLP: Necrosis-inducing proteins

npp1: gene for necrosis-inducing protein

NPP1: *P.cinnamomi* necrosis inducing protein

ORF: open reading frame

PCR: polymerase chain reaction

PDA: Potato dextrose agar

PR: Pathogenesis-Related

PTGS: post transcriptional gene silencing

qRT- PCR: quantitative real time PCR

RISC: RNA-induced silencing complex

RNA: Ribonucleic acid

Rpm: rotation per minute

SDS: Sodium dodecyl sulfate

shRNA: short/small hairpin RNA

siRNA: small interfering RNA

SOC: Super Optimal broth with Catabolite repression

TAE: buffer solution of Tris-Acetate and EDTA

Tail-PCR: Thermal Asymmetric Interlaced PCR

TGS: Transcriptional Gene Silencing

T_m: melting temperature

Tris-HCL: Tris-Hydrochloride

U: enzyme unit

V: volt

YEPD: yeast extract peptone dextrose

ABSTRACT

Ink Disease is considered one of the most important causes of the decline of chestnut orchards. The break in yield of *Castanea sativa* Mill is caused by two species: *Phytophthora cinnamomi* and *Phytophthora cambivora*, being the first one the foremost pathogen of ink disease in Portugal. *P. cinnamomi* is one of the most aggressive and widespread plant pathogen with nearly 1,000 host species. This oomycete causes enormous economic losses and it is responsible for the decline of many plant species in Europe and worldwide. Up to now no efficient treatments are available to fight these pathogens. Because of the importance of chestnut at economical and ecological levels, especially in Portugal, it becomes essential to explore the molecular mechanisms that determine the interaction between *Phytophthora* species and host plants through the study of proteins GIP (glucanase inhibitor protein) and NPP1 (necrosis-inducing *Phytophthora* protein 1) produced by *P. cinnamomi* during the infection. The technique of RNA interference was used to knockdown the *gip* gene of *P. cinnamomi*. Transformants obtained with the silenced gene have been used to infect *C. sativa*, in order to determine the effect of gene silencing on the plant phenotype. To know more about the function of GIP and NPP1 involved in the mechanism of infection, the ORF's of *gip* and *npp1* genes have been cloned to the pTOR-eGFP vector for a future observation of *P. cinnamomi* transformants with fluorescent microscopy and determination of the subcellular localization. Moreover the prediction by bioinformatics tools indicates that both GIP and NPP1 proteins are secreted. The results allow to predict the secretory destination of both GIP and NPP1 proteins and confirm RNAi as a potential alternative biological tool in the control and management of *P. cinnamomi*.

Keywords: Chestnut, *Phytophthora cinnamomi*, ink disease, GIP, NPP1, RNA interference, subcellular localization.

RESUMO

A doença de tinta do castanheiro é considerada uma das mais importantes causas do declínio dos soutos. A quebra da produção de *Castanea sativa* Mill é causada por duas espécies: *Phytophthora cambivora* e *Phytophthora cinnamomi*, sendo o principal causador da doença da tinta em Portugal. *P.cinnamomi* é um dos patógenos de plantas mais agressivo e generalizado, com cerca de 1.000 espécies de hospedeiros. Este oomiceta provoca enormes perdas económicas e é responsável pelo declínio de muitas espécies de plantas na Europa e no mundo. Até ao momento não se conhecem tratamentos eficazes para combater esses patógenos. Devido à importância do castanheiro a níveis económico e ecológico, especialmente em Portugal, é importante explorar os mecanismos moleculares que determinam a interação entre espécies de *Phytophthora* e plantas hospedeiras através do estudo das proteínas GIP (proteína inibidora de glucanases) e NPP1 (proteína indutora de necroses em *Phytophthora*) produzidos por *P.cinnamomi* durante a infeção. A técnica de RNA de interferência foi usada para o silenciamento do gene *gip* de *P.cinnamomi*. Os transformantes obtidos com o gene silenciado foram utilizados para infectar *C. sativa*, a fim de determinar o efeito de silenciamento de genes no fenótipo da planta. Para saber mais sobre a função de GIP e NPP1 no mecanismo da infeção, estes genes foram clonados no vector pTOR-eGFP para uma posterior observação de transformantes de *P.cinnamomi* com microscopia de fluorescência a fim de determinar a sua localização subcelular, apesar de, a previsão com ferramentas bioinformática indicarem que tanto GIP como NPP1 são proteínas secretadas para o meio extracelular. Os resultados permitem prever o destino celular de ambas as proteínas e confirmar a técnica de silenciamento de genes por iRNA como uma potencial ferramenta biológica alternativa no controle de *P. cinnamomi*.

Palavras-chave: Castanha, *Phytophthora cinnamomi*, doença da tinta, GIP, NPP1, RNA de interferência, localização subcelular

1-INTRODUCTION

1.1-The problem of ink disease

Ink disease is one of the most ruinous diseases altering *Castanea sativa*. It leads to root and collar rot of adult trees and of seedlings in nurseries, plantations and forests. Manifestation of the disease on adult trees comprises: chlorotic leaves shrinking, thinning of the crown, and immature peel stick around the tree after leaf-fall.

Generally, it is the large roots that are affected; they generate a black exudate that tints the surrounding soil. After trees debarking, dark necrosis are visible on the collar, but for young trees with fine bark, the necrosis is visible without debarking.

An extensive necrosis of the tap-root that reaches the lateral roots is observable in the root system and reaches the stem for some centimeters. A rapid or gradual withering of the leaves is noticeable in infected plants (**Figure 1**) (Vannini and Vettraino, 2001).



Figure 1.Symptoms of ink disease in infected *Castanea sativa* (Professor A. Choupina)

The disease was recorded in Portugal since 1838, although it is believed to have been registered in Spain in 1726 (Crandall, 1950), after that it has been reported in many European

countries, however information from several European countries has provided an updated distribution map of ink disease in Europe. Ink disease has been recorded in Italy, Spain, Portugal, United Kingdom, France, Greece, Switzerland, Turkey, Hungary, Macedonia, Slovakia, Romania and Azerbaijan (**Figure 2**) (Vannini and Vettraino, 2001).



Figure 2. Distribution map of ink disease on *Castanea sativa*. Countries where the disease has been registered are marked in grey. (Vannini and Vettraino, 2001)

The existence of ink disease coincides with the distribution maps of *Phytophthora cinnamomi* and *Phytophthora cambivora* the two main species responsible for the induction of the disease; generally, they can provoke the death of adult trees in one to three years (Vannini and Vettraino, 2001).

Phytophthora species caused several billion dollars of damage on crops, ornamental, and native plants and until now no efficient solutions have been found to control the disease, for that reason, it becomes very important to understand the mechanism of action of these species (Kamoun, 2003).

1.2-The genus *Phytophthora*

1.2.1-*Phytophthora* species

Phytophthora derives from the Greek word “phyto” (plant) and “phthora” (destroyer). *Phytophthora* genus was so named by Anton de Bary in 1876, when *Phytophthora infestans* was described as the type species of this genus (Cortizo et al, 1999).

Phytophthora species are a diverse group of filamentous, eukaryotic plant pathogens that are associated to other stramenopile (heterokont) eukaryotes within the chromalveolate super-group. The stramenopile group contains golden-brown algae, diatoms, brown algae, and oomycetes, only lately after phylogenetic analysis, these organisms which vary from autotrophic algae to pathogenic, fungus-like oomycetes were arranged together. The oomycete lineage (non-photosynthetic stramenopiles, including the genus *Phytophthora*) was demonstrated to be evolutionarily ancient. The oomycota lineage is believed to have diverged from the photosynthetic stramenopiles in the ochrophyta lineage near the Proterozoic-Phanerozoic boundary about 570 million years ago (Baldauf, 2003; Brown and Sorhannus, 2010; Fahlgren et al, 2013).

Phytophthora includes some of the most ravaging plant pathogens and contains more than 100 formally described species. Practically all eudicot plants and various monocot species can be contaminated by *Phytophthora* species, which are dependent on conditions of humidity for their survival but capable of adopting various strategies to adapt to climate change (Blair et al, 2008; Fry, 2008; Hardham, 2005).

For a long time, the genus *Phytophthora* was included in the fungi kingdom as they use similar infection strategies, are heterotrophic organisms, having hyphal growth polarization and possess vegetative spores adapted to dispersion by currents of air or by water. On the other hand, *Phytophthora* genus has many characteristics that cannot be found in fungus:

- It has zoospores that produce a cell wall during the process of encystment required to germinate and provoke infection
- The cell wall is formed by β -glucans and some cellulose unlike fungi whose main component is chitin
- The zoospores possess two flagella with different morphology, they are diploid during the vegetative phase, unlike the fungi that are haploid
- Produce only a spore in each oogonium

- Accumulate as a substance of reserve, the β 1-3-glucans and mannitol while in fungi the main substance of reserve is mannitol
- Cannot synthesize sterols and for that reason are not sensitive to the fungicides that interfere with its biosynthesis (Gouveia, 2004).

1.2.2-*Phytophthora cinnamomi*

P. cinnamomi Rands was first isolated in 1922, on the island of Sumatra, from the tree of cinnamon (*Cinnamomum burmanii*). The pathogen is believed to have emerged near Papua New Guinea but now has a worldwide distribution in over 70 countries and has almost 1000 hosts which are predominantly woody plants. The main hosts include the avocado, eucalyptus, pineapple, chestnut, several pine species, many ornamental plants, and over 2500 Australian native species. *P. cinnamomi* was introduced in Western Australia, America, Western Europe and Africa (Hardham, 2005; Roberts and Boothroyd, 1972; Zentmyer, 1981).

P. cinnamomi can grow saprophytically in the soil for long periods, rapidly capitalizing on the advent of advantageous conditions to sporulate and produce vast numbers of asexual, biflagellate zoospores. The motile zoospores are attracted to suitable infection sites, where they attach and invade the plant. Within a few days, the hyphae ramify throughout the tissues of susceptible plants, forming sporangia on the plant surface and rapidly expanding the disease inoculum. *P. cinnamomi* is known to survive for as long as 6 years in moist soil, and it is obvious that moisture is a key factor in the establishment, spread and longevity of *P. cinnamomi* diseases. Asexual sporulation needs a liquid environment, both for the formation of sporangia and for the release and activity of motile zoospores. Disease development is enhanced after heavy rain and in waterlogged soils (Hardham, 2005; Reglinski et al, 2009; Zentmyer and Mircetich, 1966).

Phytophthora species present two types of reproduction in their life cycle: asexual reproduction (with formation of chlamydospores and sporangia containing zoospores) and sexual reproduction (with production of oospores).

1.2.3-Asexual reproduction of *Phytophthora*

The coenocytic hyphae is the basic component of asexual reproduction in *P. cinnamomi*, by the cleavage of its cytoplasm, hyphae produce chlamydospores and sporangia (Zentmyer, 1983).

In the presence of moisture and optimum temperature between 20 to 28°C the sporangium germinate directly through a germ tube or indirectly through a process known as zoosporogenesis. This requires the cleavage of multinucleate sporangium producing uninucleate zoospores cells (Hyde et al, 1991).

Chlamydo spores can be also produced during the asexual phase by *Phytophthora* which represent a component of resistance and survival. Generally, chlamydo spores are hyaline but they change to yellowish or slightly brownish marks with the passing of time. In conditions of ideal temperature, chlamydo spores can germinate by cleavage of its cytoplasm and generate numerous germinative tubes or produce sporangia (Erwin et al, 1983).

1.2.4-Sexual reproduction of *Phytophthora*

P. cinnamomi is heterothallic in normal conditions and needs the presence of mycelium of opposite sexual compatibility (A1/A2) to have sexual reproduction with the formation of oospore. The process starts by sexual contact and penetration of the antheridium precursor by the precursor of oogonium without fusion of cytoplasm between gametes. After that the expansion phase of the oogonium takes place due to the flow of cytoplasm via oogonium rod that remains open and functional. When the expansion phase ends, the rod of oogonium is jammed with material similar to the cell wall. After that a diploid oospore is formed which will develop into sporangia and the cycle will continue as is would asexually (Gouveia, 2004).

1.2.5-Mechanism of infection

The mycelium, the oospore or the chlamydo spore, have the ability to survive for a long time in soil, or remain in dead plant tissue in the absence of the host. They can germinate in the presence of moisture and produce sporangium (Erwin et al, 1983).

The sporangium can germinate directly or indirectly, releasing zoospores that will follow the course of water in soil, find the plant tissue and perforate the root of the host (**Figure 3**). The attraction of zoospores by chemical stimulus (chemotaxis) of the host roots presents the first step in the process of infection (Khew and Zentmyer, 1973).

The attack of the pathogen against the host organism starts with a mechanical lesion or by direct action of *Phytophthora* enzymes capable of causing destruction of the roots cell walls. *P. cinanmomi* begins by invading the thinner roots preferably from the ends not yet lignified provoking cell lysis, passing then to colonize the roots more interior and thicker in centripetal expansion, until it reaches the neck of the root (Abreu, 1992; Cortizo et al, 1999).

