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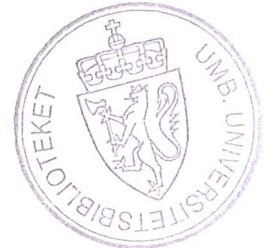
Cloning and characterization of effector genes from the strawberry crown rot pathogen *Phytophthora cactorum*



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Yang Hu

Abstract

Effectors play important roles in plant-microbe interaction, they manipulate host cell structure and function, thereby facilitating infection sometimes also triggering defense responses. Four candidate effector genes from strawberry crown rot pathogen *Phytophthora cactorum* were selected for full length cloning. One transglutaminase gene and phenylalanine-4-hydroxylase gene were cloned. This transglutaminase is from a gene family and contains PEP-13 motif, which was Ca2+ dependent and predicted important for both transglutaminase and elicitor activity. The function of this protein was predicted as pathogen-associated molecular patterns (PAMPs), and the structure of sequence predicted it is cell wall protein, working as cross-linking proteins, or cell-cell adhesion proteins. *Phytophthora* transglutaminase can be defined as apoplastic effector, so further study was carried on this gene. The gene expression pattern of this gene shows that this transglutaminase gene is hyphae specific expression gene, which also support it is the cell wall protein.

Abbreviation:

cDNA	complementary Deoxyribonucleic acid
Da	dalton
ddNTP	dideoxynucleotides
dH_2O	sterile water
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
dpi	days past infection
EDTA	ethylenediaminetetraacetic acid
ETI	effector tigger immunity
hpi	hours past infection
LB	lysogeny broth
MAPK	mitogen activated protein kinase
MLV	murine leukemia viruses
mRNA	messenger Ribonucleic acid
PAMP(s)	pathogen-associated molecular pattern(s)
PCR	polymerase chain reaction
PRRs	pattern recognition receptors
PTI	PAMPs trigger immunity
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
TGnase	transglutaminase
X-gal	bromo-chloro-indolyl-galactopyranoside

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Abbreviation: V

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1 Introduction

1.1 Strawberry

The strawberry is a perennial herb of temperate climates all over the world. Producing around a quarter of world total yield in 2005, USA is the biggest strawberry producer in the world, followed by European countries such as Spain, Russia and Poland (FAO). In USA, the annual consumption of strawberry per person has increased dramatically from 0.9 kilograms in 1970 to 3.1 kilograms in 2004 (Folta & Davis 2006). In Norway, strawberry is the most important fruit crop, with around 10,000 tons fruit yield from approximately 1,700 hectares commercial field in 2000 (Eikemo et al. 2000).

The modern cultivated strawberries in commercial production are cultivars of *Fragaria × ananassa.* The species is octoploid (2n=8x=56) and it originated in Brittany, France in 1740, via crossing of two naturally occurring octoploid species, *F. chiloensis* and *F. virginiana*, both native to the Americas (Folta & Davis 2006). The new hybrid quickly replaced other cultivars because of its large, sweet, flavorful berries (Darrow 1966).

Fragaria × ananassa is one of the most genomically complex crop species. Accordingly, it is of considerable interest to identify and develop an appropriate diploid *Fragaria* species as a model for strawberry genetic and genomic research (Folta & Davis 2006). Developing a diploid model system for *Fragaria* has focused on *F. vesca*, which has the widest geographic distribution of the diploid *Fragaria* species (Folta & Davis 2006). Phylogenetic research done by DiMeglio and Davis supported *F. vesca* to be the a good diploid model for genetic study since *Fragaria × ananassa*-derived Simple Sequence Repeat (SSR) primer pair functionality in various diploid species, as measured by amplification success rate (=100%-failture rate) in each diploid, was ranked as follows:

F. vesca(98.4%) >*F. iinumae*(93.8%) =*F. nubicola*(93.8%) >*F. mandshrica*(87.5%) >*F. nilgerrensis*(75%) >*F. viridis*(73.4%) (Davis et al. 2006).

F. vesca was chosen as a model species because of these reasons: first, this plant is easy to grow and propagate through seeds or runners, and also easy to

transform genetically (Oosumi et al. 2006). Secondly, *F. vesca* genome is small (~200 Mb), and genetic maps exist for both the diploid and octoploid strawberry. Finally, no major chromosomal rearrangement seems to have occurred between diploid and octoploid strawberry (Cipriani et al. 2006; Eikemo et al. 2010; Oosumi et al. 2006; Rousseau-Gueutin et al. 2008; Weebadde et al. 2008).

1.2 Strawberry crown rot disease caused by *Phytophthora cactorum*

1.2.1 Significance and disease features

Strawberry crown rot caused by *Phytophthora cactorum* is a disease of long term consequences in strawberry production, because of the pathogen's ability to survive indefinitely in soil and its capacity for rapid reproduction. These two traits prevent its eradication from strawberry production systems (Bhat et al. 2006; Shaw et al. 2006). Infection of the crown by *P. cactorum* can cause rot and dysfunction of the vascular system and it may also cause leather rot of the fruit. The pathogen causes loss primarily by killing plants, but reduction of growth and yield through sub-lethal infection of *P. cactorum* is also serious (Bhat et al. 2006; Shaw et al. 2006). The pathogen can be found all over the world (Jones & Benson 2001). In Norway, the first detection of crown rot pathogen of strawberry was in 1992. In 1996 and 1997, surveys were taken for detection of *P. cactorum* in Norwegian certified strawberry plant production and regular strawberry production. An enzyme-linked immunosorbent assay (ELISA) was used to investigate isolates that were recovered from plant material. *P. cactorum* was not detected in any of the samples from certified growers. However, *P. cactorum* was detected at 35 different strawberry-producing farms in 11 of the 19 counties of Norway from a survey of the distribution of *Phytophthora fragariae* var. *fragariae* and some other samples (Stensvand et al. 1999).

P. cactorum can cause a number of symptoms such as root and collar rots, fruit rots, cankers, leaf blights, wilts, and seedling blights (Jones & Benson 2001). Symptoms on strawberry vary with plant stages and time of the year. Early in the

season, infected plants may be stunted either at nurseries or in fields (Bhat et al. 2006; Shaw et al. 2006). Typical symptoms develop during early- to mid-summer when the weather becomes warmer. Young leaves turn bluish and often wilt suddenly. Wilting will spread throughout the plant, causing plant death. The necrosis occurs throughout the crown, and the crown may break at the upperpart. In most cases, brown discoloration of the crown, first appearing at the upper part and spreading downward, is diagnostic for the disease (Maas 1984). However, especially in later stages of disease, it is difficult to distinguish *P. cactorum* crown rot from crown necrosis caused by other pathogens like *Colletotrichum acutatum*. Furthermore, in early stages of the infection, crown rot may be limited to outer regions or sectors of the plant crown. It also causes runner lesions in addition to crown and root rot at nurseries, later dark necrosis on daughter plant will develop. Sometimes the disease also can be carried on nursery stock without clear symptoms (Bhat et al. 2006; Shaw et al. 2006).

Leather rot of the fruit can happen at any stage of berry development. On green berries, diseased areas appear dark brown or natural green outlined by a brown margin. The entire berry becomes brown, looks leathery, and maintains a rough texture as the rot spreads. On fully ripened berries, color change may range from little to significant discoloration, brown to dark purple, and infected fruit are usually softer to touch than healthy fruit. Generally, mature fruit is dull in color and is not shiny or glossy. When diseased berries are cut across it can be observed that the water-conducting system to each seed is noticeably darker. A white, moldy growth can be observed on the surface of infected fruit if the environment condition fits the pathogen. At the end, the infected fruits will dry up to form stiff shriveled mummies (Rebollar-Alviter et al. 2006)

1.2.2 Causal agent

P. cactorum was first identified on *cacti* in 1870 by Lebert and Cohn. It is an oomycete which is member of the kingdom *Chromista* with mycelium containing mainly cellulose and glucans but without cross walls, except to separate living parts of hypha from old parts from which the cytoplasm has been withdrawn. Oomycetes produce oospores as their resting spores and zoospores or sporangia

as their asexual spores (Agrios 2005) The most important plant pathogenic Oomycetes belong to several groups: downy mildew, *Phytophthora* and *Phytium*(also is important animal pathogen). The name *Phytophthora* is from Greek, and means plant destroyer (Agrios 2005). A genus-wide phylogeny for 82 *Phytophthora* species was presented by using seven of the most informative loci (approximately 8700 nucleotide sites). The result supported division of the genus into 10 well-supported clades. Both *Phytophthora infestans* (the potato late blight pathogen that caused the Irish potato famine in the eighteenth century) and *P. cactorum* belong to the clade 1(Blair et al. 2008).

P. cactorum is capable of infecting an extremely large number of hosts, more than 200 species in 160 genera, including strawberry and a number of woody ornamentals and fruit crops such as apple, pear, rhododendron and azalea. It occurs worldwide but is most common in temperate regions.

P. cactorum strains isolated from different host plants are genetically different. For example *P. cactorum* isolates from birch caused clear lesions on non-wounded bark of birch, while the same isolates were not detrimental to strawberry. Random Amplification of Polymorphic DNA (RAPD) analysis revealed variation within *P. cactorum*, isolates from silver birch having different banding patterns than those from strawberry. UPGMA analysis also clustered isolates from silver birch and strawberry plants into separate groups (Lilja et al. 1998). The *P. cactorum* in North America and Europe develops different symptoms in strawberry. In North America, leather rot of fruit is more common than in Europe. Analysis of *P. cactorum* from strawberry using Random Amplified MicroSatellite (RAMS) markers showed that leather rot of strawberry fruit and crown rot were not caused by genetically different strains of this species (Hantula et al. 2000). UPGMA-analysis also showed that the populations of *P. cactorum* on strawberry differed between North America and Europe, but no clear genetic separation between isolates from different plants species could be made. Slight morphological variations existing in the diameters of oogonia and oospores between the isolates from leather rot and crown rot were insufficient for the identification of genetic groups or host specificity of *P.*

cactorum isolates. Pathogenicity experiments proved that strains show a tendency towards host specialization (Hantula et al. 2000).

1.2.3 Disease cycle and epidemiology

P. cactorum survives the winter as oospores, thick walled resting spores, which form in the plant residues and can remain viable in soil for long periods of times. In the spring, oospores germinate in the presence of free water and may form sporangia. Zoospores which originate from sporangia are the most important propagules (Jones & Aldwinckle 1990). In many cases, the pathogen enters a field through infected transplants; it is also spread by splashing or wind-blown water from rain or overhead irrigation (Erwin & Ribeiro 1996; Jones & Aldwinckle 1990; Maas 1984). Infection by *P. cactorum* usually occurs at wet periods of at least one hour, and the optimum temperature is between17-22°C. Motile zoospores are released from sporangia during saturated soil conditions. Once the zoospores reach a host, they infect through wounds and develop fungus-like hyphae that colonize the host.

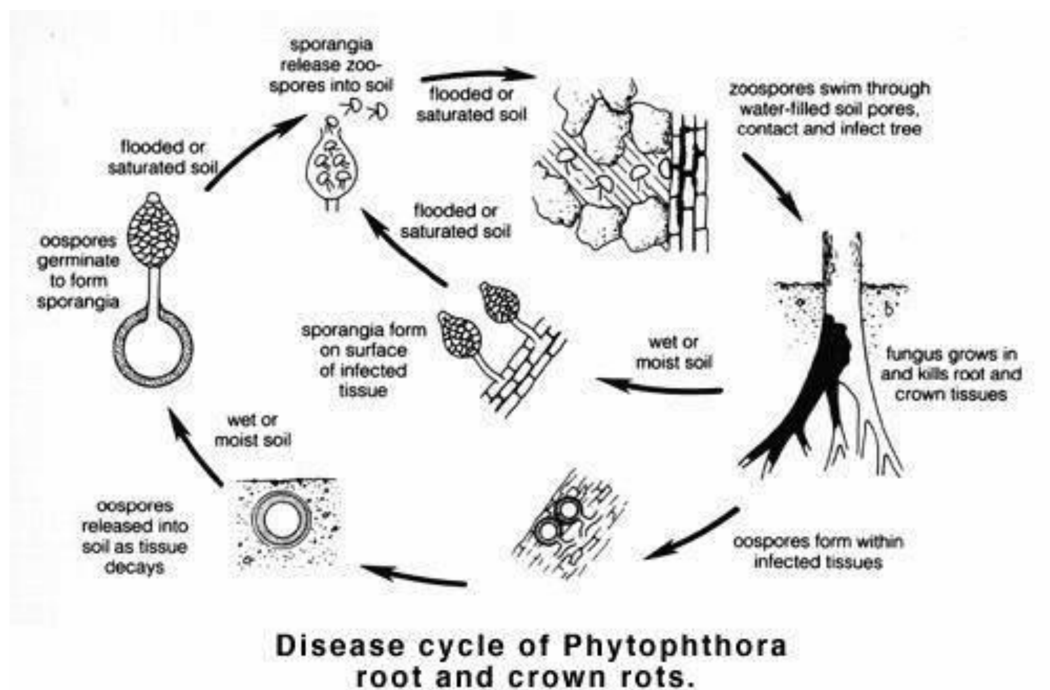


Figure 1.2.3: The figure shows the disease cycle of strawberry crown rot (Cornell University, NYSAES).

1.3 Microbe-plant interaction

1.3.1 Oomycete pathogenicity

Plant pathogenic oomycetes have a number of virulence factors to help them establish successfully as parasites. The two flagella enable zoospores to swim in the soil water and reach the potential host. Vesicles formed in the zoospores are important for adhesion and encysting (Lehnen & Powell 1989; Robold & Hardham 2005). The gene *PcVsv1* that encode a 200-kDa protein which can be found in vesicles in *Phytophthora cinnamomi* is one of 47 proteins which contain the thrombospondin type I repeat (TSR1). TSR1 is a conserved, 50 amino-acid motif found in a range of adhesive molecules secreted by both mammalian and malarial parasites (Robold & Hardham 2005). Also, the apoplastic effector Cellulose-binding elicitor lectin (CBEL) contributes to adhesion. The secreted protein *PnCcp* from the large peripheral vesicles is also a potential adhesive protein (Skalamera & Hardham 2006). After adhesion, oomycetes may develop appressorium-like swellings to help penetrat the plant surface. The formation of appressorium-like swellings, similar to fungi, is induced by forces such as surface topography and hydrophobicity (Bircher & Hohl 1997; Grenville-Briggs et al. 2005; Slusarenko & Schlaich 2003; Soylu & Soylu 2003). Like fungi, oomycete hyphae also synthesize and secrete enzymes to degrade plant cell wall components such as pectin, cellulose, and xyloglucans (Boudjeko et al. 2006). During biotrophic growth, haustoria are formed to uptake nutrients and deliver effector proteins which can suppress plant immunity and manipulate host cell structure and function (Kamoun 2006; Whisson et al. 2007).

1.3.1.1 Effectors

The term 'effector' is defined by Kamoun (2006) as 'pathogen molecules that manipulate host cell structure and function, thereby facilitating infection and/or triggering defense responses. Effectors can be elicitors and/or toxins' (Kamoun 2006). This term became popular in the field of plant-microbe interactions (Hogenhout et al. 2009) with the discovery of a specialized machinery that the plant pathogenic Gram-negative bacteria utilize, the type III secretion system (T3SS), to deliver proteins inside host cells (Abramovitch et al. 2006; Block et al.

2008). These proteins were considered 'avirulence proteins' at first because of their ability to trigger hypersensitive response in resistant plants. Later it was found that these protein also contribute to virulence in susceptible plants (Hogenhout et al. 2009). Today a number of effector genes have been identified and characterized. More and more common concepts have emerged from the study of cellular plant pathogen effectors.