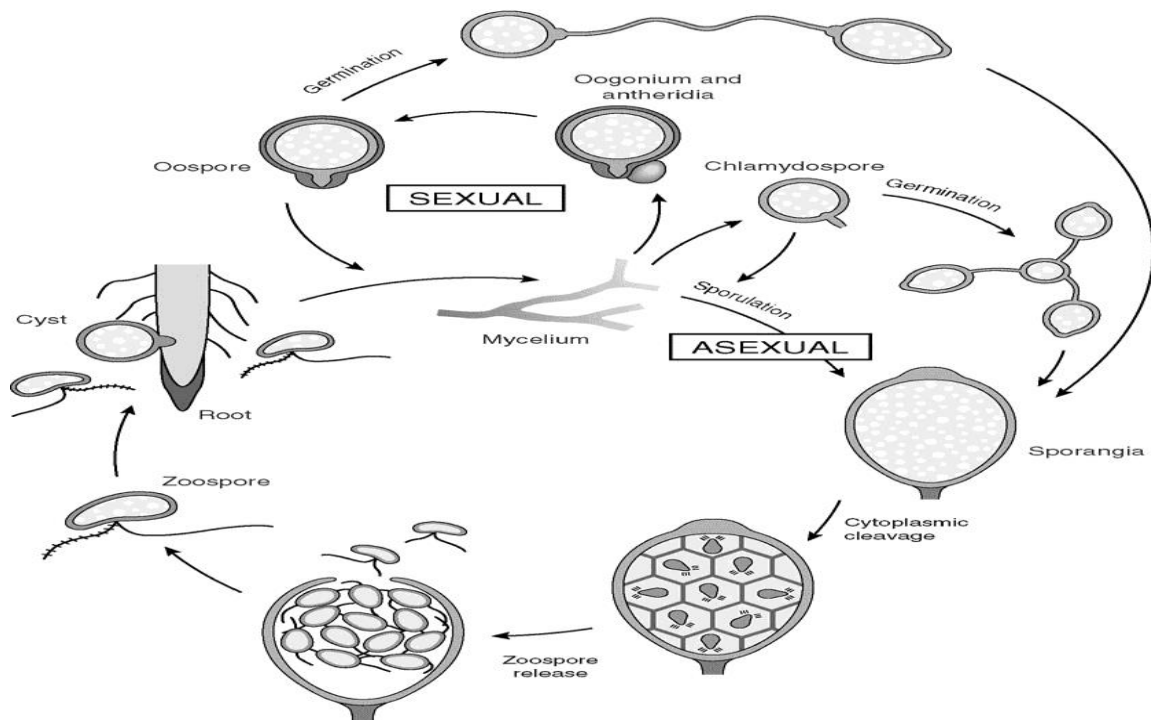


Figure 3. A diagram depicting the life cycle of *Phytophthora cinnamomi* (Diagram Professor A Hardham, The Australian National University, Canberra, A.C.T)

1.3-Traditional methods used to control ink disease

Traditional fungicides are not effective in controlling *Phytophthora* diseases, because there is a significant evolutionary distance between *Phytophthora* and the true fungi, so it is possible to have a chemical compound that can inhibit fungi and with no effect on *Phytophthora*. Therefore to control *Phytophthora* diseases it is better to develop other approaches since such root pathogens can survive as chlamydospores in soil and in the roots of symptomless plants (Hardham, 2005).

Phosphite is a valuable inhibitor of the pathogen spread, but its potency fluctuates with different *P. cinnamomi* isolates (Wilkinson et al, 2001a) and environmental conditions, such as the phosphorus levels in the soil (Hardham, 2005; Guest and Grant, 1991). On the other hand phosphite normally shows low phytotoxicity, recent research has proved that in some plant phosphite can cause foliar damage, reduction of pollen viability and pollen tube growth, an increase in the frequency of abnormal mitotic and meiotic cell divisions and a decrease in root growth (Barrett et al, 2002; Fairbanks et al, 2001; Hardham, 2005; Nartvaranant et al, 2004).

In the best cases, phosphite can just reduce zoospores production and indeed the pathogen's fecundity may not be altered as many researches have proved; that zoospore production is possible on phosphite treated hosts (Hardy et al, 2001; Wilkinson et al, 2001b).

The development of resistant varieties and the resort to chemical methods has been shown to be useless by which the development of alternative ways to control the disease assumes great importance (Abreu et al, 1995; Salesses et al, 1993).

Recently, the research at the level of molecular biology has given much information that can help to understand the molecular mechanisms responsible for pathogenicity and to eradicate the diseases caused by this pathogen.

1.4-Gene *gip*

1.4.1-Gene *gip* in *Phytophthora* species

There are many strategies used by plants to defend against microorganism pathogens, the development of defense mechanisms like the secretion of hydrolytic enzymes that degrade the polysaccharides cell wall represent an essential role in reducing the invasion of pathogens. The interaction between plants and pathogens has as important characteristic; the secretion of glucanase inhibitor proteins (GIP) by the pathogens as a response to plants hydrolytic proteins, endo- β -1,3-glucanases (Hein et al, 2009; Kamoun, 2006).

Because of the great interest of GIPs at the level of pathogenicity and response of the host plant, it becomes required to understand the mechanism of action of these proteins.

1.4.2-Gene *gip* in *Phytophthora cinnamomi*

The *gip* gene of *P. cinnamomi* (GenBank GIP, code AM259384) presents a total size of 1171 bp, an ORF of 810 bp and 269 amino acids. The amplification of the gene was achieved using the generic primers designed on the basis of homology of *gip*'s (*Phytophthora sojae*) ORF and by using the technique of TAIL-PCR (Michiels et al, 2003).

Significant homologies have been shown after alignments of *P. cinnamomi* GIP sequence with sequences of *P. sojae* and *P. infestans*. In these pathogens GIP bind and inhibit the activity of plant extracellular endoglucanases implicated in defense responses, thereby suppressing the degradation of glucan in the Oomycetes cell wall, and blocking the release of glucan elicitors and their triggered defense responses. Despite their potential importance as suppressors of EGase, the biological significance of GIP proteins has not yet been well understood. Many questions remain at the molecular level, such as the identity of the domains

and key residues of the inhibitor proteins that contribute to the specificity of recognition (Damasceno et al, 2008; Martins et al, 2014).

1.5-Gene *npp1*

1.5.1-Gene *npp1* in *Phytophthora* species

Phytophthora species secrete the necrosis-inducing protein (NPP1) recently identified among a class of a necrosis-inducing proteins, known as Nep1-like proteins (NLPs). The function of NLPs is not well understood, but it is believed that they act as positive virulence factors, accelerating disease and pathogen growth in plant hosts. This protein has been discovered in many other plant pathogens which raise the interest of their study (Gijzen and Nurnberger, 2006; Martins et al, 2010).

1.5.2-Gene *npp1* in *Phytophthora cinnamomi*

The *npp1* gene of *P. cinnamomi* (GenBank NPP1, code AM403130.1) presents a total size of 1328 bp, an ORF comprising 770 bp encoding a 256 amino acids protein with a molecular weight of approximately 25 kD. Polymerase chain reaction was utilized to amplify a fragment of the *npp1* gene, using degenerate oligonucleotides which were designed taking into account the homology of previous published *Phytophthora* species NLP's sequences. NPP1 is believed to cause necrosis on leaf and roots of the plant, leading to the plant death and it is produced later during the nectrophic phase (unlike the protein GIP produced during the infection phase) (Kanneganti et al, 2006; Martins et al, 2010; Rose et al, 2002).

Plant cells respond to NLPs by entering into a hyper-defensive state prior to death. They liberate ethylene, enhance the activity of MAP kinases, produce phytoalexins, induce PR gene transcription, increase cytoplasmic Ca²⁺ levels, and show many other rapid and prolonged changes in physiology and gene expression (Gijzen and Nurnberger, 2006).

1.6-RNA interference

1.6.1-RNA interference discovery

RNA interference (RNAi) process was recently discovered showing a powerful sequence specific gene silencing by degrading complementary mRNA transcripts in the cytoplasm after insertion of exogenous RNA sequences in cells. In 1998 Fire and colleagues have proved by injecting of dsRNAs into *Caenorhabditis elegans* that the dsRNA was effective in gene silencing, but neither antisense nor sense RNA separately, led to an efficient

loss of the target mRNA. Since then, the use of double stranded RNA to silence gene has been called RNA interference (Fire et al, 1998; Guo and Kemphues, 1995).

RNAi discovery was preceded by observations of transcriptional inhibition by antisense RNA expressed in transgenic plants and more directly from reports on the surprising results of experiments performed in 1990s when in order to produce more intense purple colored *Petunias*, Richard Jorgensen and colleagues introduced additional copies of a transgene encoding chalcone synthase (a key enzyme for flower pigmentation). Surprisingly, the experiment produced an unpredictable result, in which the *Petunias* instead of the darker flower were either variegated or completely white. This phenomenon was called co-suppression of gene expression (Mittal et al, 2012; Napoli et al, 1990).

Similar post transcriptional gene silencing (PTGS) like effect was also seen in fungi *Neurospora crassa* (Quelling). Another RNAi related phenomenon; coat protein mediated protection (CPMP) in plants gave insight into the mechanism of PTGS. In 1995 Guo and colleagues first studied RNA silencing in animals. They used an antisense RNA technique to silence *par1* mRNA expression in *C. elegans*, but found that *par1* mRNA itself suppressed *par1* gene and deduced that both sense and antisense RNA could cause silencing. Their observation inspired the experiment of Fire, Mello and colleagues (Mittal et al, 2012).

1.6.2-RNA interference silencing mediators

RNAi is mediated through a number of molecules including; microRNA (miRNA), small interfering RNA (siRNA) and short hairpin RNA (shRNA).

- miRNA

These are an endogenous single-stranded RNAs that are expressed in all higher eukaryotes with 19-25 nucleotides; they are generated in the nucleus and converted from 70 nucleotides hairpin precursors by Rnase III nuclease Dicer. They can induce gene silencing through destruction of homologous mRNA in plants or blocking its translation in plants and animals (Cullen, 2004; Mittal et al, 2012; Novina and Sharp, 2004).

- siRNA

The mechanism of RNAi can be activated via the delivery of chemically synthesized exogenous siRNA to the cell, or by the introduction of dsRNA (Dorsett and Tuschl, 2004). The dsRNA inserted is converted by Dicer into duplexes of 21 to 24 nucleotides (siRNA). SiRNAs are rapidly taken up into RISC (RNA-induced silencing complex) while

synthetic siRNA does not go through Dicer processing and it is directly incorporated into the RISC. RISC unwinds the duplex siRNA and one strand bind to the target mRNA causing specific gene silencing (Singh et al, 2011).

- shRNA

shRNAs can be generated from plasmids or viral vectors, exogenous vectors encoding a shRNA construct is transfected into the cell, where it translocates to the nucleus and integrate the genome. After that the pri-shRNA will be converted into pre-shRNA by Drosha. The transfer of pre-shRNA to the cytoplasm is accomplished by the nuclear membrane protein Exportin5 then Dicer transforms it to siRNA capable of incorporating into a RISC to degrade or block the target mRNA (De fougères et al, 2007; Paddison et al, 2002).

1.6.3-RNA interference mechanism

The process of RNAi can be divided into four stages:

- (i) double-stranded RNA cleavage,
- (ii) silencing complex formation,
- (iii) silencing complex activation
- (iv) mRNA degradation and recycling of the RISC complex

The cleavage of dsRNA into double-stranded fragments of 21-24 nucleotides long is the first step generating siRNAs. The integration of siRNAs into the protein complex called RISC is the second step of the process, however this complex is inert in this form and cannot induce RNAi.

The third step is characterized by the unwinding of the siRNA duplex and the remodelling of the complex to create an effective form of RISC. The recognition and cleavage of mRNA complementary to the siRNA strand present in RISC represent the final stage (**Figure 4**) (Kulińska- Szweykowska et al, 2003).

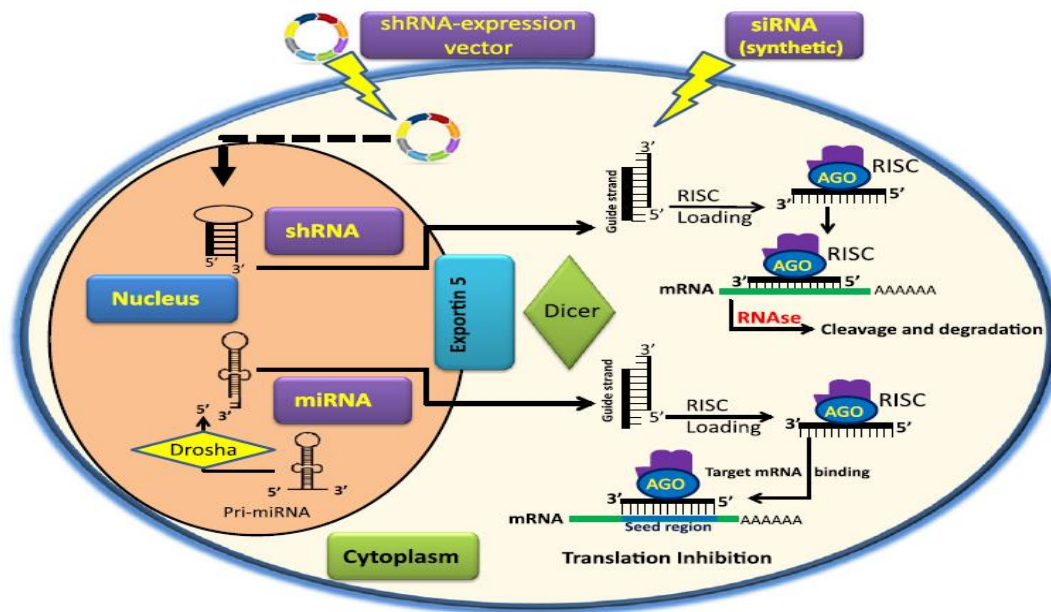


Figure 4. Mechanism of RNAi (Singh et al, 2011)

1.6.4-Biological functions of RNAi

- RNAi protects against viral infections and silences mobile elements

The information collected until now indicate that RNA silencing derive from an ancestral mechanism that controlled nucleic acids invasions. In different eukaryotic kingdoms RNA silencing is responsible for transposons, viruses and/or transgenes silencing. This supposition was verified by researches showing that silent transposons are reactivated in PTGS or RNAi impaired *Arabidopsis*, *C. elegans* and *Chlamydomonas* mutants (Hirochika et al, 2000; Ketting et al, 1999; Kulińska- Szweykowska et al, 2003; Wu-Scharf et al, 2000).

- RNAi-like mechanisms repress protein synthesis and regulate the development of organisms

Lately other type of endogenous RNA, called microRNA present in many organisms was discovered and led to many investigations on the nature and mechanism of these RNA molecules. These miRNAs bind to mRNA and regulate the gene expression by mRNA degradation or suppression of translation.

It is believed that there are approximately 500 miRNAs in mammalian cells, and about 30% of all genes are regulated by miRNAs playing an important role during development in plants, *C. elegans* and mammals. Thus, the miRNA-dependent control of gene expression

represents a new major principle of gene regulation. (Daneshmandi, 2006; Lagos-Quintana et al, 2001; Lee and Ambros, 2001).