Plant pathogen effectors frequently have more than one host target. The *Pseudomonas syringae* T3SS effector AvrRpt2 works against at least five *Arabidopsis* proteins, including the negative defense regulator RIN4 (Chisholm et al. 2005; Takemoto & Jones 2005). Another *P. syringae* T3SS effector, AvrPto, can inhibit two pathogen recognition receptors from tomato (Shan et al. 2008; Xiang et al. 2008; Xing et al. 2007). Each interaction between the effector and host protein can have positive, negative or neutral effects. It becomes important to distinguish the operative targets from other targets. Some proteins that are not operative targets, but when perturbed by effectors, trigger host recognition by cognate R proteins are thought of as decoys (van der Hoorn & Kamoun 2008). The functions of many effectors have been characterized (Hogenhout et al. 2009). Many effectors have functions that suppress plant innate immunity. Some effectors can alter plant behavior and development. Coronatine is one elegant example, it triggers stomatal reopening in *Arabidopsis* and facilitates *P. syringae* entry into the plant apoplastic space (Melotto et al. 2006). Gibberrellins produced by *Gibberrella fujikuroi*, is an example of effectors that mimic plant molecules and cause the foolish seedling diseases of rice (Tudzynski 1999).

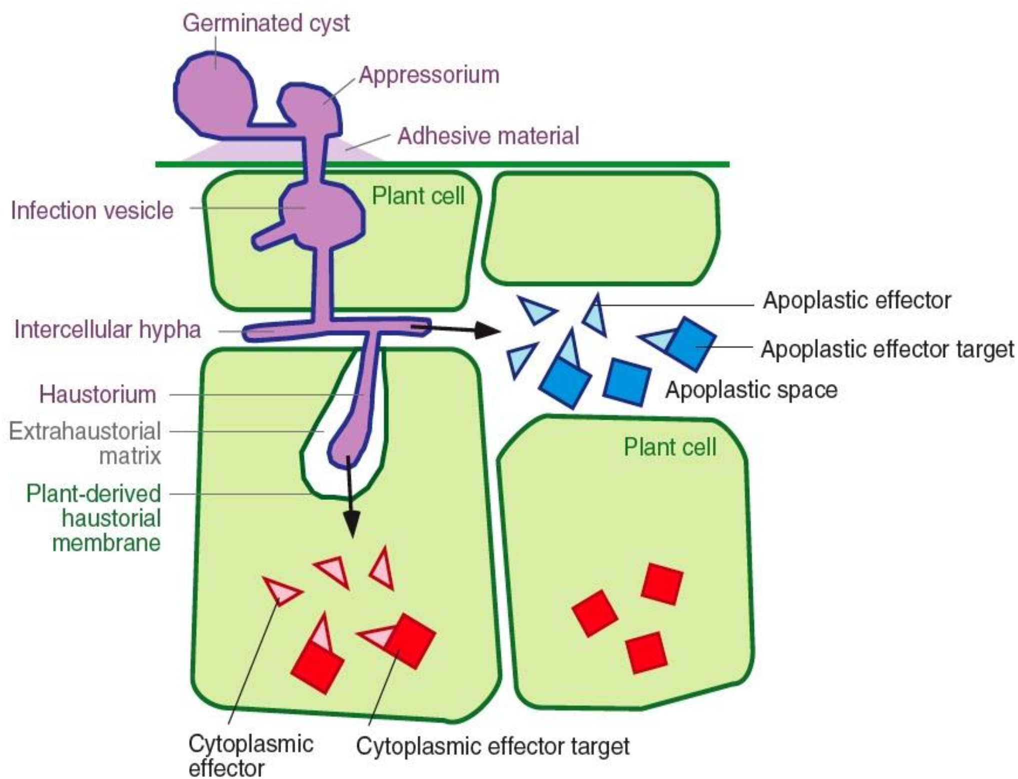


Figure 1.3.1.1 Effectors can be divided to apoplast and cytoplasm (Kamoun 2006).

1.3.1.2 Oomycete cytoplasmic effectors

Like other plant pathogens, such as bacteria, fungi, and nematodes, pathogenic oomycetes also produce two classes of effectors which target distinct sites in the host plant. Apoplastic effectors interact with extracellular targets or surface receptors in the plant extracellular space, while cytoplasmic effectors are delivered inside the plant cells.

Hiller et al. (2004)and Marti et al. (2004) observed that an N-terminal PEXEL (*Plasmodium* export element) motif (RxLxE/Q) was required for effectors of the malaria parasite *Plasmodium* to enter into the cytoplasm of red blood cells (Hiller et al. 2004; Marti et al. 2004). These observations encouraged the hypothesis that a similar motif in oomycetes, the RxLR-dEER motif, was the translocation domain of oomycete effectors. This has been confirmed experimentally forboth *Phytophthora sojae Avr1b* and *P. infestans Avr3a* (Dou et al. 2008a; Whisson et al. 2007). Bioinformatics analysis of the genome sequences of *P. sojae, Phytophthora ramorum, P. infestans* and *Hyaloperonospora. arabidopsidis* revealed a large

numbers of genes encoding proteins with sequence similarity to effector genes: nearly 400 each in *P. sojae* and *P. ramorum* (Jiang et al. 2008; Tyler et al. 2006), 500–600 in *P. infestans* and 100–200 in *H. arabidopsidis* (Jiang et al. 2008). In most cases, there is at most 10–30% identity among individual pairs of effector gene family members, but in some case, the sequence similarity is substantial (40–50% identity), even across species (Jiang et al. 2008). Emerging findings indicate that some oomycete RxLR effectors can suppress plant immunity by suppressing programmed cell death (Hogenhout et al. 2009). *P. infestans* effector protein *Avr3a* suppresses the hypersensitive response induced by another apoplastic effector (INF1 elicitin) also from *P. infestans* (Bos et al. 2006). *H. arabidopsidis ATR13* can suppress pathogen-associated molecular patterns (PAMPs)-triggered defense responses in *Arabidopsis* plants including callose deposition and production of reactive oxygen species, and then enhance the *P. syringae* virulence (Jones & Dangl 2006; Nurnberger et al. 2004; Sohn et al. 2007). Recent studies have shown that the defense suppression functions of RxLR effectors require their C-terminal domains. Three conserved motifs in the C-terminus: W, Y and L motifs were indentified from *P. sojae Avr1b*, which also suppressed the program cell death induced by the mouse protein *BAX* in yeast and plants (Dou et al. 2008b). Investigation of all of the cloned oomycete avirulence proteins suggest that at least one function of the W and Y motifs is to suppress program cell death (Dou et al. 2008b).

Crinkling- and necrosis-inducing proteins (CRN proteins) are another family of cytoplasmic effectors. As their names indicate, these proteins can trigger crinkling and necrosis of leaves when over expressed in *Nicotiana. benthamiana* transient expression assays (Torto et al. 2003). In this group of effectors, some proteins contain both RxLR and 'FLAK' motif (LxLFLAK), some contain one of the RxLR or 'FLAK' motif, and some contain overlapping RxLR motifs (sometimes the RxLR or 'FLAK' motif wre modified or unrecognized); these motifs actually play the role in cell entry (Gaulin et al. 2008; Win et al. 2007).

1.3.1.3 Oomycetes apoplastic effectors

In the apoplastic space, there are several plant pathogenesis-related (PR) proteins, such as glucanases and proteases. Some of the oomycete effectors disrupt the activities of these PR proteins. GIP1 and GIP2 are secreted proteins of *P. sojae* that inhibit the soybean endo-β-1, 3 glucanase (EgaseA) (Rose et al. 2002), and four genes indentified in *P. infestans* are similar to the *P. sojae* GIPs. *P. infestans* GIPs and tomato EGases are present in the apoplast and form stable complexes *in planta*. Structural modelling of GIP has revealed that they are in close proximity to rapidly evolving EGase residues, suggesting that the interaction between GIPs and EGases has the hallmarks of tight molecular co-evolution (Bishop et al. 2005; Damasceno et al. 2008). Also, secreted Kazal-like proteins, which work as protease inhibitors, have been found in five plant pathogenic oomycetes, including the downy mildew *Plasmopara halstedii* (Tian et al. 2004).

A number of oomycete effectors are small cysteine-rich proteins, such as elicitins, *PcF, PcF* like proteins from *P. cactorum* and *P. infestans* (Bos et al. 2003; Liu et al. 2005; Orsomando et al. 2001), and *PRAT*12, 14, 23, and 24 from *Hyaloperonospora parasitica* (Bittner-Eddy et al. 2003). They induce necrosis and may act as phytotoxin (Orsomando et al. 2001).

Nep1-like proteins and transglutaminases have also been identified as apoplastic effectors; both of them have the ability to trigger necrosis (Kamoun 2006). Cellulose-binding elicitor lectin (CBEL) is an oomycete-specific apoplastic effector. It was first isolated from *Phytophthora parasitica* var. *nicotianae* cell wall, and thus far 42 CBEL-like domains have been identified in 28 putative proteins from *P. sojae, P. infestans, P. ramorum, and P. parasitica*. These proteins are contributing to adhesion which is an important part of virulence (Gaulin et al. 2002), but they also elicit necrosis and defense gene expression in tobacco plant (Mateos et al. 1997; Torto-Alalibo et al. 2005).

1.3.1.4 Phytophthora transglutaminase

Phytophthora transglutaminases were among the first proteinaceous oomycete PAMPs to be identified. Although *Phytophthora* transglutaminases are structurally unrelated to transglutaminases from many other eukaryotes, bacteria, and archaea (Brunner et al. 2002; Makarova et al. 1999), they share the biochemichal characteristics of mammalian Ca^{2+} dependent transglutaminases. *P. sojae* transglutaminases were strictly dependent on Ca^{2+}, which could not be replaced by other ions like Mg^{2+} or Mn^{2+}. The transglutaminase inhibitors iodoacetamide, cystamine, N-ethylmaleimide and Cu^{2+}, can block this Ca^{2+} dependent transglutaminase activity efficiently, but GTP, the human tissue transglutaminase inhibitor, does not affect it significantly (Brunner et al. 2002). Brunner et al. (2002) also identified a Pep-13 motif with the sequence 'VWNQPVRGFKVYE', which is highly conserved among *Phytophthora* transglutaminases. Transcripts containing the Pep-13 encoding sequence were detected using RT PCR in most *Phytophthora* species except *P. undulata.* Detection of the peptide in the culture filtrate confirmed the results (Brunner et al. 2002). Two homologous enzymes were reported in *P. ramorum* cell walls (Meijer et al. 2006), and five members of the family were described in *P. infestans* (Fabritius & Judelson 2003). The Pep-13 motif seems to be important for both elicitor activity and transglutaminase activity. Treatment of potato cells with Pep-13 showed that Pep-13 can induce the accumulation of defense-related transcripts encoding lipoxygenase, 4-coumarate: CoA ligase and pathogenesis-related protein1. Increased transcript levels of the same genes were also detected in intact potato leaves upon infiltration of Pep-13 (Brunner et al. 2002).

1.3.2 Plant immune system

1.3.2.1 Plant innate immunity

The plant primary immune system has evolved to recognize microbe features and to translate this recognition into a defense response that is specifically directed against the pathogens encountered (Jones & Dangl 2006). Plant innate immunity can occur when one plant cultivar resists one or a few pathogenic strains, and also can work against several pathogenic species or several strains

(races, biotypes, and pathovars)(Kiraly et al. 2007). Gene for gene resistance is one of most thoroughly investigated types of plant immunity, and the current view of the mechanism can be included in the zig-zag model of the plant immune system. Here two other types of specific plant immunity are discussed: the plant resistance against pathogen toxins and gene silencing against virus infection. There are few results published about plant resistance against pathogen toxins. The corn gene HM1 was isolated by Johal and Briggs (1992), and it encodes a reductase that is able to inactivate the HC toxin of *Cochliobolus carbonum* (Johal & Briggs 1992; Meeley et al. 1992). In addition to detoxification, plants can also avoid toxins by lacking specific receptor and/or transporting the toxins out of plant cells (Balzi et al. 1994; Huang et al. 1990; Qin et al. 2004; Rhoads et al. 1995). Gene silencing is the mechanism that plant employ against viral infection. Foreign double-stranded RNA triggers ribonucleases that degrade it and also other RNA with the same sequence.

1.3.2.2 Plant acquired immunity

Plant acquired systemic immunity is analogous to animals' 'immune memory.' The surrounding tissues or remote parts of the plant can become immune to a subsequent infection after a primary infection. This kind of plant immunity used to be called systemic acquired resistance (SAR) and was first demonstrated by Ross (1961) in relation to tobacco infected by *tobacco mosaic virus* (TMV) (Ross 1961). Now it also has been found in rice against rice blast, tomato against *P. syringae* and acts nonspecifically and reduces the severity of disease caused by all classes of pathogen (Agrios 2005; Walters & Heil 2007). Systemic acquired resistance is triggered by hypersensitive necrosis (Durrant & Dong 2004). Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) are recognized as key regulators in the signaling pathways involved (Howe 2004; Lorenzo & Solano 2005; Pozo et al. 2004; van Loon et al. 2006; von Dahl & Baldwin 2007). Some other plant hormones have been implicated in plant defense, but their significance is less well studied (Koornneef & Pieterse 2008).Usually SA induces resistance to biotrophic pathogens, and JA and ET induce resistance against

necrotrophic pathogens. The plant protein NPR1 was shown to be necessary to transduce the SA signal for SAR (Glazebrook 2005).

1.3.2.3 Receptors

Plants lack an adaptive immune system and rely solely on innate immune mechanisms, which are activated by molecular surveillance that resides at the cell surface or within the cytoplasm. Membrane bound plant Pattern Recognition Receptors (PRRs) include receptor-like kinases (RLKs) (Shiu & Bleecker 2003), receptor-like proteins (RLPs) (Wang et al. 2008), and polygalacturonase inhibiting proteins (PGIP) (Di Matteo et al. 2003; Federici et al. 2006). Intracellular plant PRRs are NB-LRR proteins (nucleotide binding site–leucine-rich repeats) (Meyers et al. 2003). RLP-type receptors possess an extracellular LRR domain and a C-terminal membrane anchor but lack the cytoplasmic kinase domain. Lack of the cytoplasmic catalytic domain indicates that it relies on others to communicate the message. Some studies suggest that they may function in combination with RLK-type receptors (Shpak et al. 2005; Waites & Simon 2000). RLK-type receptors have an extracellular domain such as leucine rich repeats (LRRs), lectin, lysine motif (LysM) or wall associated kinases (WAK) with a single transmembrane-spanning region and a cytoplasmic kinase domain. They are considered primary communicators, although the diverse structures in the receptor domains suggest that there are likely to be several biological functions of these proteins (Tor et al. 2009). Once cytoplasmic signaling molecules receive the message from RLKs, they are distributed further within the cell via a canonical MAPK signaling cascade (Karlova et al. 2009; Russinovaa et al. 2004; Shiu & Bleecker 2003; Shpak et al. 2005; Tor et al. 2009; Trotochaud & Wassarman 2004; Waites & Simon 2000; Wang et al. 2008; Wang et al. 2005). These receptors are under the strict regulation of phosphorylation inhibitors which have only an extracellular LRR domain, phosphatases such as KAPP (kinase associated protein phospatase), endocytosis, ubiquitin-mediated protein degradation, and possibility of autophagy. Once the message is conveyed, they are down-regulated by some of the same mechanisms (Park et al. 2008; Robatzek et al. 2006; Todde et al. 2009; Tor et al. 2003; Trujillo et al. 2008; Wang

et al. 2006). NBS-LRR proteins (nucleotide binding site–leucine-rich repeats) are encoded by one of the largest plant gene families so-called disease resistance genes. Functions for several PRRs have been assigned for a number of plants including rice, tomato, and *Arabidopsis thaliana*. These proteins reside within the cytoplasm, but can be translocated into the nucleus, chloroplast or mitochondria (Shen & Schulze-Lefert 2007). Nearly all NBS-LRR proteins have been reported to function as disease resistance proteins, however recent research indicates they may also have diverse roles (Sweat et al. 2008). NBS-LRR proteins are strictly regulated by mechanisms including repression by the chromosomal structure, feedback amplification from the receptor protein, and repression by their negative regulators at the transcriptional level or ubiquitin-mediated degradation (Li et al. 2007; Tor et al. 2003; Tor et al. 2009).