- RNAi-like mechanisms keep chromatin condensed and suppress transcription

It was proved after plant studies that gene silencing could occur at the transcriptional level (TGS). After the discovery of RNAi, it was indicated that TGS in plants operates via RNAi-like mechanisms. In the fission yeast *Schizosaccharomyces pombe*, and next on in *Drosophila* and vertebrates, it was discovered that similar processes keep heterochromatic regions condensed and transcriptionally suppressed (Daneshmandi, 2006).

1.6.5-Applications

- Functional genomics

The technique of RNAi is used to discover novel genes involved in disease processes. In order to study the function of genes, siRNAs are used to knock down/ knockout the expression of these specific genes. Various investigations have been undertaken to clarify the role of specific genes in basic cellular processes like DNA damage response and cell cycle control general cell metabolism, signaling, the cytoskeleton and its rearrangement during mitosis, membrane trafficking transcription and, DNA methylation. Thus, this method has become the most commonly used technique for gene annotation (Mittal et al, 2012).

- Genetic improvement of crop plants

In order to improve the quality of crop plants the RNAi technique is used to enhance nutritional values or make plants resistant to pests; like the barley, crop that establishes a resistance against the barley yellow dwarf virus after utilization of RNAi (Wang et al, 2000).

RNAi has been confirmed to improve rice plants by decreasing its glutenin level and producing low glutenin content-1. The product LGC-1 is a low-protein rice and it is helpful for patients with kidney disease whose protein intake is restricted (Angaji et al, 2010).

Invention of food plants that originate lower levels of natural plant toxins is also feasible through RNAi. For example, cotton seeds are full in dietary protein but incorporate the toxic terpenoid product gossypol, making them inappropriate for human consumption. RNAi has been employed to produce cotton stocks whose seeds comprise reduced levels of delta-cadinene synthase, an important enzyme in gossypol production while maintaining a normal production of this enzyme in the rest of the plant for preserving its defensive capabilities against insects and diseases (Sunilkumar et al, 2006).

- RNA interference as a novel therapeutic agent

The ability to operate this native RNAi pathway has been identified as one the most exciting biotechnology advances in the last decade. RNAi is a way to control the development of diseases earlier in their process. The field of RNAi is progressing at an impressive rate, generating good results and their use has been documented for many diseases (Mittal et al, 2012).

In cancer research, various oncologic targets have been published in the literature, such as the employment of transferin with nanoparticles to target Ewing's sarcoma cells in a mouse xenograph model. This study confirmed the feasibility of using non-lipid based nanoparticles for the targeted delivery of siRNAs in a cancer model, and gives a powerful proof of principle of systemic delivery of siRNAs to a metastatic cancer (Ambesajir et al, 2012; Aagaard and Rossi, 2007).

HIV infection can also be stopped by targeting either viral genes (such as; *gag*, *rev*, *tat* and *env*) or human genes (for example, *cd4*, the principal receptor for HIV) that are implicated in the HIV life cycle. The strategy was used to suppress the central structural protein in the virus, p24, and the human protein *CD4*, which the virus needs to enter the cells (Mittal et al, 2012; Paddison et al, 2002).

1.7-The green fluorescent protein (GFP)

The gene encoding GFP was cloned from the jellyfish *Aequoria victoria* which is being used as a marker for gene expression and protein targeting in intact cells and organisms (Tsien, 1998). GFP's normal function is the conversion of the blue chemiluminescence of the Ca^{+2} -sensitive photoprotein aequorin into green light emission (Cody et al, 1993).

GFP is a relatively small protein of only 238 amino acids that does not interfere with biological processes, GFP was often used in transgenic investigations because it is a small and inert molecule that does not affect any biological process. The active protein does not need a cofactor or exogenous substrates to fluoresce, and it does not require the destruction or the fixation of organisms to be visualized in living cells (Chalfie, 2009; Ward et al, 1980).

The most effective class of GFP applications has been as a genetic fusion partner to host proteins to follow their localization and fate. The combination between the gene encoding the GFP and gene encoding the endogenous protein result in a fluorescent chimera expressed in the cell or organism of interest, conserving the normal functions and localizations of the endogenous protein (Tsien, 1998).

2-OBJECTIVE

The main goal of this work is to contribute to a better understanding of the molecular interactions between *P. cinnamomi* and *C. sativa* by examining the effect of pathogen gene silencing on the host phenotype and studying the subcellular localization of GIP and NPP1 proteins involved in the mechanism of infection, which is essential for the implementation of control strategies.

To reach this aim, these specific objectives will be performed:

- 1/ Design of gene constructs for silencing the *gip* gene by RNAi and transformation of *P. cinnamomi* by the genetic construct
- 2/ *C. sativa* infection with *P. cinnamomi* transformed and non-transformed in order to determine the effect of gene *gip* silencing
- 3/ Fusion of the ORFs of *gip* and *npp1* genes into the pTOR-eGFP plasmid for subsequent *P. cinnamomi* transformation and observation of protein subcellular localization using fluorescent microscopy
- 4/ Prediction of the subcellular localization of GIP and NPP1 proteins using Bioinformatics tools.

3-MATERIALS AND METHODS

3.1-Biological materials

- *Phytophthora cinnamomi*

Phytophthora cinnamomi were isolated from soil samples associated with *Castanea sativa* Mill trees, affected by ink disease in Trás-os-Montes region (northeast of Portugal), characterized by molecular methods and deposited in the Spanish Type Culture Collection (CECT) with CECT 20919 code.

- *Escherichia coli*

The NZY5 α competent *Escherichia coli* cells (NZYTech, Portugal) which have similar properties to DH5 α were used throughout this work. The NZY5 α competent cells allow high efficiency transformation in a wide variety of application.

The NZY5 α competent cells offer the following benefits:

- Increased performance in extracting plasmids due to endA1 mutation
- Blue/White screening capability due to lacZ M15
- Ensure the stability of the insert due to ecA1 mutation.

- *Castanea sativa* (Chestnuts)

Chestnuts of one year age have been used. The stratification of chestnuts was made in advance in the sand, and maintained in humid conditions in order to have germination. The germinated Chestnuts are planted in alveoli trays with a size of 6 cm x 6 cm x 20 cm for normal growth of the plants.

3.2-Culture media

The media were prepared in ultra-filtered water. Solid media were prepared by adding agar to the corresponding liquid media. All media were autoclaved at 120 °C for 20 minutes. In order to select clones resistant to antibiotics, media were supplemented with the corresponding antibiotic in a suitable concentration before pouring into plates.

3.2.1-Culture medium used for *E. coli*

To grow *E. coli* cells after transformation, Luria Bertani (LB) broth was prepared as follows: 1% (w/v) of tryptone, 1% (w/v) sodium chloride and 0.5% (w/v) of yeast extract. All were dissolved in ultra-filtered water and the volume was made up to 1000 ml.

The solution was then autoclaved. After cooling, the respective antibiotic was added at a concentration of 100 µg/ml, then the solution was aliquoted in sterile bottles, 5 ml in each, covered with the lid and stored at room temperature.

For agar plates, 1.5% (w/v) of agar was added, and the plates were poured while the medium was liquid. Then the plates were kept at room temperature until the agar set and then they were stored at 4 °C.

3.2.2-Culture media for *P. cinnamomi*

- V8 liquid medium

V8 medium was prepared by adding to 330 ml of V8 juice (Campbell Soup Co, Camden, NJ), 4.5 g of calcium carbonate (CaCO₃) and stirred for 30 min. Culture media was transferred to 1000 ml centrifuge flasks and centrifuged at 2500 x g for 15 min at 20 °C.

The supernatant was then, poured into a new flask without disturbing the pellet. The cleared V8 juice (V8 broth) was then, diluted 10 fold with ultra-filtered water and autoclaved at 120 °C for 20 min.

- V8 solid medium

It was prepared as aforementioned, but 15 g of agar per 1000 ml was added to the diluted V8 broth before autoclaving. After sterilization, the culture media was poured into Petri dishes, allowed to solidify at room temperature and kept at 4 °C.

- YEPD (Yeast Extract Peptone Dextrose Medium)

The liquid YEPD medium was prepared by adding the following: yeast extract 1%, peptone 2% and D-glucose 2%.

The solid YEPD medium was prepared as above, but adding 2.0% Agar.

- PDA medium

PDA (Potato Dextrose Agar – HIMEDIA) was prepared by adding 39 g of commercial PDA powder to 1 L of ultra-filtered water, then autoclaved at 120 °C for 20 min.

3.3-Culture conditions and maintenance of microorganisms

The growth of the microorganisms was carried out in solid medium (Petri dishes) or liquid medium (Erlenmeyer flasks). Routine incubation temperatures were: 25 °C for *P. cinnamomi* and 37 °C for *E. coli*. The cultures in liquid medium were generally grown

under 150-200 rpm agitation in orbital shaker (Stuart®, S150). Additionally, to maintain viable strains for a long time, the microorganisms were stored in glycerol 15-30% at low temperatures -70 °C.

The bacterial strain used throughout this work was stored at -70 °C, in 2 ml cryovials in their culture medium with 1/3 volume glycerol 100%. After subcultures in Petri dishes in agar LB medium *E. coli* were stored at a temperature of 4 °C.

3.4-Vectors

The vectors used were :

- pTOR-eGFP vector

Howard S. Judelson (University of California, Riverside, USA) has made this vector with approximately 7 kb in size, containing the neomycin resistance gene. This vector is regulated by promoters and terminators from the *hsp70* and *ham34* genes of *Bremia lactucae*. This vector was used to clone the PCR products of *gip* and *npp1* genes for further transformation of *P. cinnamomi* and observation of the subcellular localization of GIP and NPP1 proteins under fluorescent microscopy (**Figure 5**).

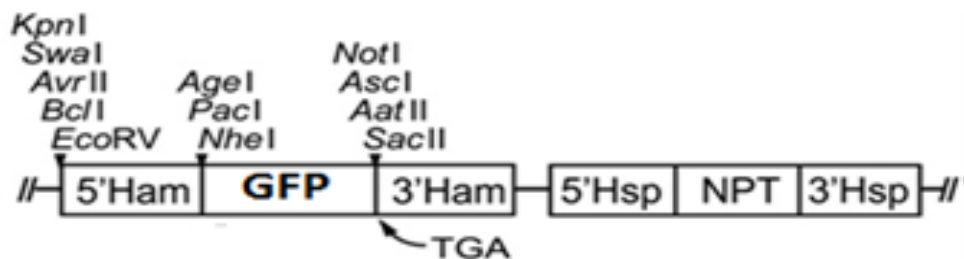


Figure 5.Linearised map of the pTOR-eGFP vector

- pUC57 vector

The pUC57 is a common used cloning vector in *E. coli*. The vector length is 2710 bp. It contains the ampicillin resistance gene. This plasmid was used for cloning the *gip* silencing cassette and therefore transforms *E. coli* to propagate the construct (**Figure 6**)

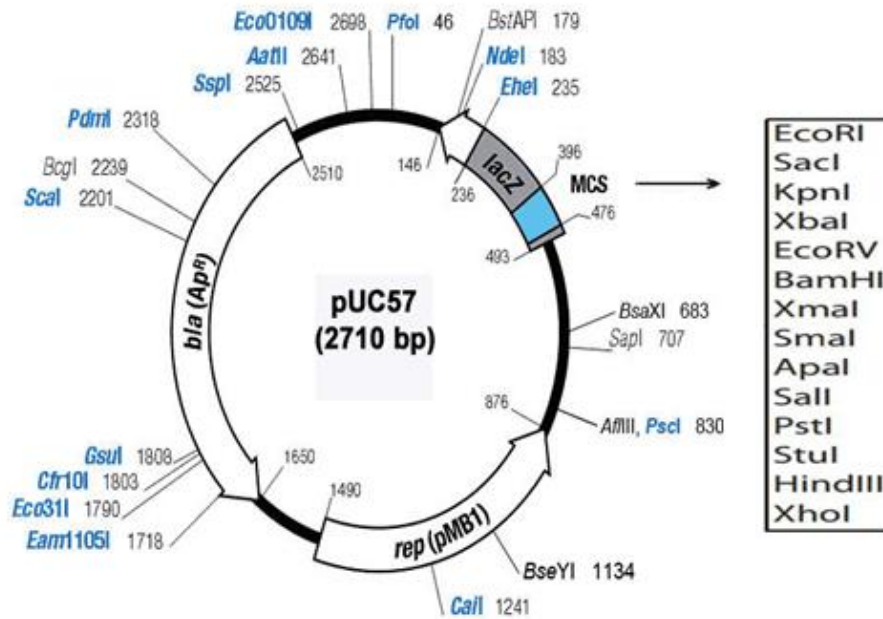


Figure 6. Map of the pUC57 vector

- pTH210 vector

This expression vector was made by Howard S. Judelson with 5030 bp driven by *hsp 70* promoter and containing the hygromycin resistance gene. This vector was used in order to insert the *gip* silencing cassette into the genome of *P. cinnamomi* (Figure 7)

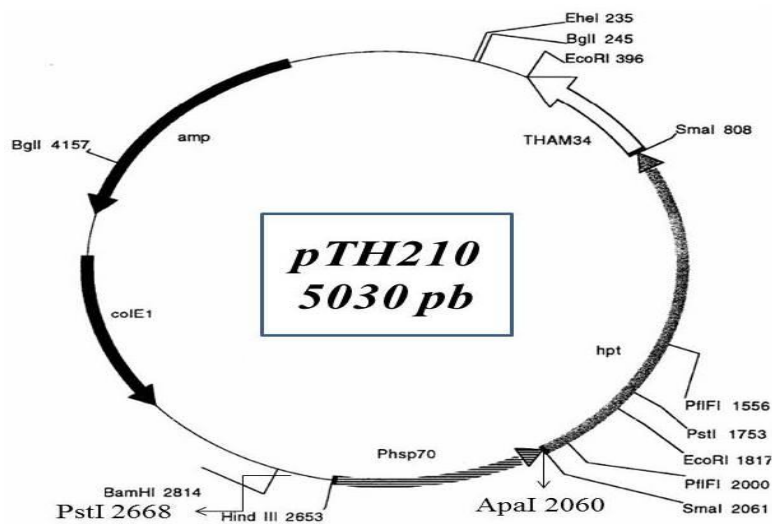


Figure 7. Map of the pTH210 vector

3.5-DNA extraction

There are several methods for the extraction of genomic DNA (depending on the type of the biological sample), although all methods involve cell lysis, followed by deproteinisation and DNA purification.

For the extraction of *P. cinnamomi*'s genomic DNA, the oomycete has been grown in PDA medium, covered with aluminum foil at 25°C and after six days of mycelial growth, DNA was extracted.

The process of extraction consisted in the use of a Lysis Solution, 200 mM Tris-HCL; 25 mM EDTA; 250 mM NaCl and SDS 0.5 % (w/v) followed by a deproteinisation with phenol / chloroform / isoamyl alcohol (25: 24: 1) and precipitation of DNA by washing with ethanol (100 % - 70 %) at -20°C, and the DNA pellet was then dissolved in ultra-filtered water. The treatment of DNA was done with RNase 5 mg/ml for 5 minutes at 37 °C.

3.6-Visualisation and purification of nucleic acids in agarose gels

The visualization and separation of DNA fragments were performed by electrophoresis in agarose gel low melting 0.8 % (w/v) in TAE (Tris-Acetate 40 mM, 1 mM of EDTA), with 0.5 µg/ml GreenSafe Premium (NZYtech,Portugal), for 40 minutes at room temperature, at 80 V.

After irradiation of the gel using the ChemiDoc™ XRS+ imaging system (BioRad) it was possible to view the size and intensity of the desired band by comparison with DNA ladders (**Figure 8**).

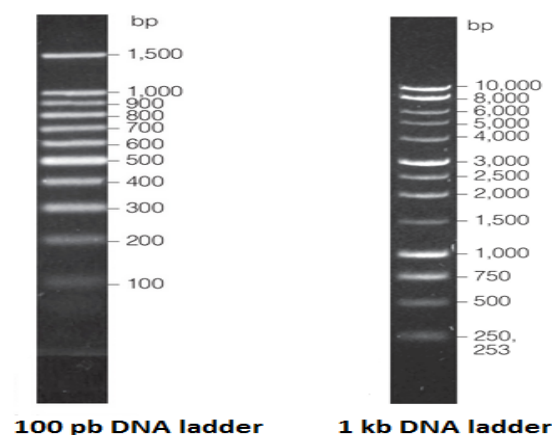


Figure 8. The DNA ladders used to determine the size of bands in agarose gels (Promega)

The Isolation and purification of DNA fragments from agarose gel were made by cutting the agarose band containing the desired fragment from gel prepared in TAE with a clean razor blade. DNA molecules present in the band were purified by the QIAquick® Gel Extraction Kit (QIAGEN, Germany) following the manufacturer's instructions.

3.7-Design of silencing cassette (shRNA based vector) for *gip* gene

Several methods are used for constructing silencing cassettes; including annealed oligonucleotide and PCR-based cloning. The PCR-based method has been chosen because it is cheaper and reliable. The silencing shRNA construct was made by joining a 5' sequence fragment to another fragment from the 3' end of the same sequence in an inverted orientation (by performing two separate PCR reactions) separated by a spacer or loop DNA (**Figure 9**). Transcripts generated from such constructs will have regions of self-complementarity that have the potential to form shRNA duplexes that generate a siRNA in the cell capable of degrading specific sequences of mRNA.



Figure 9. Structure of the silencing cassette (red for sense and antisense sequences, green for the loop sequence, grey for terminator).

The amplification of the sense sequence selected within the ORF of *gip* gene has been done using specific primers designed following Tiscornia et al protocol (**Table 1**) (Tiscornia et al, 2007).

The forward primer includes: (1) 4–5 extra nucleotides to assist in digestion following PCR; (2) a 5' restriction site recognition sequence in order to insert the PCR product into the pUC57 vector; and (3) approximately 20 nucleotides of 5' sense strand sequence, the reverse primer contains only 20 nucleotides complementary of the 3' antisense strand sequence.

The PCR product was purified following the protocol of the commercial Wizard® SV PCR Clean-Up System (Promega, USA), which consists in passing the sample through a chromatography column, in which, the nucleic acids together with a membrane binding

solution are retained on the silica membrane then with a washing solution the components of PCR mix are eliminated. Finally, the DNA is eluted in a nuclease free water.

The ligation to the loop sequence (provided by Professor Sophien Kamoun - The Sainsbury Laboratory (TSL), Norwich, UK) was done using T4 DNA Ligase (Promega) and following the manufacturer's instructions.

For the amplification of the antisense sequence; forward primer with approximately 20 nucleotides and a reverse primer including: (1) 4–5 extra nucleotides to assist in digestion; (2) 3' restriction site recognition sequence; (3) AAAAA termination sequence complementary strand; and (4) 20 nucleotides complementary of 3' antisense strand sequence were used. The PCR product was purified as aforementioned and ligated with T4 DNA Ligase (Promega) to the fragment containing the sense sequence and the loop.

Table 1.List of primers used for *gip* silencing cassette construction

Name	Sequence	Tm °C	GC %
FPCR1	5'GATAGGGCCC ATGTGCACGACATGTTTACT3'	65.6	50
RPCR1	5'GCGACGGTGAAGACAACCGT3'	62.4	60
FPCR2	5'GCGACGGTGAAGACAACCGT3'	62.4	60
RPCR2	5'CTAGGGGCCCAAAAAAATGTGCACGACATGTTTACT3'	66.4	44.44

The construct with approximately 551 bp length, was purified after ligation and then digested with *Apa*I restriction enzyme in a final volume of 30 µl with a reaction buffer (1x) (Promega), 1.5 uL (7U / microl) of *Apa*I enzyme, 1 ug / µl DNA, for 4 hours at a temperature of 37 °C.

In order to approximate the concentration comparing with the 500 bp band of the DNA ladder (Promega 100 bp ladder), the digested construct was after that visualized on a 1% agarose gel, excised with a clean razor blade and purified using the QIAquick® Gel Extraction Kit according to manufacturer's instructions (QIAGEN, Germany) so that can be ligated directly by T4 DNA ligase (Promega) to the pUC57 vector digested previously by *Apa*I restriction enzyme (**Figure 10**).

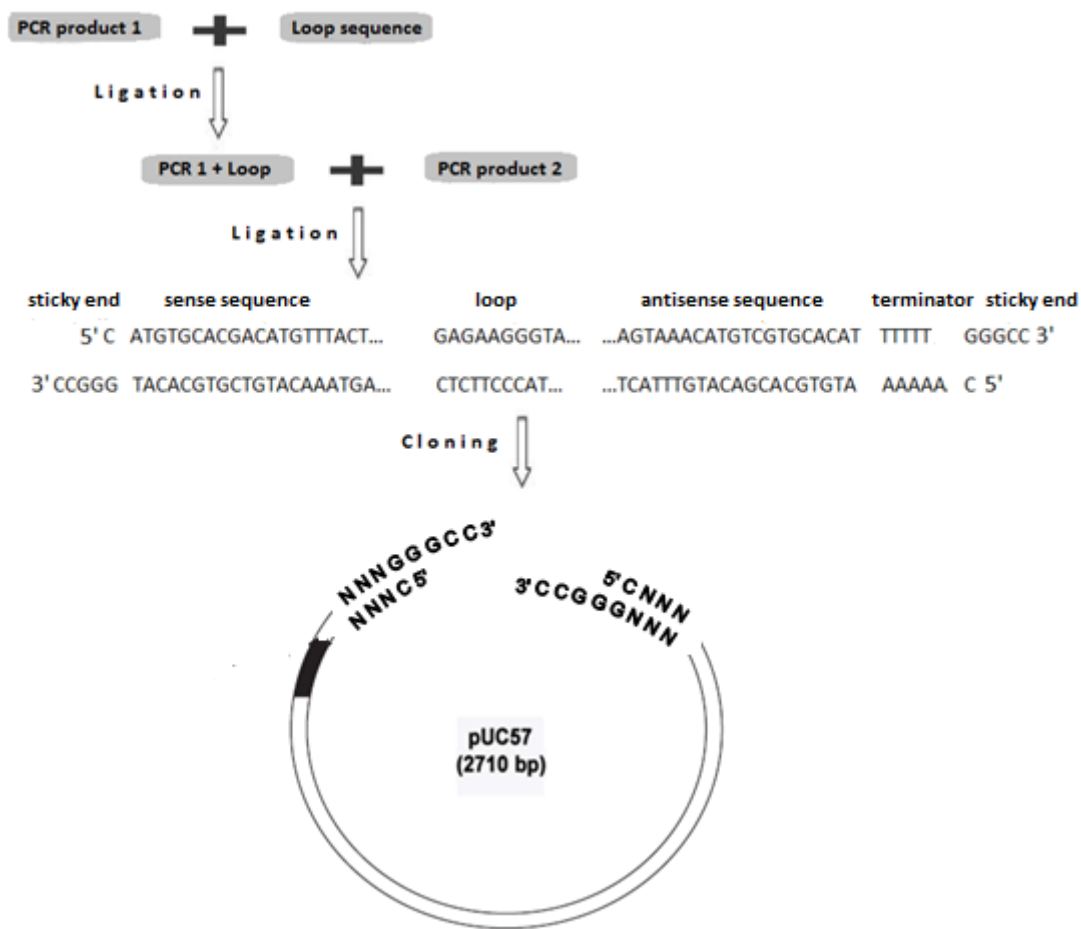


Figure 10.Methodology for shRNA silencing cassette design

3.8-Transformation of *E. coli* with the silencing construct

In order to propagate the ligated DNA, the ligation mixture was transformed into competent *E. coli* cells by mixing 100 µl of competent cells of the strain NZY5 α with 10 µl of the ligation reaction. The mixture was placed on ice for 30 minutes, followed by thermal shock at 42 °C, in a water bath for 40 seconds. The bacteria were transferred to ice during 2 minutes and 0.9 ml of SOC Medium was added. The incubation was for one hour at 37 °C with agitation exceeding 200 rpm, then 100 to 250 µl of the cells transformed with the ligated DNA were scattered on LB agar dishes containing 100 µg/ml ampicillin followed by an overnight incubation at 37 °C.

3.9-Extraction of the plasmid DNA of the transformed *E. coli*

The bacterial colonies, that have grown during the incubation period and which were expected to contain recombinant plasmids, have been picked off and transferred to the LB liquid media containing 100 µg/ml of ampicillin antibiotic. The cultures were incubated overnight on the shaker at a shaking rate of 200 rpm at 37 °C.

The plasmid miniprep has been made, using the NZYMiniprep kit (NZYtech,Portugal) according to manufacturer's instructions which is based on alkaline lysis of bacterial cells followed by adsorption of DNA onto silica. Afterward, the recombinant plasmid was digested by *ApaI* restriction enzyme then, the digested products were subjected to electrophoresis in agarose gel. The insert was purified from the gel by QIAquick® Gel Extraction Kit according to manufacturer's instructions (QIAGEN, Germany).

3.10-Cloning the silencing cassette into the pTH210 vector

The pTH210 vector was extracted from *E. coli* previously transformed, digested with *ApaI* enzyme; as described for the cassette digestion and then dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIAP).

Dephosphorylation was performed to remove phosphate groups from the 5' ends of the linearized plasmid and prevent recircularization. The linearized plasmid was incubated with CIAP, "Calf Intestine Alkaline Phosphatase" (Promega,USA) (0.01u/µl) with 5 µl of the appropriate buffer CIAP 10X and ultra-filtered water to a final volume of 50 µl for 30 minutes at 37 °C.

The fragments of the silencing cassette and the vector pTH210 were excised with a clean razor blade and purified using QIAquick® Gel Extraction Kit according to manufacturer's instructions (QIAGEN, Germany) then ligated using T4 DNA Ligase enzyme.

To clone the cassette into the pTH210 vector the ligation of DNA fragments was carried out in a 10 µl standard ligation reaction. The reaction contained DNA fragments with compatible ends, 100 ng of plasmid were used for a typical ligation reaction, 0.5 µl of T4 DNA ligase, and a 1µl of ligation mix buffer (Promega). Reactions were incubated at 4 °C overnight.

The recombinant vector was used to transform *E. coli* NZY5 α competent cells. After plasmid extraction, digestion with restriction enzymes, was done to confirm the cloning of the cassette into the pTH210 vector.

3.11-Transformation of *Phytophthora cinnamomi* zoospores with the recombinant pTH210 vector

The production of zoospores was made according to an adaptation of the protocol prepared by Edgar Huitema (Huitema et al, 2011). The zoospores used for transformation were subjected to centrifugation and the pellet was resuspended in 1.0 M mannitol / V8 medium (4/1) and regenerated for 16 hours at 25 °C in the dark.

After incubation, the zoospores were collected again by centrifugation at 3300 xg for 5 minutes, washed with a regenerating solution, and the recombinant pTH210 vector was added together with the zoospores.

After achieving the transformation procedures, *P. cinnamomi* with the recombinant pTH210 vector was grown in medium containing hygromycin (200 ug / ml), the growth was incubated for 10 days. At the same time non-transformed *P. cinnamomi* was cultured as a negative control in medium containing hygromycin (200 ug / ml) for the same incubation time.

3.12-Genomic analysis of the transformed *Phytophthora cinnamomi* (PCR & DNA sequencing)

The mycelium of transformed *P. cinnamomi* was selected in order to extract the genomic DNA and make a screening PCR to confirm the integration of the pTH210 into *P. cinnamomi* genomic DNA. The screening PCR was performed with the primers listed in **Table 2**.

Table 2.List of primers used for PCR of the cassette and the hygromycin fragment

Name	Sequence	Tm °C	GC %
hygF	5'CGACAGCGTCTCCGACCTGATGCAG3'	67.9	64.00
hygR	5'TGTGGACGAGAAGTGTGCCACCAAG3'	65.1	56.00
casF	5'GATAGGGCCC ATGTGCACGACATGTTTACT3'	65.6	50
casR	5'CTAGGGGCCCAAAAAAATGTGCACGACATGTTTACT3'	66.4	44.44

Amplification products were checked by agarose gel electrophoresis and samples were sent to the Department of Microbiology and Genetics, University of Salamanca for

sequencing with respective primers and using an automated sequencer ABI PRISM 377W that perform the electrophoretic separation and detection of DNA fragments labeled with fluorescence.