1.3.2.4 Strawberry resistance genes

Strawberry is susceptible to many diseases, and in severe cases, crop loss approaches 100%. To reduce massive amounts of fungicide applications in strawberry production, it would be of great benefit to the grower, the consumer and the environment to develop lines that would effectively resist pathogen invasion. Resistance gene candidates have been identified, in addition to molecular markers, and these genes and markers may prove useful when developing new resistant varieties

In strawberry, resistance gene analogs (RGAs)that encode a nucleotide binding site (NBS) (Meyers et al. 1999) have been identified using PCR with degenerate primers which were targeted to conserved motifs within the NBS region (Martinez Zamora et al. 2004). Like other species studied, strawberry also maintains a family of TIR (Toll/Interlukin I Receptor) like RGAs, and these genes are present in both wild and cultivated species (Folta et al. 2005).

Several dominant loci that segregate with disease resistance have been identified. The resistance to the oomycete *P. fragariae* var. *fragariae* in strawberry is associated with a gene-for-gene pattern, and it has been estimated that there are at least five avirulence genes present in various European races of

the pathogen (Haymes et al. 1997). For resistance to *Phytophthora* root rot caused by *Phytophthora fragaria* var. *rubi* in the closely related diploid red raspberry (*Rubus ideaus*), a two-gene model with dominance has been suggested (Pattison et al. 2007).

No simple model for *P. cactorum* resistance in *Fragaria X ananassa* can be supported by recent findings. Shaw et al. (2006, 2008) indicated an additive, polygenically inherited resistance (Shaw et al. 2006; Shaw et al. 2008), and five putative quantitative trait loci for resistance were found in an experimental *Fragaria X ananassa* population (Denoyes-Rothan et al. 2004). Focusing on a simpler diploid model system than the octoploid strawberry, it appears more likely to get an understanding of the nature and inheritance of the resistance of *Phytophthora* crown rot.

1.3.3 'Zigzag' model in oomycete-plant interaction and effectors and R gene coevolution

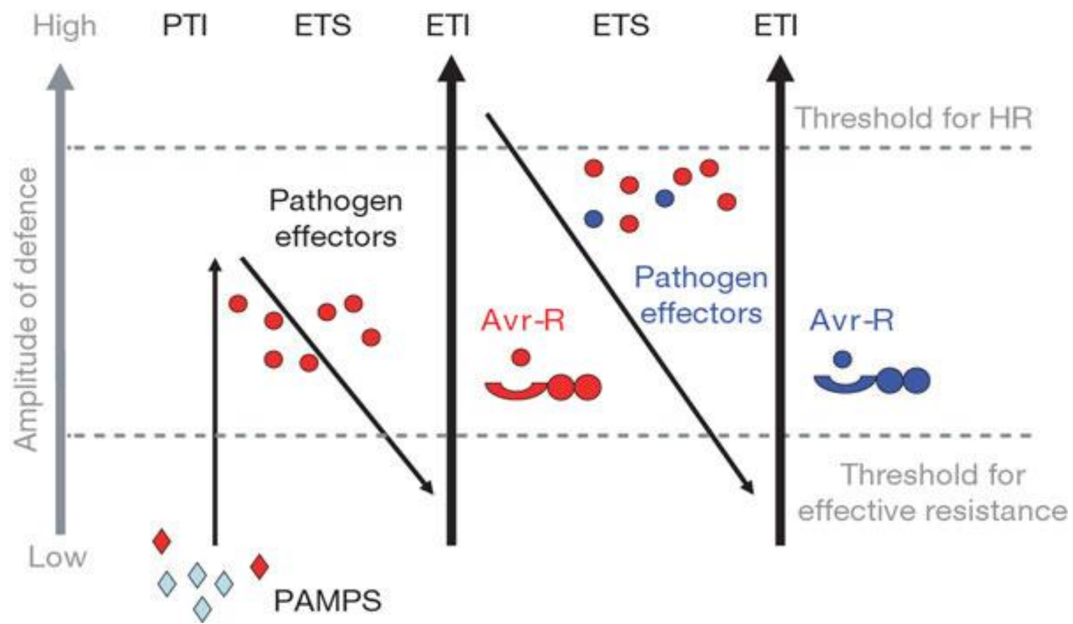
Jones and Dangl (2006) constructed a four phased 'zigzag' model to represent the current view of plant immune system against biotrophic or hemibiotrophic plant pathogenicity bacteria, fungi, and oomycetes. Plants face a constant barrage above and below ground from invading microorganisms, including bacteria, fungi, and oomycetes. The majority of plant species are resistant to invasion by all isolates of any given microbial species. However, a small percentage of plant–pathogen interactions lead to successful disease development. The first limitation of pathogen host range is thought to be the ability of a microbe to penetrate preformed barriers, such as the cuticle and cell wall, and to detoxify constitutively accumulating host antimicrobials, such as phytoanticipins, which vary between plant species (Ingle et al. 2006). The plant non-self surveillance system that perceives attempted invasions will be encountered when the pathogen that overcomes these obstructions and activates a diverse array of effective, broad-range defenses (Ingle et al. 2006; Zipfel et al. 2006). This plant non-self surveillance is also called PAMP-triggered immunity (PTI). Perception initially involves the detection of conserved molecules that are secreted or displayed on the surface of microorganisms. Pathogen-associated molecular

patterns (PAMPs) are molecules which can be described as small molecular motifs conserved within a class of microbes, associated with groups of pathogens that are recognized by cells of the innate immune system. For successful colonization of plant tissues, microorganisms must overcome PAMP-triggered immunity (PTI) that constitutes a front-line defense (Ingle et al. 2006; Schwessinger & Zipfel 2008). Both the apoplastic and cytoplasmic effectors can act as the secreted virulence determinants to suppress or otherwise manipulate plant innate immunity. This effector-triggered susceptibility (ETS) includes the suppression of PTI, representing the first level of molecular co-evolution between host and pathogen. It has also been shown to suppress immunity via direct molecular interactions with host defense-associated proteins (Block et al. 2008; Chisholm et al. 2006; Grant & Lamb 2006; Jones & Dangl 2006). The second line of defense of plants is resistance (R) proteins, which directly or indirectly detect effectors (termed avirulence proteins; AVRs). These confer effector-triggered immunity (ETI) to pathogens which are successful in suppressing PTI. Effector-triggered immunity (ETI) represents a second level of host–pathogen molecular co-evolution, as effectors evolve to evade detection and R proteins (NB-LRR proteins) evolve to establish or retain detection (Jones & Dangl 2006). For pathogens, natural selection drives the effector gene either by shedding or diversifying to avoid ETI, or by acquiring additional effectors to suppress ETI. For plants, natural selection results in new R specificities so that ETI can be triggered again. Microbial and plant co-evolution in response to ETI and ETS can be explained by the model which is similar to the boom and bust model (Jones & Dangl 2006). For example, in the flax/flax rust system, the pathogen carries an effector gene that is recognized by an R gene. This results in selection for an elevated frequency of R gene in the plant population. Pathogens with this mutated effector are then selected, because they can grow on R gene-containing plants and erode this R gene effectiveness. Also because at least some R genes have associated fitness costs (Tian et al. 2003), plants carrying R genes can have reduced fitness, resulting in reduced R gene frequencies. However, the pathogen population will still contain individuals with this effector. In the absence of R genes, this effector will confer increased fitness, increase its frequency in the pathogen population, and lead to resumption of selection for R

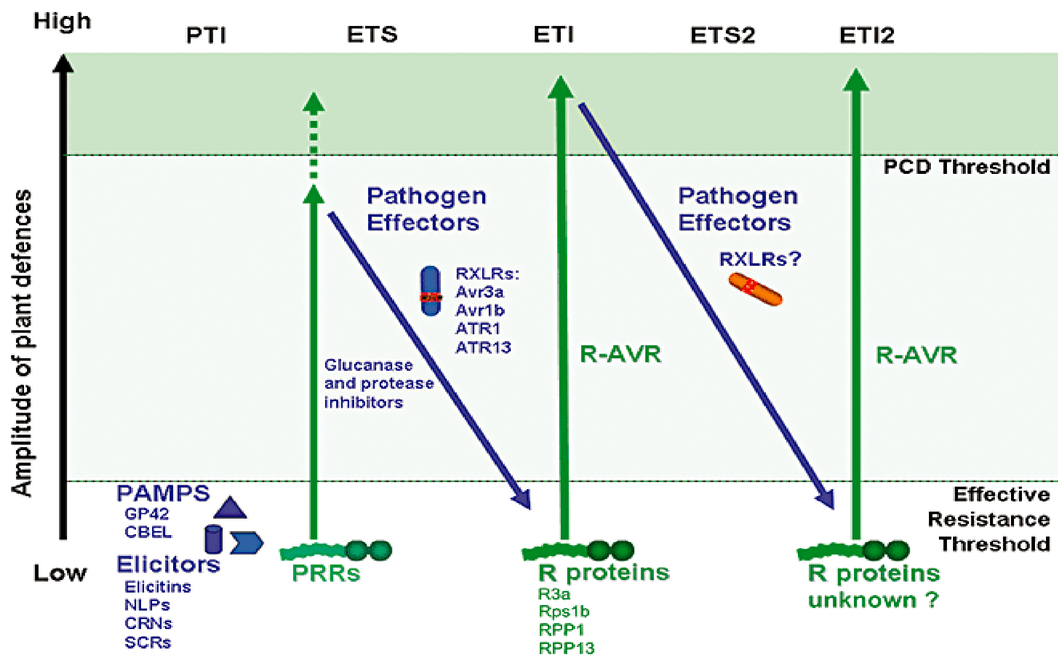
gene. This cycle is continuously turning, with many effectors and alleles at various R loci in play in populations of plants and pathogens (Jones & Dangl 2006). This paper will not discuss the small RNA-based plant immune system that is active against viruses or the active response of plants to herbivores (Jones & Dangl 2006).

Zigzag in oomycete–plant interactions was recently described by Hein *et al.* in fig1.3.3(b). The first phase is characterized oomycete pathogen-associated molecular patterns (PAMPs) and other elicitors triggering PAMP-triggered immunity (PTI). Some will cause necrosis [represented by a dotted arrow extending PTI beyond the threshold for host programmed cell death (PCD)]. The second phase is oomycete effectors, both some RxLRs and apoplastic effectors, which contribute to effector triggered susceptibility (ETS). The second phase also includes host resistance proteins, such as R3a, Rps1b, and RPPs that detect oomycete effectors to trigger immunity (ETI). The further phases of this model are still not clear. The amplitude of defense is shown on the *y* axis, and the threshold for activation of host PCD is also indicated. The role of T115B5 encoding protein *Phytophthora* transglutaminases are the PAMPs (GP42) which trigger the PAMP-triggered immunity (PTI). They induce program cell death in potatoes but not in parsley. (Hein et al. 2009)

(a)



(b)



(c)

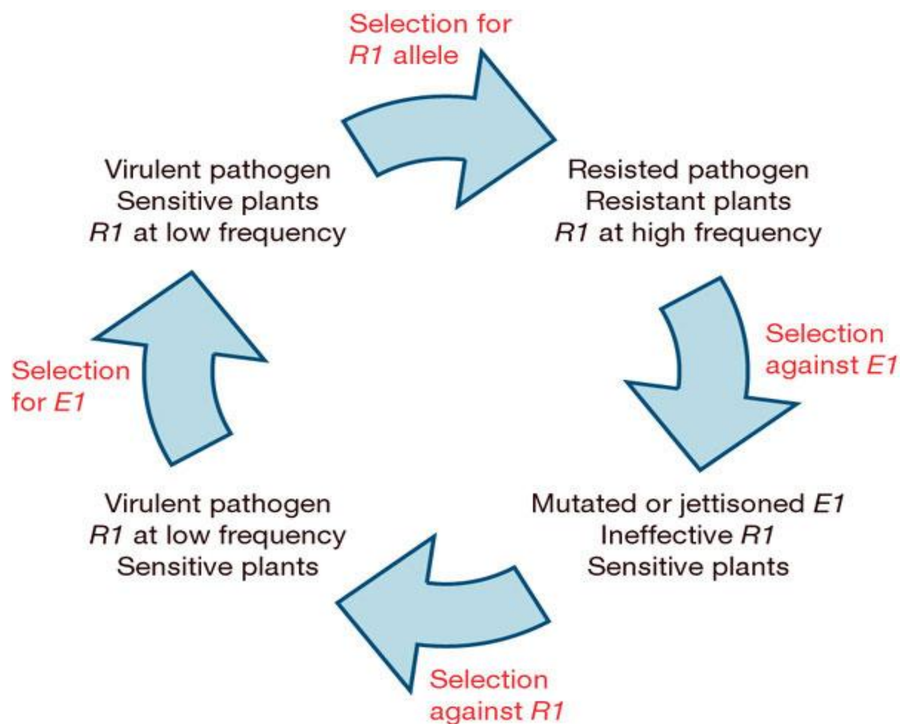


Figure 1.3.3 The zigzag model, it in oomycete–plant interactions and the model of effector R gene coevolution. (a)The ultimate amplitude of disease resistance or susceptibility is proportional to [PTI – ETS + ETI] in this scheme. one effector (indicated in red) is recognized by an NB-LRR protein, activating effector-triggered immunity (ETI), pathogen isolates lose the red effector, and perhaps gain new effectors through horizontal gene flow (in blue) to suppress ETI. New plant NB-LRR alleles that can recognize one of the newly acquired effectors are selected, resulting again in ETI(Jones & Dangl 2006). (b)This scheme shows zig-zag-zig in oomycete–plant interactions which is modified from Jones and Dangl (2006). In the first phase, some of PAMP-triggered immunity (PTI) will cause necrosis [represented by a dotted arrow extending PTI beyond the threshold for host programmed cell death (PCD)]. In the second phase, both some RxLRs and apoplastic effectors, contribute to effector triggered susceptibility (ETS). Host resistance proteins, such as R3a, Rps1b, and RPPs, detect oomycete effectors to trigger immunity (ETI). The further phases of this model are still not clear. The amplitude of defense is shown on the *y* axis, and the threshold for activation of host PCD is also indicated (Hein et al. 2009). (c)Here is the coevolution model based on the flax/flax rust system. This cycle is continuously turning in populations of plants and pathogens (Jones & Dangl 2006).

2 Materials and methods

2.1 Inoculum production

Phytophthora cactorum (Leb. and Cohn) J. Schröt (Bioforsk isolate ID 10300) was originally isolated from a diseased strawberry crown from a strawberry field in Ås, Norway.

Procedure:

1. Cultivated the *P. cactorum* in petri-dishes with V8 agar medium. Incubated them at room temperature (18 °C) around 10 days.

2. Cut the V8 agar medium into 6 equal pieces, and put 3 of them into a new empty plate. Filled the plate with the sterile pond water from UMB campus (Because this pond water is most close to the nature water during the development stages of the *P. cactorum*). Let the water just cover the V8 agar medium with the *P. cactorum* mycelium. Incubated them at room temperature around 3 days.