Four different colors of fluorescence can identify the four dideoxynucleotide incorporated into the extension reaction (A, G, T or C). After analyses with BioEdit program the nucleotide sequences were blasted on (www.ncbi.nlm.nih.gov/BLAST/) to investigate the ID of the sequenced genes. The alignment of similar genes, was analysed and compared using the Muscle tool server (CLUSTAL multiple sequence alignment) from EMBL-EBI database (<http://www.ebi.ac.uk/Tools/msa/muscle/>).

3.13-Castanea sativa infection with *P. cinnamomi* strains

Infection of *C. sativa* roots was performed using mycelium of *P. cinnamomi* transformed and non-transformed in order to compare the effect of gene silencing on the plant phenotype. The roots were covered with fully colonized V8 medium, the plants were placed in sterile vermiculite and incubated for 72 hours at 25°C.

3.14-Fusion of the ORF's into the pTOR-eGFP vector

3.14.1-Primer design to amplify the ORF's of *gip* and *npp1* genes of *Phytophthora cinnamomi* by PCR

For the amplification of *gip*'s ORF, primers were designed with adapters having enzyme restriction sites to allow the insertion of DNA fragments in the pTOR-eGFP vector.

For *gip* gene, NotI (**gcggccgc**) and AatII (**gacgtc**) restriction sites were chosen to be incorporated in primers sequences since this restriction sites are present in the vector pTOR-eGFP.

Based on the sequence of gene *gip* in the GenBank database (AM259384) the forward and reverse primers were designed. In addition to the sequence of primers and the restriction sites, protection sequences were added to the primers (**Figure 11**).

```

tttttgaaat tcagtagcaa atatgtgcac gacatgttta cttccggaag ggctaaacgt
tcaatccaat gggcgtttqt gaaccgctct ttaactctatc taacgtccac ctcctcaagc
aatggttata caccattgcg gaatttaccg cgcactgctc actccatttc gccatcttcc
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agtcccacc acgtgcttac cactgcgtca tgcacggcat acgaggaggg ctgctccacc
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at ttgcaaaa cgattgcgag ctccaaccac ctccgactat cctgataatt cgacgattta
cgcaagggac gatataggtg cacttgaagc cggatacca cccaccttat cgtgctgcat
aatatccata tcgcccgcaa cttctgactc g

```

Figure 11. The sequence of *gip* gene (in black the non coding sequence of the gene , in red the ORF of gene , in blue based sequences for primers design)

The specific characteristics of the primers such as the melting temperature (T_m) and the efficiency of primers were determined with the program FastPCR.

The Restriction Mapper website (<http://www.restrictionmapper.org/>) was used to assure the absence of restriction sites for NotI and AatII in the ORF since both of these restriction enzymes will be used for cloning the PCR product into the pTOR-eGFP vector.

The same principle was used to design primers for *npp1*'s ORF with restriction enzymes sites for NotI (**gcggccgc**) and SacII (**ccggcg**) (**Table 3**).

Table 3.List of primers used for PCR of *gip* and *npp1* genes

Name	Sequence	T _m °C	GC %
GIPpvF	5'AGCGGCCGCATGTTTACTTCCGG 3'	67.0	60.87
GIPpvR	5'GCGGACGTCTTATGCCTTGATGATAGAG 3'	62.7	50
NPP1pvF	5'CGCGGCCGCATGGTATCGGCTGTG 3'	72.0	70.83
NPP1pvR	5'G CCGCGG TGT TTC GGA TGC TGG ACA GG 3'	72.6	66.67

3.14.2-PCR amplification of *gip* and *npp1*

The PCR technique is a methodology that is based in in-vitro amplification based on an in-vivo process (replication). This allows an exponential and selective synthesis of a reduced amount of DNA. The technique consists in a cycle of three phases, which comprises the DNA denaturation, hybridisation (annealing) of primers and polymerization of DNA by Taq DNA polymerase enzyme.

The thermal cycler "my cycler" (Bio-Rad) was used to amplify a 810 bp fragment of the *gip* gene, with the primers designed as described in **Table 3**. The PCR cycling conditions were; 94 °C /5 min, followed by 36 cycles of 94 °C /1 min; 61 °C /1 min; 72 °C /30 s, and ending with 72 °C /5 min. Each 50 µl PCR contained 0.8 mM dNTP, 0.2 mM of each primer, 100 ng genomic DNA, and 1 U Taq DNA polymerase in the appropriate buffer.

The PCR of *npp1*'s ORF was done as mentioned for *gip* gene but with 66 °C as annealing temperature.

3.14.3-Enzymatic digestion and dephosphorylation of pTOR-eGFP

Digestion with restriction enzymes is an essential step for the construction of recombinant vectors. Enzymes were selected according to the restriction sites in the DNA sequence, or from the plasmids map.

Digestions using simultaneously two enzymes need universal buffers that are appropriate for both enzymes; if the buffer was not available, digestions should be done separately. After purification with Wizard® SV PCR Clean-Up System (Promega,USA), the PCR products (ORF of *gip* and *npp1* genes) were digested by respective restriction enzymes for their insertion into the vector pTOR-eGFP.

The first digestion of *gip*'s ORF was done in a final volume of 30 µl with a reaction buffer (1x) (Promega), 1.5 µl (7U / microl) of NotI enzyme, 1 ug / µl DNA, for 4 hours at a temperature of 37 °C. After incubation and purification, the insert was digested with AatII enzyme as described for NotI enzyme.

The ORF of *npp1* gene was digested first by NotI enzyme, purified and digested again with SacII enzyme.

On the other hand the plasmid pTOR-eGFP was extracted from *E.coli* previously transformed, digested separately with respective restriction enzymes, and then dephosphorylated with CIAP as described in **section 3.10**.

The insert and the plasmid were after that visualized on agarose gel 0.8% (w /v) in order to approximate the concentration of both fragment. The estimation of the insert and the plasmid concentrations is a very important step for the efficiency of the ligation reaction. The approximation was done after visualization on agarose gel and comparing with the 500 bp band of the 100 bp, DNA ladder (Promega) which has a known concentration, (the same volume was used for the DNA ladder, the vector and the insert in electrophoresis).

The amount of the insert in nanograms required for the reaction was estimated according to the following ligation equation :

$$\text{ng of insert} = \frac{(\text{ng of vector} \times \text{kb size of insert})}{\text{kb size of vector}} \times \left(\text{molar ratio of } \frac{\text{insert}}{\text{vector}} \right)$$

3.14.4-Fusion of the PCR products into the pTOR-eGFP plasmid

The insert and the plasmid were excised from agarose gel with a clean razor blade, purified using the QIAquick® Gel Extraction Kit and then allowed to ligate at 3:1 ratio by incubating the reaction mixture as reported in **section 3.10** overnight at 4 °C.

3.15-E. coli Transformation with the recombinant pTOR-eGFP vector

This task was done as described in **section 3.8**, but using the neomycin antibiotic.

3.16-Extraction of the plasmid DNA of the transformed E. coli

This task was done as described in **section 3.9**. After extraction, the recombinant plasmid with *gip* gene was digested by NotI and AatII restriction enzymes while for the

recombinant plasmid with *npp1* gene, the digestion was made using NotI and SacII enzymes and then, the digested products were subjected to electrophoresis in agarose gel.

3.17-Prediction of the subcellular localization of the GIP and NPP1 proteins

Protein subcellular localization prediction foretells the destination of a protein in the cell through computational methods that take as input the protein sequence for example and produce a prediction of the protein localization as output.

There are several softwares publicly available using different methods for predicting the localization of proteins (Amino Acid Composition, N-peptide Composition, Physico-chemical Composition ...) which is a very important part of bioinformatics based prediction of protein function and genome annotation.

The softwares used for the protein localisation predictions can be accessed through the URL addresses as follows:

- SignalP 3.0: (<http://www.cbs.dtu.dk/services/SignalP-3.0/>)
- Cello: (<http://cello.life.nctu.edu.tw/>)
- LOCtree: (<https://roslab.org/services/loctree2/>)
- EuK-mPLoc 2.0: (<http://www.csbio.sjtu.edu.cn/bioinf/euk-multi-2/>)
- Esl pred: (<http://www.imtech.res.in/raghava/eslpred/index.html>)

4-RESULTS AND DISCUSSION

4.1-DNA extraction of *Phytophthora cinnamomi*

After six days of mycelial growth in the dark at 25°C, the genomic DNA of *Phytophthora cinnamomi* was extracted to design the *gip* silencing cassette and to amplify the ORF's of *gip* and *npp1* genes (Figure 12).

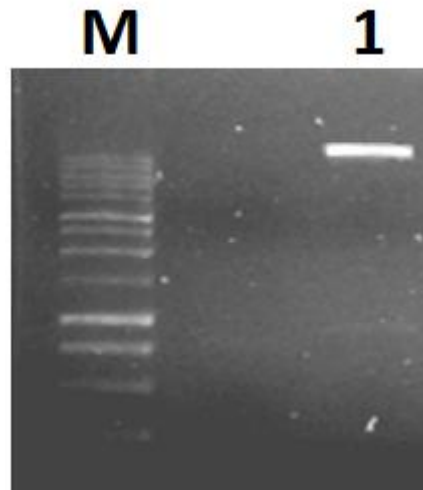


Figure 12. Visualization of *Phytophthora cinnamomi* genomic DNA in agarose gel 0.8 % (w/v) **M**) DNA ladder of 1 kb (Promega); **1**) genomic DNA of *Phytophthora cinnamomi*

The gel showed that the extracted DNA is concentrated, not degraded and does not contain proteins neither RNA molecules.

4.2-Synthesis and preparation of the *gip* silencing cassette

In order to synthesize an efficient conventional silencing cassette that targets the corresponding mRNA of the *gip* gene, many measures should be taken into consideration:

- The potential construct sequence should not have a perfect match of more than 16 nucleotides to an off-target gene of *P. cinnamomi* (verifying by BLAST search) (<http://www.ncbi.nlm.nih.gov/BLAST/>).
- The construct should not have an internal repeat or palindromes and must include low-to-medium G/C content (Cheng and Chang, 2007).
- It is recommended to use a silencing cassette fragment between 300 and 1000 bp length as a suitable size to maximize the efficiency of silencing obtained (Wesley et al, 2004).

The sense and anti-sense sequences were produced by performing two separate PCR reactions then the PCR products were ligated by T4 DNA ligase (**Figure 13**).

```
ATGTGCACGACATGTTTACTTCCGGAAGGGCTAAACGTTCAATC
CAATGGGCGTTTGTGAACCGCTCTTTACTCTATCTAACGTCCAC
CTCCTCAAGCAATGGTTATCCACCATTGCGGAATTTACCGCGCA
CTGCTCACTCCATTTTCGCCATCTTCCGCTAACCATGACGGTTGT
CTTCACCGTCGCGAGAAGGGTAAGCCAAGACATTTGACTTCCAC
AAAAGTAAGAGTACTCACGCACCACGCTGGCACCCAGTTGATCG
GCGCGAGATTTAATCGCCGCGACAATTTGCGACGGCGCGTGCAG
GGCCAGACTGGAGGTGGCAACGCCAATCAGGCGACGGTGAAGAC
AACCGTCATGGTTAGCGGAAGATGGCGAAATGGAGTGAGCAGTG
CGCGGTAAATTCCGCAATGGTGGATAACCATTGCTTGAGGAGGT
GGACGTTAGATAGAGTAAAGAGCGGTTACAAACGCCCATTTGGA
TTGAACGTTTAGCCCTTCCGGAAGTAAACATGTCGTGCACAT
```

Figure 13.The sense,loop and antisense of the *gip* silencing cassette

The generated cassette was purified, digested with *Ap*I enzyme and then visualized on 1% agarose gel, as shown in **figure 14**.

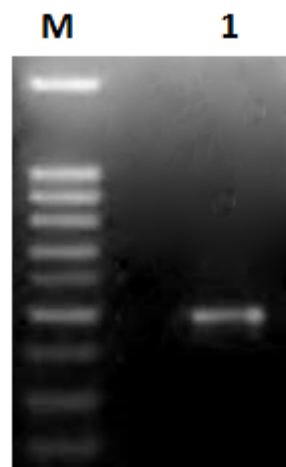


Figure 14.Visualization of the synthesized cassette on agarose gel **M**) DNA ladder of 100 bp (Promega); **1**) the *gip* silencing cassette

The gel showed a specific band of 543 bp length with a good DNA quality and confirmed the success of producing a silencing cassette that can trigger a post transcriptional gene silencing.

RNA interference using shRNA has been chosen because previous studies have shown that shRNA-expression system is more efficient than siRNA expression systems (Cheng and Chang, 2007). In addition, the cost of shRNAs construction is cheaper compared with synthesized siRNAs and for stable and enforceable expression it is also recommended to use shRNAs because it integrates the genome and can be expressed for up to three years while synthesized siRNAs are degraded after 48 hours (Rao et al, 2009; Paddison et al, 2004).

4.3-Transformation of *E. coli* with the *gip* silencing cassette

The silencing cassette was purified using the QIAquick® Gel Extraction Kit and then cloned into the pUC57 vector in order to transform *E. coli* competent cells. The transformed bacteria that are able to grow in medium containing ampicillin have been selected for further experiences (**Figure 15**).

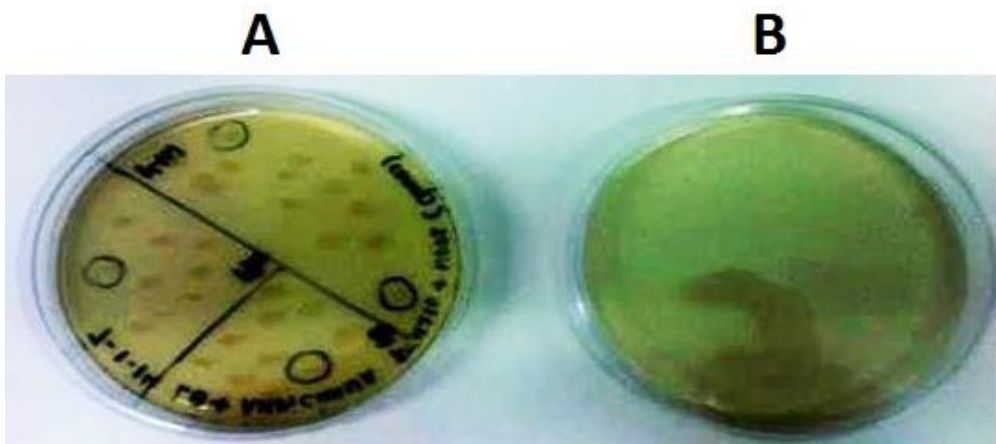


Figure 15. Screening of transformed *E. coli* resistant to ampicillin antibiotic **A)** *E. coli* transformed was able to grow in medium containing ampicillin; **B)** non-transformed *E. coli* was not able to grow in medium containing ampicillin

To confirm the insertion of the cassette in pUC57, the recombinant vector was extracted from transformants *E. coli* competent cells and digested with the *ApaI* enzyme. After visualisation on agarose gel (0,8%) two bands were observed; one with 2710 bp length corresponding to the vector pUC57 and another of 543 bp corresponding to the cassette.

At the same time, the pTH210 expression plasmid was extracted from *E. coli* cultured cells and linearized with the *ApaI* restriction enzyme with the purpose of preparing the cloning of the silencing cassette.

The products resulting from the enzymatic digestion were analyzed on agarose gel (0,8%) and a band of 5030 bp corresponding to the pTH210 linearized plasmid was observed (**Figure 16**).

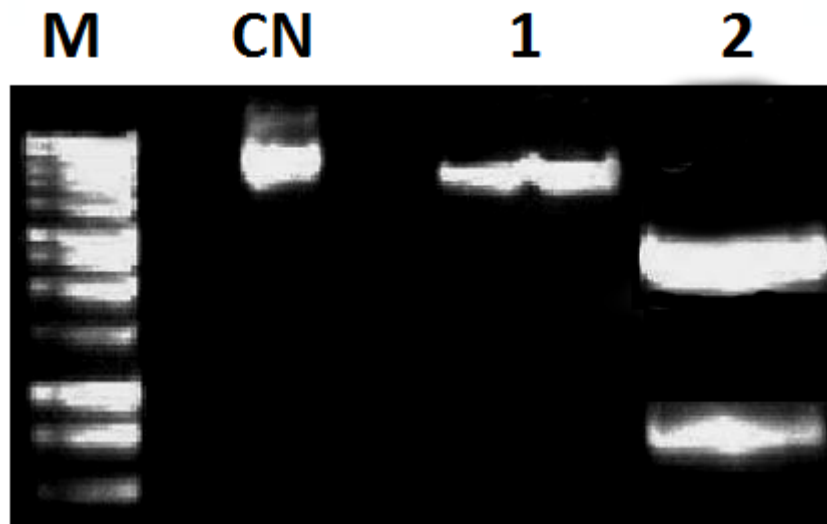


Figure 16.Enzymatic digestion of pTH210 plasmid and the recombinant pUC57 plasmid with *ApaI* **M**) DNA ladder of 1 kb (Promega); **CN**) Negative Control pTH210 supercoiled plasmid; **1**) pTH210 plasmid linearized by *ApaI* enzyme; **2**) pUC57 recombinant plasmid digested by *ApaI* enzyme generated two fragments

The two bands generated after digestion with *ApaI* enzyme; of 2710 bp length corresponding to the vector pUC57 size and of 543 pb corresponding to the cassette size confirm the success of cloning the silencing cassette into the pUC57 plasmid.

The extraction of the recombinant vector followed by digestion with respective restriction enzymes was done from different colonies to confirm the ligation of the cassette into the pUC57 cloning vector.

4.4-Cloning the silencing cassette into the pTH210 expression vector

The fragments of the silencing cassette and the vector pTH210 were excised with a clean razor blade and purified using the QIAquick® Gel Extraction Kit then ligated using T4 DNA Ligase enzyme.

The recombinant vector was used to transform competent *E. coli* NZY5 α cells. After plasmid extraction, the enzymatic digestion with ApaI, PstI and SmaI enzymes, was conducted to confirm the cloning of the cassette into the pTH210 vector.

After visualisation on agarose gel (0,8%) the digestion with the enzyme ApaI generated two bands; one for the vector pTH210, with 5030 bp length and one of 543 bp corresponding to the cassette. Digestion with PstI enzyme released a larger fragment of 4115 bp and a smaller of 1458 bp. The digestion with SmaI enzyme produce a larger fragment of 4320 bp and another smaller of 1253 bp (**Figure 17**).

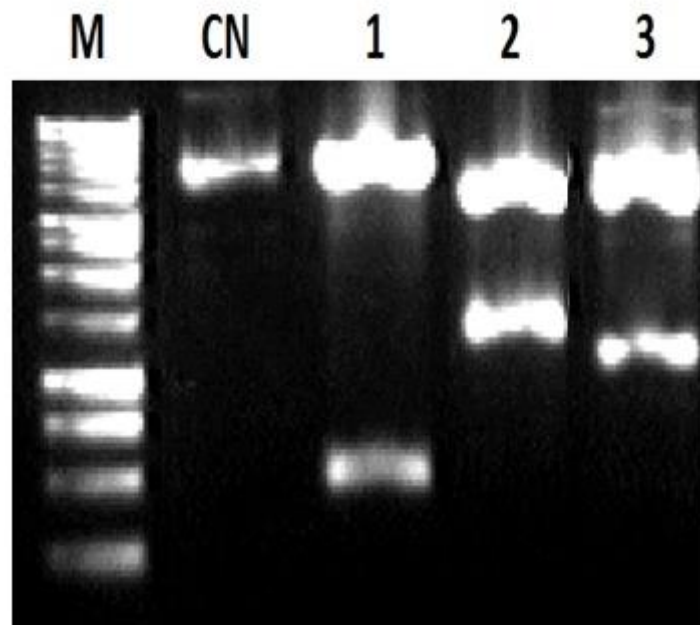


Figure 17.Enzymatic digestion of the recombinant pTH210 vector with ApaI, PstI and SmaI **M**) DNA ladder of 1 kb (Promega); **CN**) Negative Control non recombinant pTH210 supercoiled plasmid; **1**) recombinant pTH210 plasmid digested by ApaI enzyme; **2**) recombinant pTH210 plasmid digested by PstI enzyme; **3**) recombinant pTH210 plasmid digested by SmaI enzyme

The virtual enzymatic digestion based on the pTH210 sequence, performed using the Restriction Mapper website (<http://www.restrictionmapper.org/>) complies with the products of digestion showed in **figure 17**. These results allow the confirmation of cloning the cassette into the pTH210 vector.

The pTH210 vector has been used because the Hsp70 promoter from the downy mildew *Bremia lactucae* was proved to work in diverse oomycetes including *Achlya*, *Phytophthora*,

and *Pythium*. Moreover it was used in many researches for stable DNA integration (Chen et al, 2009; Judelson and Ah-Fong 2009).

4.5-Confirmation of *P. cinnamomi* transformation by culture in medium containing hygromycin antibiotic

The recombinant plasmid was used to transform *Phytophthora cinnamomi* by electroporation, then the transformants were cultivated on medium containing hygromycin (200 ug / ml) and incubated for ten days at 25 °C in the dark.

At the same time non-transformed *P. cinnamomi* were incubated in the same conditions as negative control plates in medium containing hygromycin (200 ug / ml).

After ten days of incubation at 25 °C in the dark, there was no growth of the negative control of *P. cinnamomi* in medium containing the hygromycin selection marker, while for the transformed *P. cinnamomi* only six out of ten grew in the medium with hygromycin . **(Figure 18).**

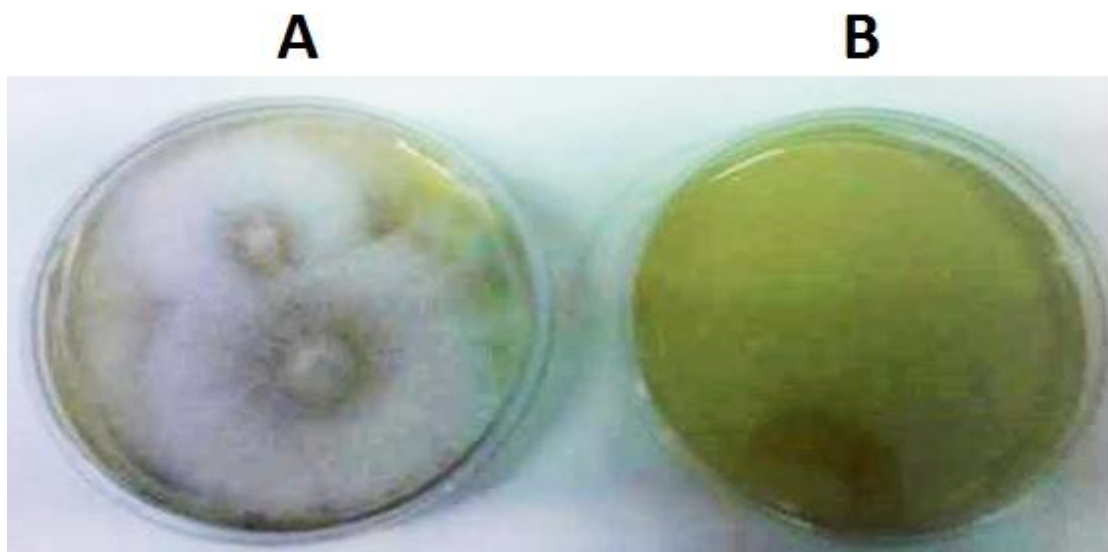


Figure 18. Screening of *P. cinnamomi* transformed with the recombinant pTH210 vector, resistant to hygromycin antibiotic **A)** *P. cinnamomi* transformants are able to grow in medium containing hygromycin; **B)** non transformants *P. cinnamomi* was not able to grow in medium with hygromycin

The growth of transformed *P. cinnamomi* in medium containing hygromycin means that they can produce the hygromycin phosphotransferase (HPT) protein that degrade the hygromycin antibiotic giving resistance to these microorganisms and that is only possible

with the transformation of *P. cinnamomi* by the pTH210 vector meaning that this transformation was successful.

4.6-Genotypic confirmation of *P. cinnamomi* transformation

In order to confirm the transformation of *P. cinnamomi* by PCR screening, the genomic DNA of transformed *P. cinnamomi* was extracted after ten days of incubation and specific primers were designed to amplify a region of approximately 551 bp corresponding to the *gip* silencing cassette sequence and other region of 1090 bp within the hygromycin gene (*hpt*). With these procedures it is expected to prove the integration of the recombinant plasmid pTH210 in the genome of *P. cinnamomi* (**Figure 19**).

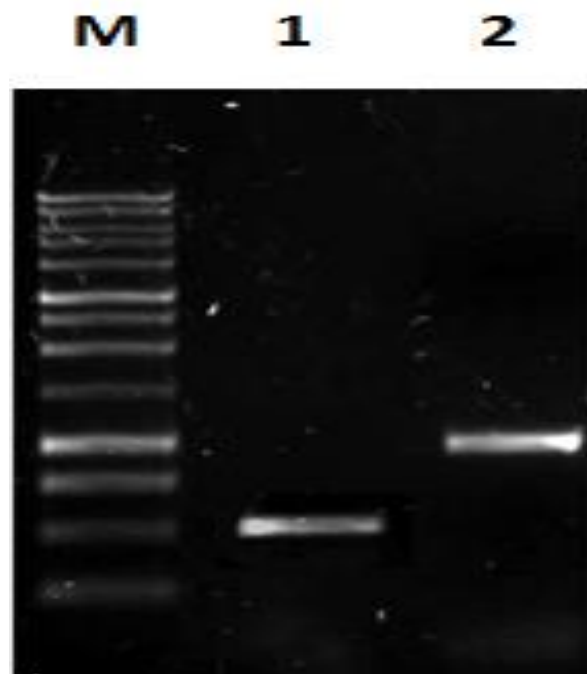


Figure 19. Visualization of the silencing cassette and the hygromycin fragment PCR products **M)** DNA ladder of 1 kb (Promega); **1)** PCR product of the silencing cassette 551 bp; **2)** PCR product of the hygromycin fragment 1090 bp

The amplification by PCR of the *gip* silencing cassette produced a band of approximately 551 bp while the amplification of the hygromycin segment produced a band of 1090 bp. The two PCR products were purified, then digested by restriction enzymes to have another proof of the fragments identity

After visualization on agarose gel the digestion of the *gip* silencing cassette fragment of 551 bp with the enzyme MseI generated two bands; one of 284 bp and another of 267 bp. The

digestion of the 1090 bp hygromycin fragment was performed with the enzyme PstI and generated a band of 282 bp and another of 808 bp (**Figure 20**).

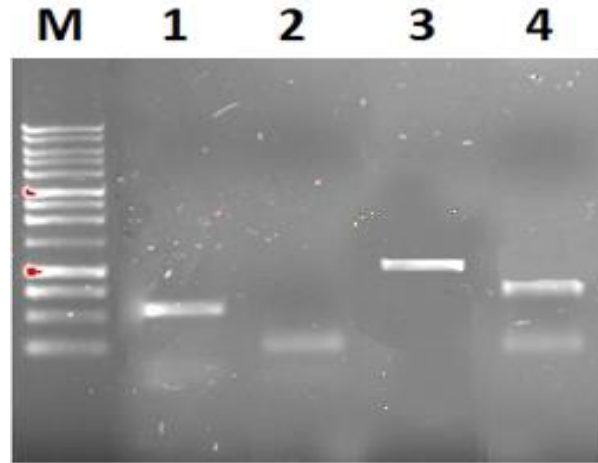


Figure 20.Enzymatic digestion of the silencing cassette and the hygromycin fragment PCR products **M**) DNA ladder of 1 kb (Promega); **1**) PCR product of the silencing cassette 551 bp; **2**) enzymatic digestion of the silencing cassette PCR product generated two superimposed bands; **3**) PCR product of the hygromycin fragment 1090 bp; **4**) enzymatic digestion of the hygromycin fragment PCR product generated two bands

For the digestion of the cassette fragment with MseI enzyme the two generated bands of 284 bp and 267 bp length are superimposed in the gel because of the close size.