3. Afterward, checked the plates under microscope. The V8 agar medium was covered by a high density of sporangia. Transferred plates to a refrigerated room or fridge (4 °C). For sporangium RNA extraction, harvested directly without refrigeration: Filtrated through four layers of cheese cloth, collected in 50mL tubes, and centrifuged at 1500 *g* for 10 minutes. Discarded most of the supernatant, leaving around 1 mL liquid together with the pellet. Mixed remaining supernatant with pellet by pipetting, and processed for RNA extraction immediately.

4. Incubated at 4 °C for 0.5 hour. Checked the plates under the microscope again. Zoospores should be fully released and swimming in the water. Filtrated with four layers cheese cloth, and collect in 50mL tubes. For zoospore RNA extraction, centrifuged at 1500 *g* for 10 minutes, and discarded most of the supernatant. Left approximately 1 mL liquid with

the pellet and mix it by pipetting. Processed for RNA extraction immediately.

5. Shook the 50ml tube at 2500-3000 rpm, for 3 minutes, which enabled zoospores encysting.
6. Added 1 volume 10% V8 liquid in order to increase the speed of cyst geminating.
7. Harvested the samples at designated time points and centrifuge at 1500 *g* for 10 minutes. Discarded most of the supernatant, leaving around 1 mL liquid with the pellet. Mix it by pipetting, and processed for RNA extraction immediately.

2.2 Inoculation of strawberry crown

The wild diploid strawberry (*Fragaria vesca* L.) variety FDP821, which is susceptible to the isolate 10300 (Eikemo et al. 2010), was maintained in a greenhouse as Eikemo et al. (2000) described: (Eikemo et al. 2000)

Procedure:

1. Produced zoospores as describe in '2.1 inoculum production.
2. At the same time, strawberry plants should be prepared.
3. Injured the strawberry crowns with sterile knives until the vessels can be seen. Covered the crown with soil again.
4. Pipetted 10mL water containing zoospores into the soil surrounding the injuried strawberry crown. One treatment included 4 plants. 3 plants received inoculations, and 1 plant was without inoculation as the control.
5. After 6 hours, 12 hours, 1 day, 3 days, 5 days, and 7days, dissected out (around 100mg) of the infected plant tissues showing symptoms. Wrapped the tissue in aluminum foil, and put it in to liquid nitrogen

immediately, later took it out from liquid nitrogen and store at -80 °C. Cut out tissue from the non-inoculated control plants near the injuries. Collected them separately from the infected sample. At the end mixed all non-infected samples together for RNA extraction.

2.3 Full length cDNA cloning by Rapid Amplification of cDNA Ends (RACE PCR)

2.3.1Introduction and overview

RACE or Rapid Amplification of cDNA Ends, is a technique used to obtain the full length sequence of an RNA transcript found within a cell. Here, the SMART™ RACE cDNA Amplification Kit was used to clone the full length cDNA of potentially interesting cDNA fragments isolated by SSH library and effector differencial diaplay This kit contains SMART II™ oligonucleotide and the Moloney Murine Leukemia Virus everse Transcriptase (MMLV RT). The MMLV RT, upon reaching the end of an RNA template, exhibits terminal transferase activity, adding 3-5 residues (predominantly dC) to the 3' end of the first strand cDNA. The SMART oligo with a terminal stretch of G residues anneal to the dC-rich cDNA tail and serves as an extended template for the reverse transcriptase. MMLV RT switch templates from the mRNA molecule to the SMART oligo, generating complete cDNA copies of the original RNA with the additional SMART sequence at the end (Clontech)

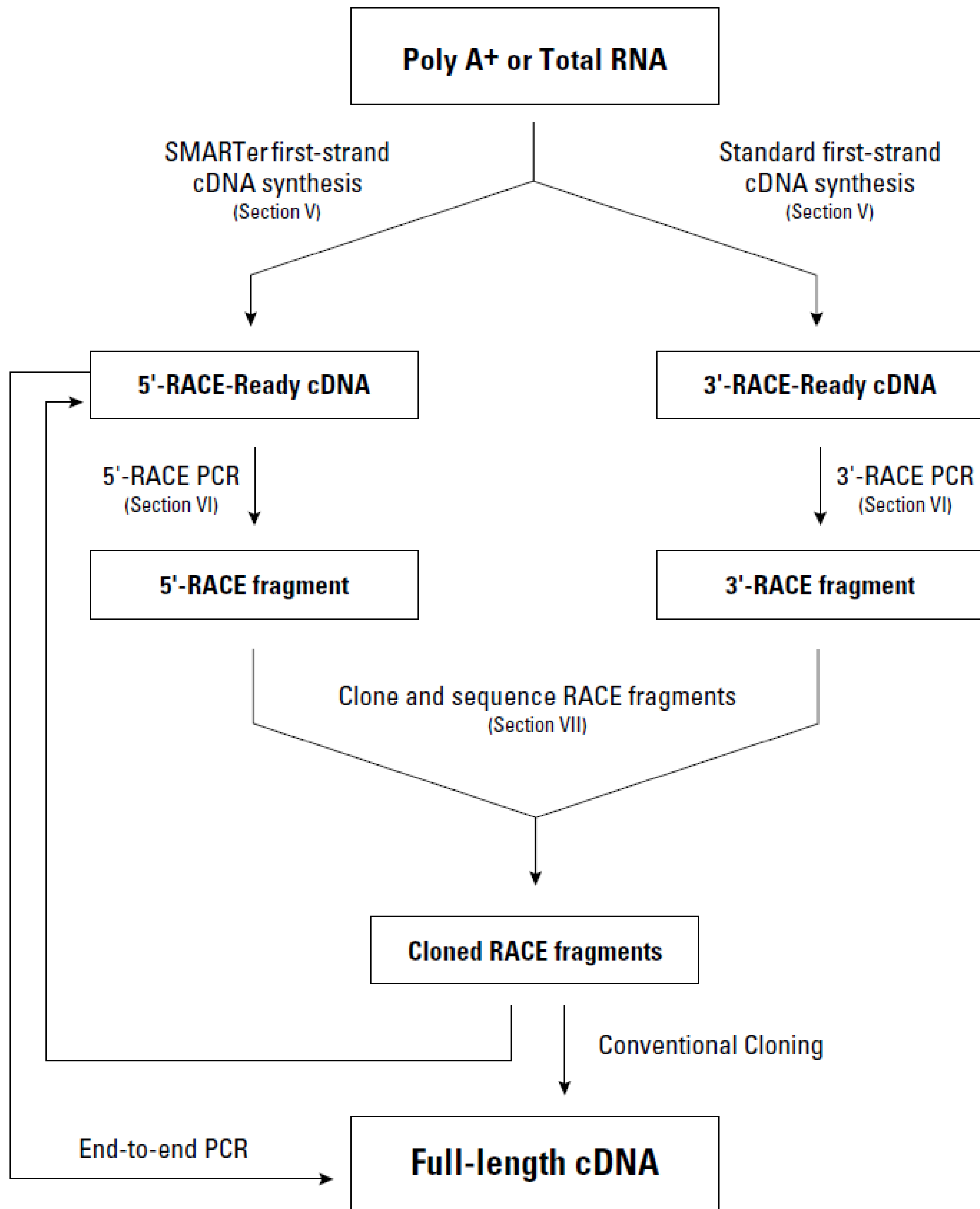


Figure 2.3.1 This figure shows procedure of RACE cloning, involving sequencing the 5' end of the 5' product and the 3' end of the 3' product to obtain the sequences of the extreme ends of the transcript (Clontech).

2.3.2 RNA manipulation

2.3.2.1 RNA extraction

Total RNA was isolated by using the QIAGEN RNeasy Mini Kit. The RNeasy method represents a well-established technology for RNA purification. This technology is based on the selective binding properties of a silica-based membrane with the microspin technology. The RNeasy silica membrane can bind up to 100 µg of RNA longer than 200 bases with help of a specialized high-salt buffer system. Biological samples are first lysed and homogenized in the presence of a highly denaturing guanidine-thiocyanate-containing buffer, which immediately inactivates RNases to ensure purification of intact RNA. Ethanol is added to provide appropriate binding conditions. The total RNA binds to the membrane and contaminants are efficiently washed away. High-quality RNA is then eluted in RNase-free water (QIAGEN)

Materials:

QIAGEN RNeasy Mini Kit

Liquid nitrogen

Samples collected from 2.1 and 2.2

Procedure:

1. Added 4.5 µL beta-Mercaptoethanol into 445µL buffer RLC and transfer into microfuge tubes.
2. (a)For samples from mycelium of *P. cactorum*, infected and non-infected plants, placed the weighed sample immediately into liquid nitrogen, and ground thoroughly with a mortar and pestle.

   (b) For liquid samples of sporangia, zoospores, and different time points of geminated cysts of *P. cactorum*, added the solution into liquid nitrogen using a pipette, and ground thoroughly with a mortar and pestle.

3. Transfered the lysate to a QIAshredder spin column (lilac) placed in 2ml collection tubes. Centrifuged for 2 min at 13000 g. Carefully transferred the supernatant of the flow-through to a new microfuge tube without disturbing the cell-debris pellet in the collection tube. Used only this supernatant in subsequent steps.

4. Added 0.5 volume of ethanol (96-100%) to the cleared lysate, and mixed immediately by pipetting. Proceeded immediately to step 5.

5. Transferred all the sample in one tube to an RNeasy spin column (pink) placed in a 2 ml collection tube. Closed the lid gently, and centrifuge 1 min at full speed. Discarded the flow through.

6. Added 700 µL buffer RW1, centrifuged for 1 min at full speed to wash the spin column membrane, and discarded the flow through.

7. Added 500µL buffer RPE twice, centrifuged for 1 min and then 2 min, at full speed, and discarded the flow through.

8. Placed the RNeasy spin column into a new 2 mL collection tube, and centrifuged at full speed for 1 min.

9. Placed the RNeasy spin column into a new 1.5mL collection tube; added 30µL RNase-free water directly to the spin column membrane. Closed the lid gently, and centrifuged at full speed for 1 min. Pipette the eluted solution to the membrane again and repeated the centrifuge to enhance the RNA concentration.

10. Store RNA at -80 °C.

2.3.2.2 DNase treatment of RNA samples, genomic DNA check by SYBR Green and RNA cleanup

TURBO DNase was applied to remove contaminating DNA from RNA preparations, and QIAGEN RNeasy mini kit was used to subsequently remove the DNase and divalent cations from the sample. The result of DNase digestion was tested by Real Time PCR.

Material:

TURBO DNase Kit

QIAGEN RNeasy mini kit

RNA samples

Procedure:

1. DNase digestion:

Added DNase digestion reagents: 5μL 10x buffer, 1μL TURBO DNase; incubated at 37°C for 25 minutes.

2. QIAGEN RNA clean up method:

(a)Adjusted the sample to a volume 100μL with RNase free water, added 350μL buffer RLT, and mixed well.

(b)Added 250μL ethanol (96-100%) and mixed well by pipetting.

(c)Transferred the sample to RNeasy mini spin column, centrifuged for 1 min at 13000 *g*, and discarded the flow through.

(d)Added 500μL buffer RPE, washed twice, centrifuged for 1 min and then 2 min, at 13000 *g*, and discarded the flow through.

(e)Placed the RNeasy spin column into a new 2mL collection tube, centrifuged at 13000 *g* for 1 min.

(f)Placed the RNeasy spin column into a new 1.5mL collection tube; added 30μL RNase-free water directly to the spin column membrane. Closed the lid gently, and centrifuged at full speed for 1 min. Pipette the eluted solution to the membrane again and repeated the centrifugation to enhance the RNA concentration.

(g)Stored RNA at -80 °C.

3. Real-time PCR was used to test the result of DNase digestion (the detail of Real-time PCR will be introduced in section 3.4).

(a)Real-time PCR was performed using DNase treated RNA samples as templates, together with undigested RNA samples, genomic DNA and water as controls. The reactions were performed as that described in section 3.4.

(b)Comparing with the controls, PCR products should not be detected from the samples, or the Ct values should be significantly higher than the controls.

2.3.2.3 Quantification and determination of quality of RNA

Before subsequent application, the quality of the RNA samples must be tested to make sure that they are intact. Their concentration is acceptable if the RNA concentration is higher than 20 ng/μL after purification, based on the requirement of RACE-Ready first-strand cDNA synthesis.

2.3.2.3.1 Agarose gel electrophoresis

The integrity and concentration of total RNA can be visually assessed by agarose gel electrophoresis. The ratio of 28S: 18S for eukaryotic RNA should be approximately 2: 1.

Agarose gel electrophoresis is a technique widely used for the separation of DNA and RNA of different molecule sizes, using an electric field applied to a gel matrix. Agarose is a linear polymer composed of long unbranched chains of uncharged carbohydrates without cross links. This results in a gel with large pores, allowing for the separation of macromolecules and macromolecular complexes, like DNA or RNA mix with loading buffer. DNA or RNA with different molecular weight will move at different speeds by electromotive force. Ethidium bromide, usually abbreviated as EtBr, is the most common dye used to make DNA or RNA bands visible for agarose gel electrophoresis. By running RNA (or DNA) through an EtBr-treated gel and visualizing it with UV light, any band containing more than 20 ng RNA (or DNA) becomes distinctly visible, because EtBr fluoresces under

UV light when intercalated into RNA (or DNA). This method is also frequently used to test PCR products, plasmids and restriction enzyme digestion.

Materials:

1X TBE buffer

6X loading buffer

Agarose

Ethidium bromide

DNA or RNA samples

Procedure:

Agarose was weighed and mixed with 1 X TBE buffer. The mixture was heated in a microwave-oven until the agarose was completely dissolved. The solution was cooled down to around 60°C. One drop (~5 μl) of ethidium bromide was added into every 50 mL agarose solution (final EtBr concentration was 0.5 μg /mL). The gel solution was subsequently transferred to a gel tray, with a well-comb in one end, for solidification. The solidified gel was transferred into an electrophoresis chamber with enough buffer TBE to cover the gel.

RNA (or DNA) samples were mixed with 0.2 volumes 6 X loading buffer and then pipetted into the wells of the gel. The electrophoresis was run at 60-90 V until the target fragments were sufficiently separated. The results were visualized by UV light and recorded by a computer with Quantity One system.

2.3.2.3.2 Spectrophotometric quantification of RNA

The concentration of RNA should be determined by measuring the absorbance at 260 nm (A260) by Pharmcia Biotech Gene Quant II RNA/DNA Calculator. 2 μL RNA was diluted by adding 100 μL 10 mM Tris Cl buffer (pH=7.0). The spectrophotometer should be zeroed using buffer before the measurement of samples. Each sample should be measured three times and the cuvette should be

rinsed with buffer between each sample. The concentration of RNA can be calculated by the formula: concentration=A260 X 44 μg /mL X dilution factors and it can be read on the machine directly.

2.3.2.3.3 Agilent 2100 Bioanalyzer

Agilent 2100 Bioanalyzer is an automated system based on microfluidic technology, which provides an accurate estimate of RNA quality and concentration.

Material:

RNA 6000 Nano Kit

RNA samples

Procedure:

Strictly follow the protocol

2.3.3 First-strand cDNA synthesis

The protocol for cDNA synthesis for 5'RACE and 3'RACE is different. The 5'-RACE cDNA is synthesized using a modified lock-docking oligo(dT) primer and the SMART II A oligo as described above. The 5'-RACE CDS Primer A (5'-CDS), has two degenerate nucleotide positions at the 3' end. These nucleotides position the primer at the start of the poly A+ tail and thus eliminate RACE-Ready the 3' heterogeneity inherent with conventional oligo(dT) priming (Borson et al., 1994).