With these results it is possible to confirm that the pTH210 recombinant plasmid was integrated into *P.cinnamomi*'s genome.

4.7-Sequencing of the transformed *Phytophthora*

In order to confirm the integration of the recombinant pTH210 vector in the genome of *P. cinnamomi*, the PCR products of the hygromycin fragment and the *gip* silencing cassette were sequenced and then analysed with BioEdit program.

After analysis with BioEdit program the nucleotide sequences were blasted using NCBI database to investigate the ID of the sequenced genes. The alignment of similar genes, was analysed and compared using the Muscle tool server (CLUSTAL multiple sequence alignment) from EMBL-EBI database. The results of the alignment are represented in **figures 21** and **22**.

CLUSTAL multiple sequence alignment by MUSCLE (3.8)

```
hpt_gene_sequence      ATCGAAAAGTTCGACAGCGTCTCCGACCTGATGCAGCTCTCGGAGGGCGAAGAATCTCGT
hygromycin_fragment_clean_sequen -----GCTCTCGGAGGGCGAAGAATCTCGT
*****

hpt_gene_sequence      GCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGAT
hygromycin_fragment_clean_sequen GCTTTCAGCTTCGATGTAGGAGGGCGTGGATATGTCCTGCGGGTAAATAGCTGCGCCGAT
*****

hpt_gene_sequence      GGTTTCTACAAAGATCGTTAIGTTTATCGGCACCTTTCATCGGCCCGCTCCCGATTCCG
hygromycin_fragment_clean_sequen GGTTTCTACAAAGATCGTTAIGTTTATCGGCACCTTTCATCGGCCCGCTCCCGATTCCG
*****

hpt_gene_sequence      GAAGTGTCTGACATTGGGGCATTGAGGAGAGCCTTACCTATTGCATCTCCCGCGTGCA
hygromycin_fragment_clean_sequen GAAGTGTCTGACATTGGGGCATTGAGGAGAGCCTTACCTATTGCATCTCCCGCGTGCA
*****

hpt_gene_sequence      CAGGGTGTACGTTGCAAGACCTGCCTGAAACCGAAGTCCCGCTGTTCTGCAGCCGGTC
hygromycin_fragment_clean_sequen CAGGGTGTACGTTGCAAGACCTGCCTGAAACCGAAGTCCCGCTGTTCTGCAGCCGGTC
*****

hpt_gene_sequence      GCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
hygromycin_fragment_clean_sequen GCGGAGGCCATGGATGCGATCGCTGCGGCCGATCTTAGCCAGACGAGCGGGTTCGGCCCA
*****

hpt_gene_sequence      TTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCT
hygromycin_fragment_clean_sequen TTCGGACCGCAAGGAATCGGTCAATACACTACATGGCGTGATTTCATATGCGCGATTGCT
*****

hpt_gene_sequence      GATCCCCATGTGTATCACTGGCAAAGTGTGATGGACGACACCGTCACTGCGTCCGTCGCG
hygromycin_fragment_clean_sequen GATCCCCATGTGTATCACTGGCAAAGTGTGATGGACGACACCGTCACTGCGTCCGTCGCG
*****

hpt_gene_sequence      CAGGCTCTCGATGAGCTGATGCTTGGGCCGAGGACTGCCCGAAGTCCGGCACCTCGTG
hygromycin_fragment_clean_sequen CAGGCTCTCGATGAGCTGATGCTTGGGCCGAGGACTGCCCGAAGTCCGGCACCTCGTG
*****

hpt_gene_sequence      CACGCGGATTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTTCATT
hygromycin_fragment_clean_sequen CACGCGGATTCGGCTCCAACAATGTCCTGACGGACAATGGCCGCATAACAGCGGTTCATT
*****

hpt_gene_sequence      GACTGGAGCGAGGCGATGTTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTCTGG
hygromycin_fragment_clean_sequen GACTGGAGCGAGGCGATGTTTCGGGGATTCCCAATACGAGGTCGCCAACATCTTCTCTGG
*****

hpt_gene_sequence      AGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAG
hygromycin_fragment_clean_sequen AGGCCGTGGTTGGCTTGTATGGAGCAGCAGACGCGCTACTTCGAGCGGAGGCATCCGGAG
*****

hpt_gene_sequence      CTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGATTGGTCTTGACCAACTCTAT
hygromycin_fragment_clean_sequen CTTGCAGGATCGCCGCGGCTCCGGGCGTATATGCTCCGATTGGTCTTGACCAACTCTAT
*****

hpt_gene_sequence      CAGAGCTTGGTTGACGGCAATTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCA
hygromycin_fragment_clean_sequen CAGAGCTTGGTTGACGGCAATTCGATGATGCAGCTTGGGCGCAGGGTCGATGCGACGCA
*****

hpt_gene_sequence      ATCGTCCGATCCGGAGCCGGACTGTGCGGCGTACACAAATCGCCCGAGAAGCGCGGCC
hygromycin_fragment_clean_sequen ATCGTCCGATCCGGAGCCGGACTGTGCGGCGTACACAAATCGCCCGAGAAGCGCGGCC
*****

hpt_gene_sequence      GTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACT
hygromycin_fragment_clean_sequen GTCTGGACCGATGGCTGTGTAGAAGTACTCGCCGATAGTGGAAACCGACGCCCCAGCACT
*****

hpt_gene_sequence      CGTCCGAGGGCAAAGGAATAGAGTAGATGCCGACCGAACAAGAGCTGATTCGAGAACGC
hygromycin_fragment_clean_sequen CGTCCGAGGGCAAAGGAATAGAGTAGATGCCGACCGAACAAGAGCTGATTCGAGAACGC
*****

hpt_gene_sequence      CTCAGCCAGCAACTCGCGCGAGCCTATGAAGGCAATGCGAGAGAACGGCCTTACGCTTG
hygromycin_fragment_clean_sequen CTCAGCCAGCAACTCGCGCGAGCCTATGAAGGCAATGCGAGAGAACGGCCTTACGCTTG
*****

hpt_gene_sequence      GTGGCACAGTTCCTGTCACAGTTCGCTAAGCTCGCTCGGCTGGGTCGCGGGAGGCCGGT
hygromycin_fragment_clean_sequen -----
```

Figure 21. Alignment between the sequenced hygromycin PCR product and the *hpt* gene sequence

```

CLUSTAL multiple sequence alignment by MUSCLE (3.8)

gip_gene_sequence      TTTTGGAAATTCAGTAGCAAATATGTGCACGACATGTTTACTTCCGGAAGGGCTAAACGT
cassette_clean_sequence -----CTTCCGGAAGGGCTAAACGT
                        *****

gip_gene_sequence      TCAATCCAATGGGCGTTTGTGAACCGCTCTTTACTCTATCTAACGTCCACCTCCTCAAGC
cassette_clean_sequence TCAATCCAATGGTGGTTGTGAACCGCTCTTTACTCTATCTAACGTCAACCTCCTCAAGC
                        *****

gip_gene_sequence      AATGGTTATCCACCATTGCGGAATTTACCGGCACTGCTCACTCCATTTCGCCATCTTCC
cassette_clean_sequence AATCGTTATCCACCATTGCGGAATTTACCGGCACTGCTCACTCCATTTCGCCATCTTCC
                        *** *****

gip_gene_sequence      GCTAACCAATGACGGTTGTCTTCCCGTCCGCACTGCACATCATTGTGGTGGTGCCTCATC
cassette_clean_sequence GCTAACCAATGACGGTTGTCTTCCCGTCCG-----
                        *****

gip_gene_sequence      AGTCCCACCCACGTGCTTACCACCTGCGTCAIGCACGGCATAACGAGGAGGGCTCGTCCATC
cassette_clean_sequence -----CGTGAAGGGTAAGCCAAGA-----
                        * * * * *

gip_gene_sequence      CCCCCTGGGCTGCAGTGGGCACGCACTACATCAACGGCGCGAAAGATGGCGAGCGGATC
cassette_clean_sequence ---CATTGACTTC-----CACAAAAG-----
                        ** * * * * *          * * * * *

gip_gene_sequence      AAGATCGTGTGACCAAGAACCACACTATACAACCTCGAGCAGTTTCTCGTACAATTC
cassette_clean_sequence -----TAAGAGT-----ACTCACGCA-----
                        ****          **** *

gip_gene_sequence      GCTGTGTTGACACTTGAGAATCCAAGCAAGTTCGCCCCCGTCAAGCTCCCTAAGGCGGAT
cassette_clean_sequence --CCAGCTGGCA-----CCCAGTTGA-----
                        * * * * *          *** * *

gip_gene_sequence      GGTTCGGACATTTTCCCGCGGGTGGTCAAGGTTATGTCGCTGGGTGATACCAGCTAC
cassette_clean_sequence -----TCGGCGCG-----AGATTAAATCGCCG-----
                        * *****          ** * * * * *

```

Figure 22. Alignment between the sequenced *gip* silencing cassette and the *gip* gene sequence

The alignment results of the hygromycin fragment sequence showed a high identity with the corresponding gene, while for the *gip* cassette sequence only the beginning of the sequence corresponding to the sense strand of the silencing construct showed a complete homology with *P. cinnamomi gip* gene, because the construction of the cassette was based only on the sense sequence selected within the ORF of *gip* gene sequence, the antisense sequence is inverted comparing with the gene sequence and the loop does not have any homology with *gip* gene sequence. These results confirm that the silencing cassette has integrated the genome of *P. cinnamomi*.

4.8-C. *sativa* infection with transformed and control *P. cinnamomi*

Six plants of *C. Sativa* were sterilized and germinated in sterile vermiculite, and then grown in a greenhouse until their root length reached approximately 30 cm. Three of these plants were infected with transformed *P. cinnamomi* mycelium having the *gip* silencing

cassette and another three were infected with non-transformed mycelium. The roots of chestnuts infected by transformed and non-transformed *P. cinnamomi* were grown at room temperature in bottles with sterile vermiculite for 72 hours. After the end of incubation the morphological analysis of the chestnuts was made to assess the effect of *gip* gene silencing on the plants (**Figure 23**).



Figure 23. Chestnuts in bottles with sterile vermiculite

The infection of Chestnut roots with transformed and non-transformed *P. cinnamomi* provided a comparative morphological analysis of the chestnut plants. The results demonstrate a clear contrast between chestnuts infected with transformed and those infected with non-transformed *P. cinnamomi*.

After 72 hours, two of the three chestnuts that have been infected by the transformed *P. cinnamomi* (transformed with the *gip* silencing cassette) revealed a smaller percentage of wilting leaves and root necrosis, while for plants infected with non-transformed *P. cinnamomi* there was a higher percentage of wilting leaves and root necrosis. As the *gip* gene is considered responsible for the suppression of plant defense responses enhancing susceptibility of disease symptoms development (Rose et al, 2002) these results seems to indicate the effectiveness of *gip* gene silencing by the cassette cloned into *P. cinnamomi* (**Figure 24**).

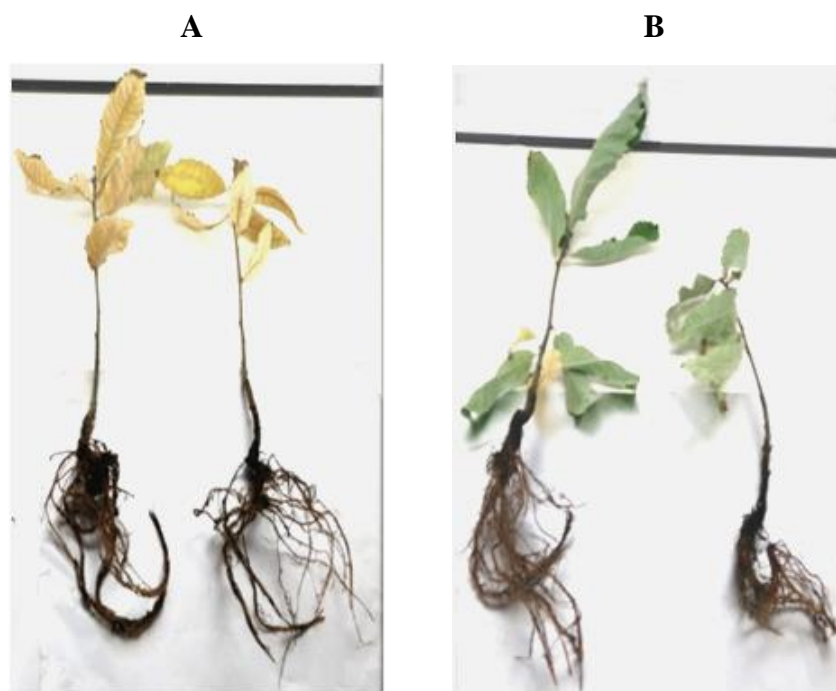


Figure 24.Aspect of chestnuts after infection with non-transformed (A) and transformed (B) *P. cinnamomi*

The protein GIP is believed to reduce the effectiveness of the plant host's surveillance system inducing disease symptoms (Martins et al, 2014) means that the silencing of the gene encoding this protein will cause the attack of the pathogen by the non altered plant defense mechanisms, thus suppressing or at least delaying the manifestation of the disease symptoms (described in **section 1.1**) which is compatible with the results obtained .

The results are considered as preliminary confirmation of the effectiveness of RNA interference in silencing genes involving in the development of ink disease caused by *P. cinnamomi*.

Recognition of the importance of RNA interference came from research that produced a range of effective applications in different fields and for various purposes, as discussed in section 1.6.5. This technique has been examined in a number of agricultural studies to control pathogens and it is a promising tool in the development of environmentally friendly solution to control pathogens.

Thus, this research study has investigated the effects of RNAi, using *P. cinnamomi* as a model organism because, as described in chapter 1.2.2, they are a serious pathogens attacking various plants and causing huge economic losses.

In this study the RNAi technique has been applied by using a shRNA based vector that generates a siRNA in the cell capable of degrading mRNA sequence of *gip* gene and the possible effects on the plant phenotype were examined.

Transformation with the *gip* silencing cassette has no effect on the phenotype and the appearance of transformants *P. cinnamomi* comparing with non transformants because the GIP protein is only produced during the infection (only secreted in the presence of the host) (Rose et al, 2002).