The 3’-RACE cDNA is synthesized using a traditional reverse transcription procedure, but with a special oligo (dT) primer. This 3’-RACE CDS Primer A (3’-CDS) primer includes the lock-docking nucleotide positions as in the 5’-CDS primer and also has a portion of the SMART sequence at its 5’ end. By incorporating the SMARTer sequence into both the 5’- and 3’-RACE-Ready cDNA populations, the Universal Primer A Mix (UPM) can be used to prime both RACE PCR reactions. UPM recognizes the SMARTer sequence, in conjunction with distinct gene-specific primers.

Procedure:

1. For 5'RACE-Ready cDNA, added 3 μL RNA sample, 1 μL primer A, 1 μL SMART II A oligo, into a PCR tube; and for 3'RACE-Ready cDNA, added 3μL RNA sample, 1uL CDS primer A, 1 μL water in another PCR tube.

2. Mixed contents and spin the tube briefly. Incubated the tubes at 70 °C for 2min.

3. Cooled down the tubes on ice for 2 min.

4. Span the tubes briefly to collect the contents at the bottom.

5. Added 2μL 5X-First-Strand Buffer, 1μL DDT, dNTP Mix(10mM) and MMLV Reverse Transcriptase. Mixed the contents by gently pipetting.

6. Span the tubes briefly to collect the contents at the bottom.

7. Incubated the tubes at 42 °C for 1.5 hours followed by 72 degrees for 7 min.

8. Diluted the first-strand reaction product with 20μL Tricine EDTA Buffer, and stored at tubes at -80 °C.

2.3.3.1 Check the synthesis of cDNA

To confirm whether the cDNAs are intact, perform PCR using tubulin primers with the cDNA samples and controls.

Material:

cDNA templates and genomic DNA templates

tubulin primers reverse and forward

Taq DNA polymerase

dNTP mix

dH2O

5X PCR buffer

Procedure:

1. Mixed each 2μL cDNA sample with 23μL master mix, and span the tubes briefly to collect the contents at the bottom. The samples were amplified using the following program: an initial step at 94 °C for 5min, followed by 32 cycles of 95 °C for 15 seconds, 60 °C for 30 seconds, 72 °C for 1 minute, and finally 72 °C for 5 minutes. Cooled down the samples to 4 °C. Controls with water and genomic DNA were included in the experiment.
2. The PCR products were run on a 1% agarose gel. One clear band whose size is around 400bp should be seen if the samples are intact. The negative and positive controls were water and genomic DNA as the template. The same band should be seen on the positive control but not on the negative control.

2.3.4 Rapid Amplification of cDNA Ends (RACE PCR)

2.3.4.1 The polymerase chain reaction (PCR)

The polymerase chain reaction (PCR), developed by Kary Mullis, is a common and often indispensable technique to amplify a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. The PCR method usually consists of a series of 20-40 repeated temperature changes called cycles. Thermal cycling consists of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. The key components to enable selective and repeated amplification are primers which contain sequences complementary to the target region along with a DNA polymerase. Almost all PCR applications have to employ a heat-stable DNA polymerase, such as the enzyme originally isolated from the bacterium *Thermus aquaticus* called *Taq* polymerase. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified.

PCR cycling usually begins with a denaturation step, heating the reaction to 94–98 °C for 20–30 seconds. (An initialization step heating the reaction to a temperature of 94–96 °C, which is held for 1–9 minutes is required by hot-start PCR before the denaturation) It causes DNA melting of the DNA template by disrupting the hydrogen bonds between complementary bases, yielding single strands of DNA. This is followed by a switch to the annealing step. The reaction temperature is lowered about 3-5 degrees Celsius below the Tm of the primers used, to allow annealing of the primers to the single-stranded DNA template. Afterwards, the extension step is performed. The temperature at this step depends on the DNA polymerase used. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. After several cycles, a single final elongation step is required, which is occasionally performed at a temperature of 70–74 °C for 5–15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended. At the end of the reaction is the final hold. Holding at 4°C for an indefinite time may be employed for short-term storage of the reaction products.

Today a wide array of extensively modified PCRs is used to perform a variety of genetic manipulations.

2.3.4.2 Primer design

Primer design is an important factor of RACE cloning. For the complete SMARTer RACE protocol, at least two gene specific primers are required, which can create around 100-200 bp overlapping 5'- and 3'- RACE. The gene specific primers should have a GC content of 50-70% and a Tm of at least 65 degrees. Longer primers with annealing temperature above 70°C give more specific results based on using 'touchdown PCR'. Self-complementary primer sequences must be avoided. All the primers are designed and picked up by the online software Primer III.

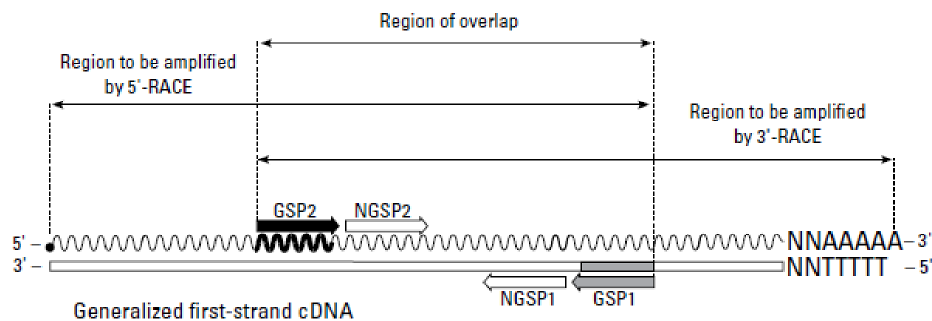


Figure 2.3.4.2 Diagram of first-strand cDNA template with theoretical location of gene-specific primers designed to produce overlapping RACE products. This overlap also permits the use of the primers together in a control PCR reaction. Additionally, it enables the construction of full-length cDNA (Clontech).

2.3.4.3 Touchdown PCR and Nested PCR

In order to increase the specificity, the RACE PCR program usually is a touchdown PCR program. Touchdown PCR is a method to avoid amplification of nonspecific sequences. The specificity of annealing is determined by the temperature at which primers anneal during a cycle of PCR. Temperatures just

below the melting point of the primer set the upper limit on annealing temperature, and only very specific base pairing between the primer and the template will occur. The primers bind less specifically at lower temperatures. Nonspecific primer binding will obscure RACE PCR results, causing multiple bands during amplification.

The earliest steps of a touchdown PCR cycle have high annealing temperatures, which will be decreased in increments for every subsequent set of cycles. The primer will anneal at the highest temperature which is least-permissive of nonspecific binding. Thus, the first sequence amplified is most likely the one of interest because of greatest primer specificity. These fragments will be further amplified during subsequent rounds at lower temperatures, and compete with the nonspecific sequences where the primers may bind at lower temperatures. If the primer initially binds to the sequence of interest, subsequent rounds of PCR can further amplify this product.

Nested PCR is another modification of intended to reduce the contamination in products due to the amplification of unexpected primer binding sites. It involves two sets of primers, used in two successive runs of PCR, the second set intended to amplify a secondary target within the first run product. During RACE amplification, sometimes multiple bands appears in the result, to reduce the untargeted bands, this nested PCR was employed to enhance the specificity.

Material:

3´RACE cDNA and 5´RACE cDNA (the cDNA templates are from 5 days after infection, mycelium and germinating cysts of *P. cactorum*. Here, germinating cysts were collected when the average length of germ tubes of 80% of the germinating cysts was 2 times longer than the average diameter of cysts.)

GSP reverse and GSP forward (GSPs for RACE amplification for each gene were in the Appendix 2, sometimes the GSP for 5´RACE cannot amplified the expected fragment, new GSP will be designed)

UPM

PCR-grade water

50X Advantage II polymerases mix

10X Advantage PCR buffer

dNTP mix

Procedure:

1. For RACE PCR:

Reaction for 3’-RACE (5´-RACE) cloning:

	1(sample1) (µL)	2(sample2) (µL)	3(gsp1+gsp2) (µL)	4(UPM) (µL)	5(gsp1) (µL)	6(gsp2) (µL)
3’-RACE (5´RACE)-Ready cDNA	2	2	2	2	2	2
UPM(10X)	2.5	2.5	0	2.5	0	0
GSP1(10µM)	0.5		0.5		0.5	
GSP2(10µM)		0.5	0.5			0.5
H_2O			2	0.5	2.5	2.5
Master Mix	20	20	20	20	20	20
Final volume	25	25	25	25	25	25

Master Mix:

PCR-grade water	15µL
50X Advantage II polymerases mix	0.5 µL
10X Advantage II PCR buffer	2.5 µL
2.5 mM dNTP mix	2 µL
Total volume	20 µL

PCR program:

5 cycles:

94°C 30sec

72°C 3 min

5 cycles:

94°C 30sec

70°C 30sec

72°C 3 min

25 cycles:

94°C 30sec

68°C 30sec

72°C 3 min

2. For nested PCR:

Templates diluted from RACE PCR products (usually 50X dilution), Nested Universal Primer A (NUP) and GSP were used as 2 primers. The reaction was done as normal PCR.

2.3.5 Gel purification of RACE products

The single strong band visible on the gel was cut and purified. PureLink™ Check Gel Extraction Kits were used for purification of DNA fragments from agarose gels. The kits were designed to rapidly and efficiently purify 40bp-10kb DNA fragments from TAE and TBE agarose gels of various percentages using cartridges without the use of organic solvents. The purified DNA fragments could be applied in various downstream experiments, including sequencing, PCR, *in vitro* transcription, restriction enzyme digestion, cloning, and labeling (PureLink™ Check Gel Extraction Kit User Manual, Version C).

Material:

PureLink™ Check Gel Extraction Kit

Gel with DNA fragments

Procedure:

1. Used a clean, sharp blade to cut the target band from the gel. Minimized the amount of surrounding agarose around the band.
2. Weighed the gel slice.
3. For <2% agarose gels, placed up to 400 mg gel into one tube. Added 30 µL Gel Solubilization Buffer (GS1) for 10mg of gel.
4. Incubated the tube at 50 °C for 15 minutes. Mixed every 3 min. After gel slice appears dissolved, incubated for additional 5 minutes.

5. Preheated an aliquot of TE buffer to 70 °C.

6. Placed a Check Gel Extraction Column into a Wash Tube. Pipette the mixture from step 4 onto the column, centrifuged at 13000 *g* for 1 minute. Discarded the flow-through.

7. Added 500 µL GS1 buffer, incubated at room temperature for 1 minute, centrifuged at full speed for 1 minute.

8. Added 700 µL Wash Buffer (W9) with ethanol to the column and incubated at room temperature for 5 minutes. Centrifuged at 13000 *g* for 1 minute. Discarded the flow-through.

9. Centrifuged the column at 13000 *g* for 1 minute to remove any residual buffer. Placed the column into a 1.5 ml Tube.

10. Added 25 µL warm TE buffer to the center of the cartridge. Incubated at room temperature for 1 minute. Centrifuged at full speed for 2 minutes.

11. Stored the DNA at -20 °C.

12. The isolated DNA products should run on 1% agarose gels, to confirm that the single and correct size of fragment was extracted.

2.3.6 TOPO TA Cloning

The DNA fragments obtained from RACE PCR and purified by gel extraction were cloned using the TOPO-TA cloning system (Invitrogen), after which the fragment can be further characterized by sequencing.

TOPO TA Cloning system provides a highly efficient, fast, one-step cloning strategy for the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector, without ligase, post-PCR procedures, or requirement of PCR primers containing specific sequences.

The *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine (A) to the 3′ ends of PCR products，and the linearized vector has single，overhanging 3′-deoxythymidine (T) residues. This

allows PCR inserts to ligate efficiently with the vector. Topoisomerase I from *Vaccinia virus* binds to duplex DNA at specific sites and cleaves the phosphodiester backbone after 5′ -CCCTT in one strand (Shuman 1991). The energy from the broken phosphodiester backbone is conserved by formation of a covalent bond between the 3′-phosphate of the cleaved strand and a tyrosyl residue of topoisomerase I. The 5′-hydroxyl of the original cleaved strand attack the phosphotyrosyl bond between the DNA and enzyme, reversing the reaction and releasing topoisomerase (Shuman 1994).

Materials:

Invitrogen TOPO TA Cloning kits

LB agarose medium with ampicillin (50 μg /mL)

X-gal

Procedure:

The reactions were conducted following the Invitrogen protocol, and all the reagents were using half amount.

2.3.7 Transformation

Transformation is the genetic alteration of a cell resulting from the uptake, genomic incorporation, and expression of environmental genetic material. Here, it is necessary to insert the information limited RACE fragments into certain vectors for sequencing. Transfer them into *Escherichia. coli* is one way to select the identical inserted vector for sequencing reaction. Both electroporation and heat shock method are used for transformation here.

2.3.7.1 Electroporation

Electroporation is using an externally applied electrical field to significantly increase the electrical conductivity and permeability of the cell plasma membrane. It is often used for the transformation of bacteria, yeast, and plant protoplasts. If bacteria and plasmids are mixed together, the plasmids can be transferred into the cell during electroporation. Bacteria also have cell walls which are made of peptidoglycan and its derivatives in addition to the lipid membranes, but the walls are naturally porous. After electroporation, the cells have to be handled carefully until they have had a chance to divide, producing new cells that contain plasmids.

Material:

Vectors carry the inserted DNA

E. coli DH5α

LB medium

10% glycerol

LB agarose medium with ampicillin (50 µg /mL)

bromo-chloro-indolyl-galactopyranoside

Procedure:

1. Inoculated *E. coli* using a single colony into 2mL LB medium, incubated at 37 °C overnight, shaking at 200 rpm.
2. Transferred the culture into 200 mL LB medium, incubated at 37 °C. Checked the cell density; harvested the cells at 0.6 OD_{600}.
3. Transferred the culture into 50mL tubes, chilled on ice 10 minutes, and pellet the culture at 4000 *g*, 4 °C, for 15 minutes.

4. Re-suspended cell pellet in 1 volume sterile cold 10% glycerol, centrifuged at 4000 *g*, 4°C, for 15 minutes.
5. Repeated Step 4 four times.
6. Re-suspended pellet in 0.002 volume sterile cold 10% glycerol.
7. Chilled 1mm gap elecroporation cuvette on ice.
8. Added 1 μL DNA into 50 μL *E. coli* cells from above steps and mixed gently. Then transferred the mixture into cuvette on ice.
9. Transferred the cuvette into the electroporation machine. Gave a pulse using the following settings: 1.9kV, 25uF, and 200 ohms.
10. Immediately after the pulse was finished, added 950 μL LB. Briefly and gently pipette up and down to mix thoroughly.
11. Transferred the cells into 2mL microcentrifuge tube, incubated at 37 °C, shaking at 200 rpm (at the beginning 10 minutes, shaking at 100 rpm), for 1 hour.
12. Centrifuged the culture at 10000 rpm for 30 seconds, kept 100 μL liquid medium with the pellet, spread on the LB agarose medium with ampicillin and Xgal.
13. Incubated at 37 °C overnight.

2.3.7.2 Chemical transformation

Chemical transformation method is also called heat shock method. Chemical competence is conferred to *E. coli* by re-suspension from the LB liquid medium in $CaCl_2$ solution at 0°C. Under these conditions, the Ca^{2+} ion is thought to create pores in the membrane, assist binding of the DNA to the cell membrane, and mask the negative charge on the DNA, easing its passage through the hydrophobic cell membrane. The DNA is forced into the cells by applying a short 42°C heat shock, which results in a thermal current that sweeps the DNA into the cells. This method is less efficient compared to electroporation in our practice.

Material:

Vectors carry the inserted DNA

E. coli DH5α^{TM}-T1^{R}

LB medium

10% glycerol

LB agarose medium with ampicillin

Xgal

Procedure:

1. Grown and harvested *E. coli* as for preparation of electroporation competent cells.
2. Transferred the suspension into 50mL tubes, chilled on ice 10 minutes, and pelleted the culture at 2500rpm, 4 °C, for 10 minutes.
3. Discarded the supernatant as clean as possible, add 0.05 mol/L cold $CaCl_2$. Re-suspended the cells, chill on ice 30 minutes, and centrifuged at 3000rpm, 4 °C, for 10 minutes.
4. Discarded the liquid. Re-suspended the cells with 0.05 mol/L cold $CaCl_2$ containing 15% glycerol on ice.
5. The following steps were performed as described for the TOPO TA cloning protocol.

2.3.7.3 Blue-white screening

The blue-white screen allows easy detection of successful ligations into the vector. The ligation mix is transformed into competent cells (bacteria) and plated

on growth medium containing X-gal. If the ligation was successful (containg insert), the bacterial colony will be white; if not, and the colony will be blue.

The molecular mechanism for blue-white screening is based on genetic engineering of the lac operon in the *E. coli* strain serving as the host cell, combined with a subunit complementation achieved with the cloning vector. The vector encodes α subunit of the LacZ protein with an internal multiple cloning site (MCS), while the chromosome of the host strain encodes the remaining Ω subunit to form a functional β-galactosidase enzyme. The foreign DNA can be inserted within the lacZα gene to disrupt the production of functional β-galactosidase. X-gal, a colourless modified galactose sugar, is metabolized by β-galactosidase to form an insoluble product (5-bromo-4 chloroindole) which is bright blue, and thus functions as an indicator. Isopropyl β-D-1-thiogalactopyranoside (IPTG), which functions as the inducer of the Lac operon, can be used in some strains to enhance the phenotype. The hydrolysis of colorless X-gal by β-galactosidase causes the characteristic blue color in the colonies; it shows that the colonies contain vectors without inserts. White colonies indicate loss of the cells' ability to hydrolyze X-gal and hence insertion of foreign DNA into the vector. However, a bacterial colony with no vector at all will also appear white in general. These are usually suppressed by the presence of an antibiotic in the growth medium. A resistance gene on the vector allows successfully transformed bacteria to survive despite the presence of the antibiotic.

2.3.7.4 Analyzing transfomants

After the blue-white screening, the transformants should be further analyzed, to confirm that the vector really contains inserted fragments. Two methods are applied: PCR analysis and restriction analysis. PCR analysis is applied initially, because it is an easy check. In one special case, when the result of PCR analysis was not as expected, the restriction analysis is done in parallel for avoiding the artifacts during PCR amplification because of mispriming or contaminated template.

2.3.7.4.1 PCR analysis of transformants

It is fast to analyze transformants using PCR. For PCR primer, M13 reverse and forward were used; for PCR templates, 10 colonies from the same transformation, were picked up and re-suspended individually into separate tunes containing PCR mix. Before PCR, incubate the reaction for 10 minutes at 94 °C to lyse the cells and inactivate nucleases. The PCR programs were same as common PCR.

2.3.7.4.2 Restriction analysis of transformants

When using PCR to analyze transformants, artifacts may be obtained because of mispriming or contaminating template.

Material:

*Eco*RI

NEBuffer 10X

QIAprep Spin Miniprep kit

dH_2O

Transformants selected by blue white screen (single white colonies)

LB liquid medium with 50 µg /mL ampicillin

Procedure:

1. Transferred colonies from the plate in storage (4 °C), to a new plate, and cultured them in 37 °C overnight.
2. Inoculated 3 mL prewarmed LB medium containing 50 µg /mL ampicillin with one single colony (37 °C, 220 rpm, 4 hours).

3. Extracted plasmid DNA using QIAprep Spin Miniprep kit.

4. Added 1 μL plasmid, 1 μL 10X buffer, 0.5 μL *Eco*RI and 7.5 μL dH_2O. Incubated at 37 °C for 2 hours.

5. Checked the result on the 1% agarose gel.

2.3.8 Plasmid isolation

Plasmids are considered transferable genetic elements, or "replicons", capable of autonomous replication within a suitable host. They are used as vectors in the vast majority of molecular cloning.

Material:

QIAprep Spin Miniprep Kit

Procedure:

Strictly follow the QIAprep Spin Miniprep Kit protocol.

2.3.9 DNA sequencing

Both plasmid and PCR products can be used for sequencing. The samples are diluted into required concentration according their protocols. The PCR products should also be purified before sequencing.

Dye-terminator sequencing permits sequencing in a single reaction by utilizing labeling of the chain terminator ddNTPs with the use of a modified DNA polymerase enzyme system and dyes that minimize incorporation variability, as well as methods for eliminating "dye blobs". In dye-terminator sequencing, each of the four dideoxynucleotide chain terminators is labeled with fluorescent dyes, each of which has different of fluorescence and emission. Owing to its greater expediency and speed, dye-terminator sequencing along with automated high-

throughput DNA sequence analyzers is now one of the main methods in automated sequencing and is being used for the vast majority of sequencing projects.

Materials:

Big Dye™ Terminator Cycle Sequencing Ready Reaction kit

125mM EDTA

96% and 70% ethanol

M13 primers reverse and forward and walking primer

DNA template

Procedure:

1. PCR product purification using ExoSAP-IT

   (a)DNA concentration was estimated by agarose gel, and diluted into around 10ng/ μL.

   (b)Added 2 μL ExoSAP-IT to each 5 μL DNA, incubated at 37 °C, for 15 minutes to degrade remaining primers and nucleotides, afterwards incubated at 80 °C, for 15 minutes to inactivate ExoSAP-IT

2. Dye termination reaction

   (a)Diluted DNA was used as template in a 20 μL sequencing reaction with 5x sequencing buffer, 4 pmol primers and 2 μL Big Dye premix.

   (b)PCR was performed as follows: 96 °C for 1 minute, and 95 °C for 10seconds, 50 °C for 5 seconds, 60 °C for 4 minutes, for 25 cycles.

3. Ethanol/EDTA precipitation

(a)The PCR product was precipitated by 5 µL 125 mM EDTA and 70 µL 95% ethanol.

(b)Sealed the tube with aluminum paper and mixed by inverting 4 times, incubated at room temperature for 15 minutes.

(C)Centrifuged at 1650 *g* and 4 °C for 45 minutes.

(d)Inverted the tube and span up to 185 *g*

(e)Added 60 µL 75% ethanol to each tube, centrifuged at 1650Xg, and 4 °C for 15 minutes.

(f)Inverted the tube and span up to 185 *g*.

(g)Put the tube in darkness without cover 5 minutes, in order to dry the products.

4. The products from ethanol/EDTA precipitation were analyzed in ABI 3730XL DNA sequencer (Applied Biosystems), and analyzed by specific software DNA star.

2.4 Gene expression profile

2.4.1 Introduction and overview

In all organism's cells, gene expression level can be estimated by the number of copies of gene transcripts (messenger RNA or mRNA), which is determined by the rates of its synthesis and degradation. Northern blotting is one method often used to estimate gene expression level by visualizing the abundance of a spesific mRNA transcript in a sample. In this method, purified RNA is separated by agarose gel electrophoresis, transferred to a solid matrix (such as a nylon membrane), and probed with a specific probe complementary to the gene of interest. The disadvantage of this technique is that it requires relatively large amounts of RNA and difficult to get the qualitative information of mRNA levels. In order to robustly detect and quantify gene expression from small amounts of RNA, amplification of the gene transcript is necessary. The RNA sample is first

reverse transcribed to cDNA with a reverse transcriptase. This enables it to be amplified by the polymerase chain reaction (PCR). Development of PCR technologies based on fluorophores permits measurement of DNA amplification at each PCR cycle. The data thus generated can be analyzed by computer software to calculate relative gene expression in several samples, or mRNA copy number. Plotting fluorescence against cycle number on a logarithmic scale determines the relative concentrations of DNA present during the exponential phase of the reaction. Theoretically, the quantity of DNA doubles every cycle during the exponential phase and relative amounts of DNA can be calculated by the threshold cycle (C_t), the cycle at which the fluorescence from a sample crosses the threshold for detection of fluorescence above background. The reaction efficiency has to be calculated first, since all sets of primers don't work equally well. It is determined by comparing the results to a standard curve produced by serial dilutions of a known amount of RNA or DNA. Thus, the precise difference in starting template can be calculated by using this as the base and the cycle difference C_t as the exponent. To accurately quantify gene expression, the amount of RNA from a housekeeping gene in the same sample is also measured to normalize for possible variation in the amount and quality of RNA between different samples to measure amount of RNA from the gene of interest. The expression of the reference (housekeeping) gene used in the normalization should be very similar across all the samples; this permits accurate comparison of expression of the gene of interest between different samples (Nailis et al. 2006; Nolan et al. 2006).

2.4.2 RNA purification

See RACE cloning.

2.4.3 cDNA synthesis

For Real-time PCR, cDNA synthesis is slightly different from the protocol used for RACE. It needs an oligo (dT) primer, which hybridizes to the poly (A) tail of the

mRNA, and extends by reverse transcriptase to synthesize the first strand of cDNA.

Materials:

SuperScript™ III Reverse transcriptase kit

RNaseOUT™ Recombinant RNase inhibitor

Oligo d(T)

dNTP mix

DEPC-treated water

Total RNA template

Procedure:

1. Added around 200ng RNA sample, 1uL random primer [oligo d(T)], 1 µL 10mM dNTP Mix, into a PCR tube. Added RNase free water to a final volume of 7 µL for each reaction.
2. Mixed contents and span the tube briefly. Incubated the tubes at 65 °C for 5minutes.
3. Cooled down the tubes on ice for 3 minutes.
4. Span the tubes briefly to collect the contents at the bottom.
5. Added 4 µL 5X-First-Strand Buffer, 1 µL DDT, RNase out and superscript III Reverse Transcriptase. Mixed the contents by gently pipetting.
6. Span the tubes briefly to collect the contents at the bottom.
7. Incubated the tubes at 25 °C for 5 minutes, then at 50 °C for 1.5 hours and finally at 72 °C for 15 min.

8. Stored at -80 °C.

Quality analysis of cDNA by PCR

See quality analysis of First-strand RACE-Ready cDNA.

2.4.4 Real-time PCR by SYBR Green

Primer pairs for real time PCR analysis were designed using Primer Express Software v 2.0 (Applied Biosystems). The PCR amplification efficiency of each primer pair was calculated from the slope of standard curves where efficiency = $10^{-1/slope}$ (Pfaffl 2001). The housekeeping genes encoding β-tubulin (Tub-b) and a protein of the BAR-domain family (WS41) were selected as real time PCR reference genes (Yan & Liou 2006). WS41 sequences of *P. cactorum* were unavailable from the public database and thus the primer pair was designed based on the conserved region of each gene between *P. parasitica, P. sojae and P. infestans*. WS41 was selected as the control for the developmental stages while Tub-b was used as the control for the infection stages. Real-Time PCR was performed in an Applied Biosystems 7900HT Real-Time PCR system (Applied Biosystems). The thermal cycling consisted of an initial heat-denaturing step at 95°C for 10 min, 40 cycles of denaturing (95°C for 15 s) and combined annealing and elongation (1 min, 60°C). All reactions were performed in two replications. Non-template controls and mock-inoculated strawberry sample were used to exclude the possibility of amplification of plant homologous genes, and genomic DNA templates were included for each primer pair as positive control. The expression level was estimated by the threshold cycler (Ct) values of each gene. The results were analyzed using software SDS v2.3 and BestKeeper. The expression of all genes in the different samples was calculated relative to their expression in sporangia using the Relative Expression Software Tool (REST) (Pfaffl et al. 2002) and presented as log10 of the relative expression values. The biological replication has not been done because of time limitations; if possible it will be completed in future studies.

Material:

SYBR GREEN PCR Master Mix

B5 Real-time PCR primer reverse

B5 Real-time PCR primer forward

WS41 Real-time PCR primer reverse

WS41 Real-time PCR primer forward

Tubulin Real-time PCR primer reverse

Tubulin Real-time PCR primer forward

dH_2O

cDNA templates

Procedure:

The 96 well plate was designed as shown in Fig3.4.4 which shows the difference of the templates and primers. Because the efficiency of the primers used was already tested, the process of making standard curves was skipped. The efficiency of B5 Real-time PCR primer pair was 113.196%, Tubulin Real-time PCR primer pair was 96.149%, WS41 Real-time PCR primer pair was 94.186%.

Sp(b)	Zo(b)	0h(b)	0.5h(b)	1h(b)	1.5h(b)	6hpi(b)	12hpi(b)	1dpi(b)	3dpi(b)	5dpi(b)	7dpi(b)
Sp(b)	Zo(b)	0h(b)	0.5h(b)	1h(b)	1.5h(b)	6hpi(b)	12hpi(b)	1dpi(b)	3dpi(b)	5dpi(b)	7dpi(b)
Sp(w)	Zo(w)	0h(w)	0.5h(w)	1h(w)	1.5h(w)	6hpi(t)	12hpi(t)	1dpi(t)	3dpi(t)	5dpi(t)	7dpi(t)
Sp(w)	Zo(w)	0h(w)	0.5h(w)	1h(w)	1.5h(w)	6hpi(t)	12hpi(t)	1dpi(t)	3dpi(t)	5dpi(t)	7dpi(t)
Ck(t)	Ck(b)	Ck(w)	W(b)	W(t)	W(w)	G(b)	G(w)	G(t)			
Ck(t)	Ck(b)	Ck(w)	W(b)	W(t)	W(w)	G(b)	G(w)	G(t)			

Figure 2.4.4 Distribution of the different templates and primers in the 96 well plate. Sp, Zo, 0, 0.5, 1, 1.5h and 6, 12hpi, 1, 3, 5, 7dpi mean cDNA templates from sporangia, zoospore, 0, 0.5, 1, 1.5hours after cyst germinated and 6, 12hours,1, 3, 5, 7 days after infection.

3 Results

3.1 Selection of genes

Four Expressed Sequence Tags (ESTs) from SSH libraries and effector specific differential display (T506C4, T511A4, T115B5, E2), were selected for further study including cloning of full length cDNA. These were selected based on their putative function as determined from sequence similarity searches (BLASTX) in public databases (*P. sojae* genome database, NBCI database), and gene expression profile (Chen et al. unpublished). Two suppression subtractive hybridization (SSH) libraries of *P. cactorum* were made by Chen et al. (unpublished): an infection library and a geminated cyst library, because these two stages are considered important in the *Phytophthora* plant interaction and suggested important for effectors delivery. T115B5 from the infection library showed most similarity with one of the *P. infestans* transglutaminase elicitors (Gene Bank accession no. EEY65316), and up-regulated expression (compared to sporangia) of this gene also suggests it may play an important role in the *Phytophthora* plant interaction. E2 was detected both in effector specific

differential display and geminated cyst library. Effector specific differential display is a method that exploits cDNA in differential display PCR using RxLR and EER motif degenerate primer together with anchored oligo-dT primer. That indicated E2 was likely to be an RxLR-dEER effector. The expression profile of E2 is similar to T115B5. Although, the BLASTX searches of *P. sojae* genome database and NBCI database gave no significant hits. T506C4 and T511A4 is from the SSH libraries. All the sequences obtained by RACE cloning were in Appendix 3.

3.2 Gene T506C4

Both 5´RACE and 3´RACE PCR were performed. One band around seven hundred bp from 3´RACE PCR was purified from the gel, and the DNA fragment was sequenced. In total 382 bp sequence was obtained from the 3´RACE fragment of T506C4. Table 3.2 shows the result of BLASTX searches from four different databases: NCBI, *P. sojae* genome, *P. infestans* genome, and the COGEME databases. The hits of BLASTX searches clearly indicate that T506C4 is from a gene encoding a ribosomal RNA processing protein. Further cloning of this gene was stopped since it was not considered particularly relevant for *P. cactorum* pathogenicity.