This particular gene was selected because of its important role in counteracting plant defense mechanisms and inducing disease symptoms development (Martins et al, 2014) meaning that the silencing of this gene will deprive the pathogen from an important weapon used to weaken the defense effectors of the plant thus facilitating the fight against the pathogen. The possibility of interference between the *gip* silencing cassette and other similar genes was not considered because the blast search reveals that there are no similarities between *gip* and other genes of *P. cinnamomi*.

Studying the phenotype of plants after they were exposed to transformed *P. cinnamomi* having the silencing construct, for three days, showed a smaller percentage of disease developments while for plants infected with non-transformed *P. cinnamomi* there was a higher percentage of disease development which confirms the silencing of *gip* gene.

Transformation of *Phytophthora* protoplasts using polyethylene glycol and CaCl₂ has historically been the method of choice but the transformation by electroporation has been chosen because it is more rapid and efficient since the motile zoospores produced by *Phytophthora* species are deprived from cell walls, which facilitate the transformation (Huitema et al, 2011).

Affecting the proliferation of *P. cinnamomi* by designing shRNAs that target specific genes could be a feasible method of limiting the spread of this pathogen and accordingly reducing their effects on the plants. In general, in order to achieve an effective knockout/knockdown method, it is important to choose the right targets which have a fatal phenotype.

4.9-Amplification of *gip* and *npp1* genes

To carry out the amplification of *gip* gene of *P. cinnamomi* by PCR, primers with NotI and AatII restriction enzymes sites have been used to allow after the digestion of the amplified product the insertion of the *gip* gene into the pTOR-eGFP Plasmid.

The results were analyzed on agarose gel 0.8% (w/v) confirming the amplification of a fragment of approximately 828 bp corresponding to the *gip*'s ORF size (810 bp) and primer sequences (**Figure 25**).

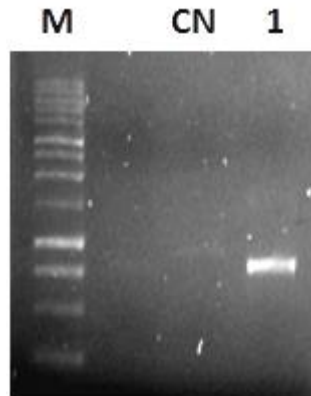


Figure 25. Amplification of *gip* gene; M) DNA ladder 1kb (Promega®); CN) negative control; 1) 828 bp band corresponding to the *gip*'s ORF size (810 bp) and primer sequences

For the amplification of *npp1* gene by PCR, primers with NotI and SacII restriction enzymes sites have been used to permit the insertion of the PCR product into the pTOR-eGFP plasmid. The results were analyzed on agarose gel 0.8% (w/v) proving the amplification of a fragment of approximately 810 bp corresponding to the *npp1*'s ORF size (771 bp) and primer sequences (**Figure 26**).

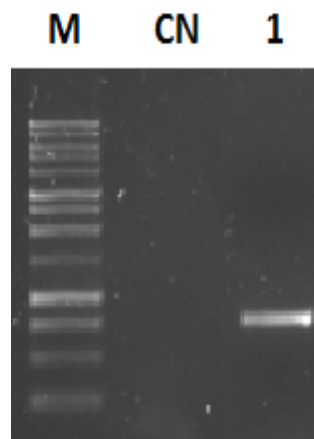


Figure 26. Amplification of *npp1* gene. M) DNA ladder 1kb (Promega); CN) negative control; 1) 810 bp band corresponding to the *npp1*'s ORF size (771 bp) and primer sequences

As it is shown in Figures 13 and 14, the amplification of *gip* and *npp1* genes was successfully accomplished, allowing the initiation of next steps.

4.10-Cloning the ORF's of *gip* and *npp1* genes into the pTOR-eGFP vector

The PCR products (ORF of *gip* and *npp1* genes) were purified using the Wizard® SV PCR Clean-Up System (Promega,USA) then digested by respective restriction enzymes.

The ORF of *gip* gene was first digested by NotI enzyme, purified and digested again with AatII enzyme while the ORF of *npp1* gene was digested first by NotI enzyme, purified and digested again with SacII enzyme.

On the other hand the plasmid pTOR-eGFP was extracted from *E. coli* previously transformed, digested separately with respective restriction enzymes, as described for the insert digestion, and then dephosphorylated with Alkaline Phosphatase, Calf Intestinal (CIAP) to ensure that the vector does not re-circularize during ligation.

The insert and the plasmid were after that visualized on agarose gel 0.8% (w /v) in order to approximate the concentration of both fragment, a very important step for the efficiency of the ligation reaction.

The insert and the plasmid were excised from agarose gel with a clean razor blade, purified using the QIAquick® Gel Extraction Kit and then ligated using T4 DNA Ligase enzyme.

The recombinant vector was used to transform *E. coli* NZY5α competent cells and after the screening of transformants, the plasmids were extracted, digested with respective restriction enzymes and visualized on agarose gel to confirm the insertion of the ORF's in pTOR-eGFP vector (**Figure 27**).

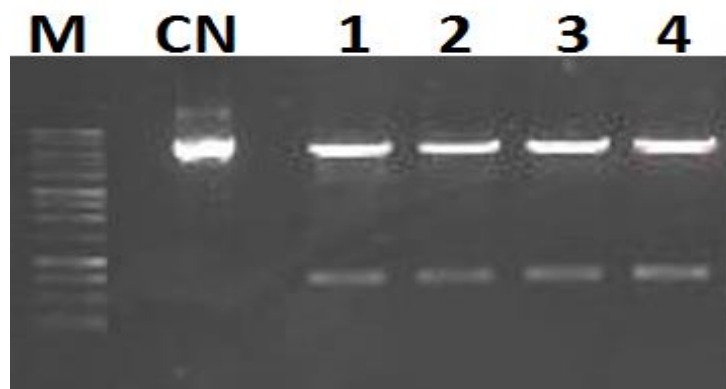


Figure 27. Visualization of the recombinant pTOR-eGFP vector digestion M) DNA ladder of 1 kb (Promega); CN) non recombinant pTOR-eGFP supercoiled; **1,2**) digestion of recombinant pTOR-eGFP with *npp1* ORF by respective enzymes generated two bands; **3,4**) digestion of recombinant pTOR-eGFP with *gip* ORF by respective enzymes generated two bands

Two bands were generated after the digestion of the recombinant plasmid with *gip* fragment one of approximately 7 kb corresponding to the pTOR-eGFP and another one of approximately 828 bp corresponding to the *gip* fragment confirming the success of the cloning.

The digestion of the recombinant plasmid with *npp1* fragment generated two bands one of approximately 7 kb corresponding to the pTOR-eGFP and another of approximately 810 bp corresponding to the *npp1* fragment.

The digestion of the insert or the vector with two restriction enzymes was done separately because the universal buffer was not available in our lab and it is not recommended to make a digestion reaction with two different buffers together.

The 100 bp DNA ladder has been used because the 500 bp band present at triple intensity comparing with the other fragments serves as a reference indicator that gives an approximation of the concentration of the vector and the insert.

Using two different enzymes to ligate the insert into the pTOR-eGFP vector is mandatory to impose the direction of the insertion that produces the GFP-GIP/NPP1 chimeric protein (the 5' sense strand of the insert must be ligated downstream the *gfp* sequence) because if using only one enzyme the 5' antisense strand of the insert can be ligated downstream the *gfp* sequence and that will not produce the chimeric protein.

Due to the short time the transformation of *P. cinnamomi* with the recombinant pTOR-eGFP and the observation with fluorescent microscopy of the protein localization would not be accomplished, hence this task will be postponed.

4.11-Prediction of the subcellular localization of the GIP and NPP1 proteins

The softwares publicly available CELLO, LOctree, EuK-mPLoc2.0, Esl pred and SignalP 3.0 have been used for the protein localization prediction.

For the protein GIP prediction, many softwares with different prediction methods (Amino Acid Composition, N-peptide Composition, Physico-chemical Composition, etc) have been used, indicating all the extracellular destination (**Figure 28**).



SignalP 3.0 Server - prediction results

Technical University of Denmark

```
>Sequence length = 70
# Measure Position Value Cutoff signal peptide?
max. C 32 0.580 0.32 YES
max. Y 32 0.469 0.33 YES
max. S 11 0.837 0.87 NO
mean S 1-31 0.536 0.48 YES
D 1-31 0.503 0.43 YES
# Most likely cleavage site between pos. 31 and 32: SNG-YP

>Sequence
Prediction: Signal peptide
Signal peptide probability: 0.595
Signal anchor probability: 0.000
Max cleavage site probability: 0.483 between pos. 31 and 32
```

CELLO RESULTS

SeqID: gi|164510745|emb|CAJ90742.1| glucanase inhibitor protein [Phytophthora cinnamomi]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Extracellular	0.684
N-peptide Comp.	Extracellular	0.790
Partitioned seq. Comp.	Extracellular	0.562
Physico-chemical Comp.	Extracellular	0.779
Neighboring seq. Comp.	Extracellular	0.892

Figure 28.Subcellular localization prediction of GIP protein

According to the program SignalP 3.0, the signal peptide located between the positions 31 and 32 of the sequence indicates that the protein is extracellular. Signal peptide is a short sequence of amino acids present at the N-terminus of proteins assuring their shipping to the ER for subsequent transport through the secretory pathway (Rapoport, 1992).

For the protein NPP1 the different softwares used (CELLO, LOctree, EuK-mPloc 2.0, Esl pred and SignalP 3.0) indicated different results; while the softwares using the peptide signal method indicate that the protein is not extracellular other softwares using other methods indicate that NPP1 protein is extracellular (**Figure 29**).



SignalP 3.0 Server - prediction results

Technical University of Denmark

```
>gi_164507578_emb_CAL length = 70
# Measure   Position   Value   Cutoff   signal peptide?
max. C      29         0.136   0.32     NO
max. Y      29         0.108   0.33     NO
max. S      4          0.630   0.87     NO
mean S     1-28       0.215   0.48     NO
D          1-28       0.162   0.43     NO

>gi_164507578_emb_CAL47421.1_
Prediction: Non-secretory protein
Signal peptide probability: 0.001
Signal anchor probability: 0.000
Max cleavage site probability: 0.001 between pos. 21 and 22
```

CELLO RESULTS

SeqID: gi|164507578|emb|CAL47421.1| necrosis-inducing protein [Phytophthora cinnamomi]

Analysis Report:

SVM	LOCALIZATION	RELIABILITY
Amino Acid Comp.	Extracellular	0.576
N-peptide Comp.	Cytoplasmic	0.478
Partitioned seq. Comp.	Extracellular	0.213
Physico-chemical Comp.	Cytoplasmic	0.574
Neighboring seq. Comp.	Extracellular	0.611

Figure 29. Subcellular localization prediction of NPP1 protein

The results of the subcellular localization of NPP1 protein suggest that it is secreted but not through the classical ER-Golgi pathway (via signal peptide) but through unconventional protein secretion processes.

During plant infection the pathogen secretes GIP proteins that bind to plant extracellular EGaseA, inhibiting its hydrolytic activity thus suppressing the induction of defense responses (Rose et al, 2002). The function of GIP protein in binding plant extracellular EGaseA suggests that GIP protein is secreted by the pathogen which is consistent with the prediction results.

On the other side the function of NPP1 proteins in initiating cell death processes and causing necrosis of host tissue require a direct contact with the host cells so the protein must be secreted which seems to be in conformity with the aforementioned results (Kanneganti et al, 2006).

Determining the localization of proteins involved in the infection process by which *P. cinnamomi* invades the plant is valuable for understanding their functions and that will facilitate the treatment of ink disease.

5-CONCLUSIONS AND FUTURE RESEARCH

In this study the effect of pathogen gene silencing on the host phenotype was interpreted by using a shRNA based vector capable of producing siRNA in cells that degrade the mRNA of the target gene.

This work has shown that a silencing cassette for *P. cinnamomi gip* gene can be produced by PCR-based cloning method which is a very cheap procedure compared with other mediators of RNA interference.

Also the transformation of *P. cinnamomi* zoospores by electroporation was successfully performed and the genome integration of the silencing construct has been verified by genotypic tests (PCR screening and sequencing).

The effectiveness of the cassette has been verified by infecting *C. sativa* with transformed and non-transformed *P. cinnamomi* and observation of the gene silencing effect on the plant phenotype. It was clear that the chestnuts infected by transformed *P. cinnamomi* revealed a smaller percentage of wilting leaves and root necrosis, while for plants infected with non-transformed *P. cinnamomi*, there was a higher percentage of wilting leaves and root necrosis, which is consistent with the *gip* gene function that trigger the development of disease symptoms.

The cloning of the *gip* and *npp1* ORF in the pTOR-eGFP vector having the gene encoding for the GFP was successfully achieved for future subcellular localization. The verification of the cloning was done by digestion of the recombinant plasmids with respective restriction enzymes and visualization on agarose gel.

The subcellular localization prediction of the proteins shows that the protein GIP is secreted through the classical ER-Golgi pathway while for NPP1 protein the secretion is assured by unconventional protein secretion processes.

This study indicates that RNA interference could be a promising approach to resolve the problem of ink disease by suppressing genes involving in disease development, a method that can be applied without any controversies and thus replace the transgenic methods used to obtain resistant crops.

The following tasks will include 1) the extraction of total RNA from *P. cinnamomi* for a cDNA synthesis in a reverse transcriptase PCR reaction followed by a quantitative real time PCR (qRT-PCR) to determine the expression level of *gip* in both non transformants and

transformants *P. cinnamomi* and 2) the observation with fluorescent microscopy of the secretory pathways of the NPP1 and GIP proteins in *P. cinnamomi* transformed by the recombinant pTOR-eGFP vector.

Moreover, more research could be performed such as 1) the transformation of *P. cinnamomi* with a vector harboring many silencing cassette for different genes, involved in the mechanism of infection and analyse the effect on the plant phenotype, the expression level of genes in *P. cinnamomi* and the proteome of the plant and 2) the examination of the interaction between *P. cinnamomi* and chestnuts by monitoring proteins tagged with different fluorescent vector.

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