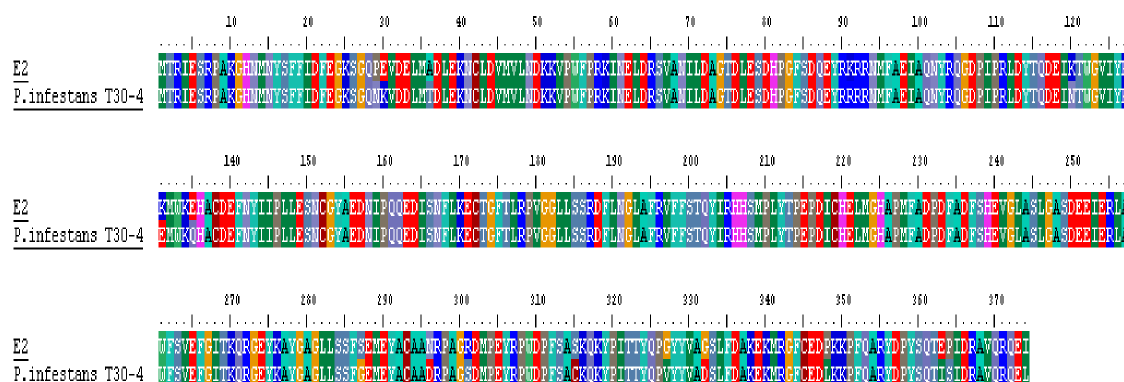
Table 3.2 BLASTX searches with T506C4 in four databases: NCBI database, *P. sojae* genome database, *P. infestans* genome database, and COGEME database.

gene name	**length**	***P. sojae* blastx**				**NCBI blastx**			
		Protein ID	**Best Hit**	**e-value**	**identify**	**GENE ID**	**Best Hit**	**e-value**	**identify**
C4	**527**	**134697**	**MGC53743 protein [*Xenopus laevis*]**	**3.00E-21**	**95 %**	**379182 MGC53677**	**MGC53677 protein [*Xenopus laevis*]**	**0.46**	**51 %**
gene name	**length**	***P. infestans* blastx**				**COGEME tblastx**			
		Protein ID	**Best Hit**	**e-value**	**identify**	**Protein ID**	**Best Hit**	**e-value**	**identify**
C4	**527**	**PITG_10725.1**	**ribosomal RNA processing protein 1**	**1.19E-17**		**no**			

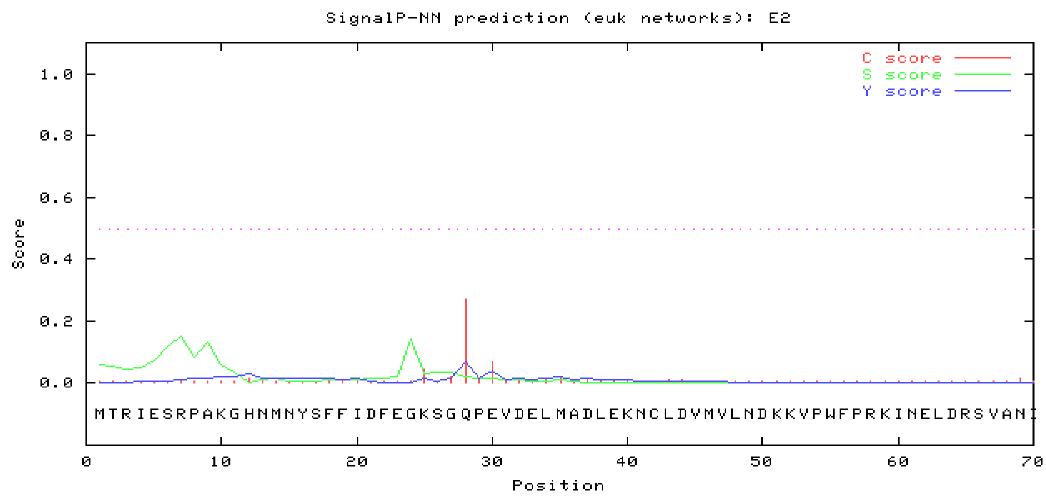
3.3 Gene E2

After sequencing of both 5´RACE and 3´RACE PCR products from gene E2, the full length of E2 was obtained. The full length clone consisted of 1122 base pairs encoding 374 amino acids. From BLASTX searches of the NCBI database and *P. infestans* genome database, the best hit was a phenylalanine-4-hydroxylase, a henna-like protein of *P. infestans.* Both the DNA sequence and the amino acid sequence of E2 were highly similar to the *P. infestans* gene and protein sequence, 96.5 and 89.6 % identity respectively. Only 13 of the 374 amino acids of the proteins were different (Fig4.4a). The conserved oomycete RxLR effector domain was not detected in the E2 protein sequence, but a dEER like motif (DEEIER) was discovered. However, this motif appeared in the C-terminal instead of the N-terminal where such effector motifs are normally located. A signal peptide analysis was performed using on line software Signal IP v3.0. The search was manipulated to select for signal peptide containing sequences using two criteria: either a hidden Markov model (Signal IP-HMM) or an artificial neural network (Signal IP-NN) which predicts a cleavage site between 10 and 40 amino acids from the N-terminal. The result did not reveal any potential signal peptide in this protein. These findings indicate that gene E2 neither encodes a cytoplasmic nor an apoplastic effector protein.

(a)



(b)



(c)

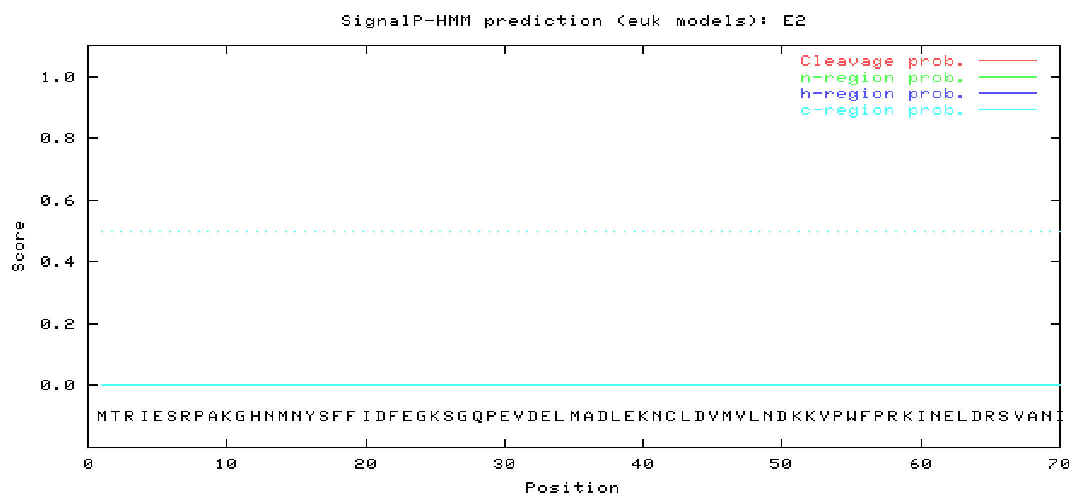


Figure 3.3 (a) Amino acid sequence alignment of E2 encoding protein and *P. infestans* phenylalanine-4-hydroxylase, henna-like protein. (b) Signal peptide prediction in the protein of E2 using SignalIP–NN. (c)Signal peptide prediction in the protein of E2 using SignalIP–HMM.

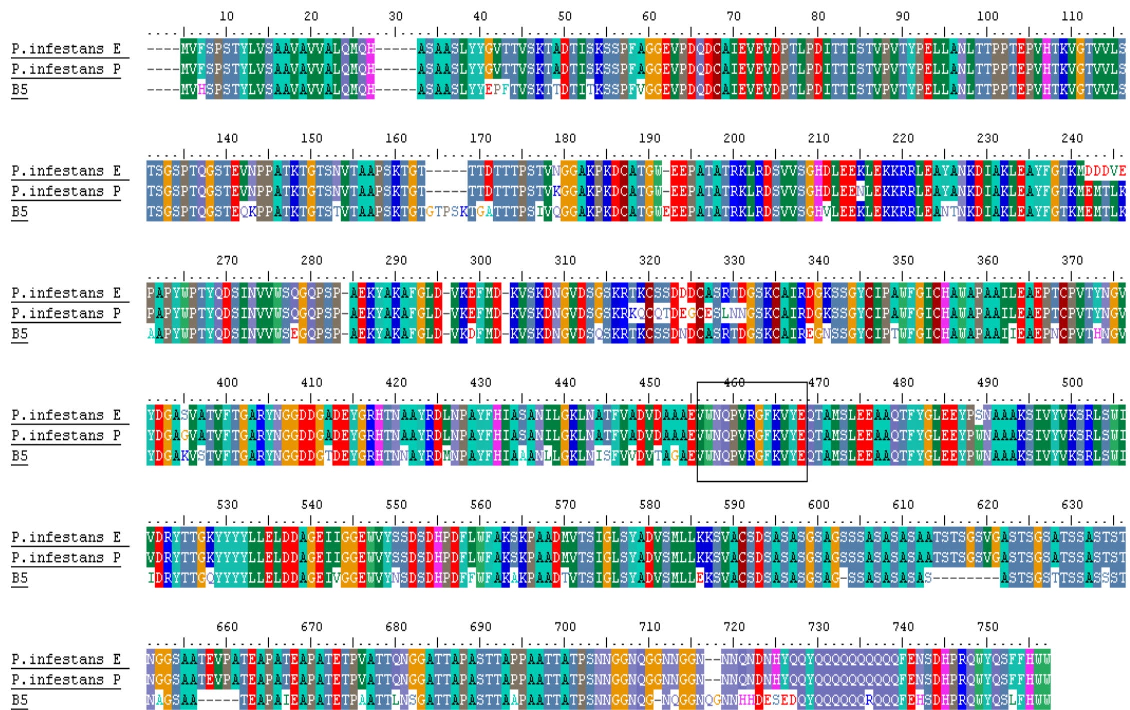
3.4 Gene T115B5

A full length sequence of the EST T115B5 was obtained using 5’ and 3’ RACE. (The full length clone consisted of 2190 base pairs encoding 730 amino acids.) The gene was predicted as a putative transglutaminase gene according the result of BLASTX searches of four databases: NCBI database, *P. sojae* genome database, *P. infestans* genome database, and the COGEME database. This gene showed 88% identity to two other transglutaminase genes from *P. infestans* (Gene Bank accession no. EEY65316.1and AAP70479.1). The identity between gene T115B5 and *P. sojae* transglutaminase gene encoding protein Gp42 is 81%.

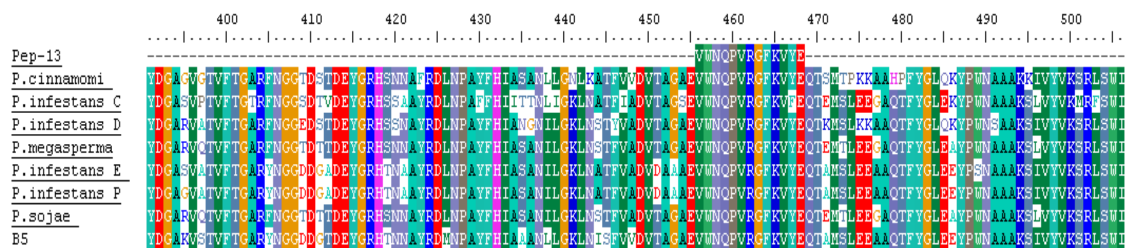
The Pep-13 motif, with the sequence 'VWNQPVRGFKVYE', is an important highly conserved feature of *Phytophthora* transglutaminases, and this motif was also discovered in the T115B5 protein. Fig3.5b shows the amino acid alignment of partial sequences of the T115B5 protein and other *Phytophthora* transglutaminases including the Pep-13 motif with the sequence 'VWNQPVRGFKVYE'. Only the *P. infestans* transglutaminase M81C has one amino acid in this motif that is different from the others. It contains phenylalanine (F) at the second position of the motif from the C-terminal, while the others contain tyrosine (Y).

The amino acid sequence of the T115B5 protein was also analysed by the on line software Signal IP v3.0. The result of both Signal IP–HMM and Signal IP–NN predicts that the T115B5 protein contains a 27 amino acid long signal peptide in the N-terminal of the protein sequence. (Fig3.4 c and d)

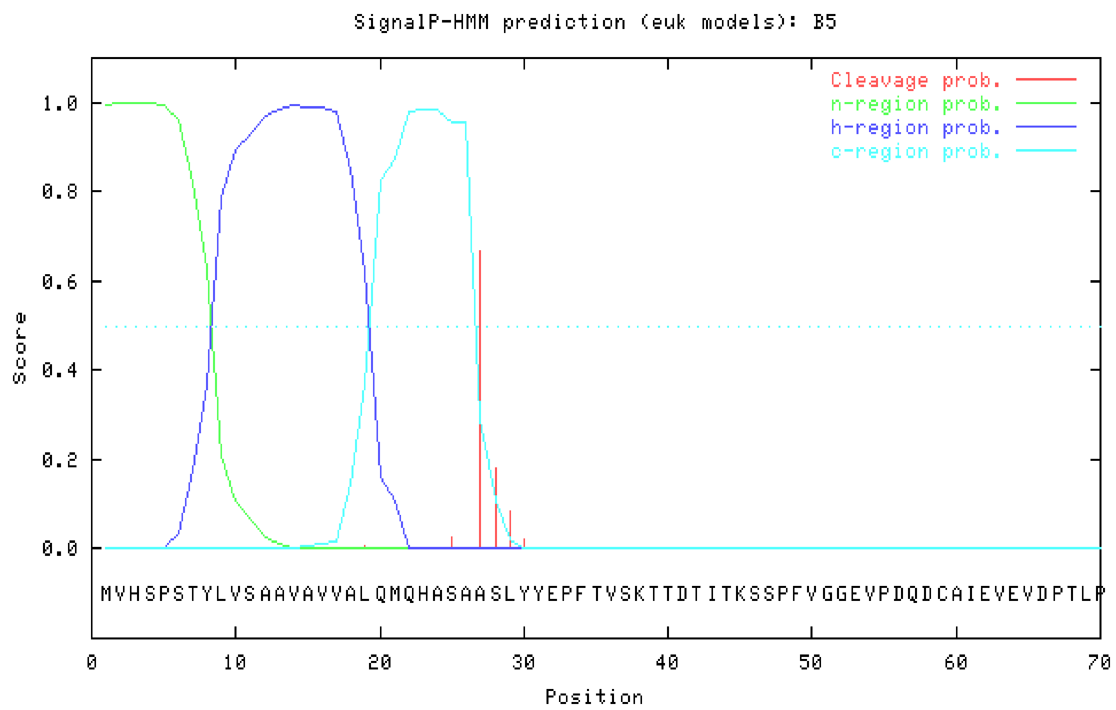
(a)



(b)



(c)



(d)

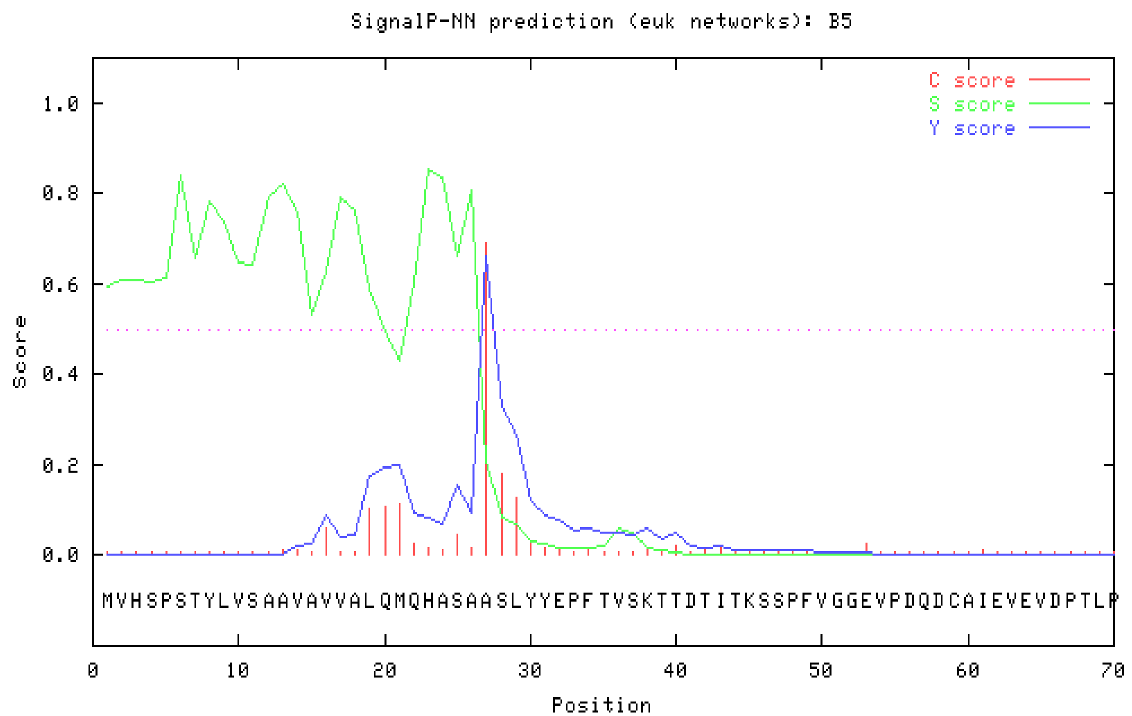


Figure 3.4 (a) Amino acid sequence alignment T115B5 protein, and two *P. infestans* transglutaminases (include accession numbers) All of them contain the Pep-13 motif, with the sequence 'VWNQPVRGFKVYE'. (b) Amino acid sequence alignment of the Pep-13 motif region from transglutaminases of different *Phytophthora* species including T115B5 from *P. cactorum*. Pep-13 motif: VWNQPVRGFKVY(F)E. (c, d) The signal peptide analyzed by Signal IP v3.0 (c) SignalIP–HMM predicts a 27 amino acids long signal peptide of gene T115B5 encoding protein. (d) SignalIP–NN predicts a cleavage site at 27th amino acid from N-terminal.

3.5 Gene Expression Patten of T115B5

Two full length cDNA clones were obtained by 5' and 3' RACE. Gene E2 encodes a putative phenylalanine-4-hydroxylase gene, which cannot be considered an effector gene, and gene T115B5 encodes a putative transglutaminase, which is a kind of apoplastic effector gene. Because of the potentially interesting function of T115B5 in pathogenicity, the rest of the study was concentrated on gene T115B5. Real-time PCR was performed to investigate its expression in five different development stages: zoospores (ZO), encystment (GC-0h), 0.5, 1 and 1.5 hour after start of cyst germination (GC-0.5h, GC-1h, GC-1.5h), and six stages after start of infection (6 hours (6hpi), 12 hours (12hpi), 1 day (1dpi), 3days (3dpi), 5 days (5dpi), 7 days (7dpi)). Expression was compared to expression in the sporangial stage (SP). The transglutaminase was expressed during all the tested stages, but the relative expression was very low at the zoospore stage and during encystment. However the expression level increased very fast during cyst germinating. The gene was also expressed quite stably from 0.5 hour after start of encystment to 3 days after start of infection. The expression level remained around half of that of the three high points except 1 hour after cyst germinated and 6 hours after infection. At 7 days after the start of infection, the T115B5 expression level decreased 22.3 fold. However the expression level was still around 20 times of the sporangium stage. In summary, the expression of T115B5 was very low in zoospores (zoospore) and newly encysted cysts (encystment), but was relatively high (22-115 fold) during cyst germination and infection.

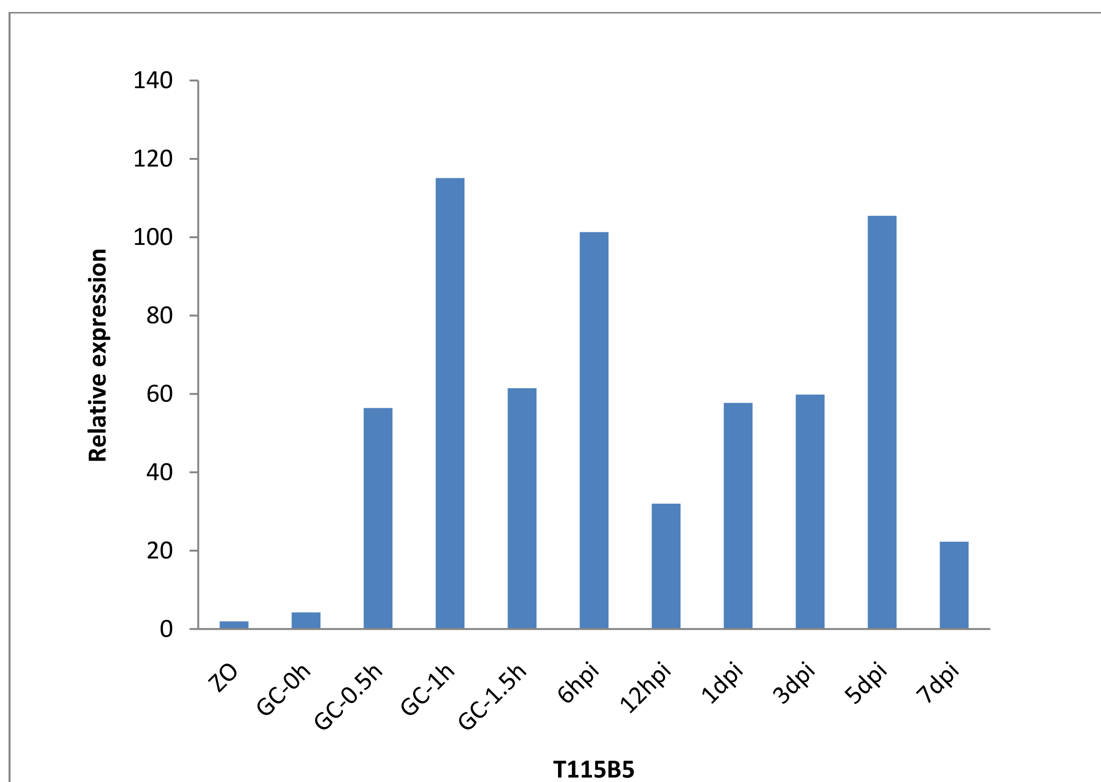


Figure 3.5 The real-time PCR was done to investigate the quantification of relative gene Expression of gene the putative transglutaminase (T115B5) in 5 development stages, zoospores (ZO), encystment (GC-0h), 0.5, 1, 1.5hours after cyst germinated (GC-0.5, 1, 1.5h), and 6 stages after infection, 6, 12 hours (6, 12hpi), 1, 3, 5, 7 days (1, 3, 5, 7dpi)after infection, comparing with sporangium stage (SP).

3.6 Gene T511A4

Only 3´RACE of gene T511A4 was obtained, the sequence obtained was 749 bp, and the full length sequence was predicted approximate 2 kp. The BLASTX searches of NCBI database, *P. sojae* genome database, *P. infestans* genome database, and COGEME database indicated that it is a transglutaminase. The sequence of 3´RACE fragment was very similar to the 3´-terminal sequence of clone T115B5, transglutaminase elicitor M81E (Gene Bank accession no. EEY65316.1) and transglutaminase elicitor, putative (Gene Bank accession no. AAP70479.1) from *P. infestans*, The BLASTX searches of NCBI database showed

that both the identities between 3´RACE fragment of T511A4 and two *P. infestans* transglutaminase are 87%.

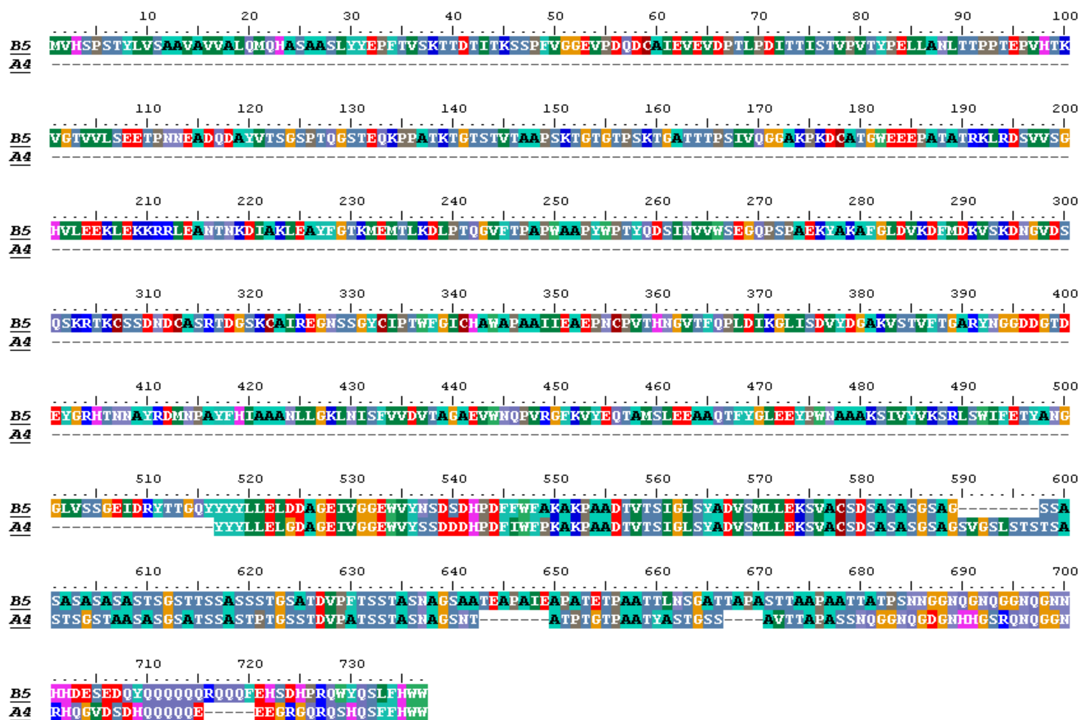


Figure 3.6 Amino acid sequence alignment of two putative transglutaminases (T511A4 and T115B5). Only partial sequence of T511A4 was obtained.

4 Disscusion

4.1 E2

The full length sequence of E2 showed that the gene encoded a putative phenylalanine-4-hydroxylase. The oomycete effector conserved RxLR domain could not be found in the protein sequence, and the signal peptide analysis did not find any potential signal peptide in the N-terminal of this protein. Both factors above indicate that E2 is not an oomycete effector. As a metabolic enzyme, phenylalanine-4-hydroxylase catalyses the reaction responsible for the addition of a hydroxyl group to the end of the 6-carbon aromatic ring of phenylalanine, such that it becomes tyrosine. In humans, liver phenylalanine hydroxylase (PAH) has an established catabolic function, and mutations in PAH

cause *phenylketonuria*, a genetic disease discovered by the Norwegian physician Ivar Asbjørn Følling in 1934 and characterized by neurological damage (Burton et al. 2007). In mosquito, it may play a potential role in immune responses (Johnson et al. 2003). Tyrosine, the product of the reaction, is the precursor to the pigment melanin. In many pathogenic microbes, melanin appears to play important roles in virulence and pathogenicity by protecting the microbe against immune responses of its host. This pigment can protect microorganisms, such as bacteria and fungi, against stresses that involve cell damage caused by reactive oxygen species, but, so far no melanin in *Phytophthora* has been reported (Hamilton & Gomez 2002). In invertebrates, a major aspect of the innate immune defense system against invading pathogens involves melanin. Within minutes after infection, the microbe is encapsulated within melanin, and the generation of free radical byproducts during the formation of this capsule is thought to aid in their killing (Cerenius & Soderhall 2004). In nematodes, Calvo found that pah-1 knockouts lack a yellow-orange pigment in the cuticle, cuticle melanin functions as an oxygen radical scavenger, can clean reactive oxygen species to suppress the program cell death (Calvo et al. 2005). But, no report about phenylalanine-4-hydroxylase in oomycete can be found. The role of this enzyme in *Phytophthora* is therefore not clear. Further study will not carry on this gene, because my interest is in effector genes.

4.2 T115B5 is a transglutaminase gene

Transglutaminases were first described in 1959, and their biochemical activity was discovered in blood coagulation protein factor XIII, 9 years later (Mycek et al. 1959; Pisano et al. 1968). Today, these enzymes are commonly used in food industry as meat glue to bind proteins together, such as in the production of imitation crabmeat, fish balls, and Chicken McNuggets. Transglutaminases are a family of enzymes that catalyze the acyltransferase reaction between a γ-carboxamide group of glutamine residues and the ε-amino group of lysine residues or other primary amines, creating intramolecular or intermolecular peptide chain bonds, which exhibit high resistance to proteolytic degradation. Transglutaminases exists in archaea, bacteria, and in eukaryotes. The

Phytophthora transglutaminases are structurally unrelated to other transglutaminases (Brunner et al. 2002; Makarova et al. 1999). One of the transglutaminases from *P. sojae* has been shown to elicits defense reactions in plants (Parker et al. 1988). Brunner et al. (2002) reported that *Phytophthora* transglutaminases were Ca^{2+} dependent. The *P. sojae* GP42 possessed transglutaminases activity as was shown by incorporation of 5-(biotinamido) pentylamine into N, N-dimethylcasein in a solid-phase microtiter plate assay. The activity of both purified and recombinant *P. sojae* transglutaminases was strictly dependent on Ca^{2+} [$K_{M\ (pentylamine)}$ = 0.249mM at 5mM Ca^{2+}], which could not be replaced by other ions like Mg^{2+} or Mn^{2+}. The transglutaminase inhibitors iodoacetamide, cystamine, N-ethylmaleimide and Cu^{2+}, can block this Ca^{2+} dependent activity efficiently, but GTP, the human tissue transglutaminase inhibitor, did not affect it significantly (Brunner et al. 2002). The T115B5 protein was predicted to have transglutaminase activity based on sequence similarity with other *Phytophthora* transglutaminases throughout the protein including the Pep-13 region which is required for enzyme activity.

4.3 PEP-13 motif is important for both transglutaminase and elicitor activity

The Pep-13 motif, with the sequence 'VWNQPVRGFKVYE', is animportant feature of *Phytophthora* transglutaminases. It was identified by Brunner et al. (2002), and is highly conserved among *Phytophthora* transglutaminases. The Pep-13 motif was detected in most *Phytophthora* species except *Phytophthora undulate* using RT PCR (ref). The peptide could also be detected in the culture filtrate of the these *Phytophthora* species (Brunner et al. 2002). Database analysis of plant sequences, including the *Arabidopsis thaliana* genome, using a complete transglutaminase sequence (Sacks et al. 1995), or the Pep-13 sequence to search with, suggested that plants possess neither orthologs of the *Phytophthora* transglutaminases nor proteins containing peptide motifs with Pep-13 elicitor activity (Brunner et al. 2002). Replacement within Pep-13 of each individual amino acid revealed that the Pep-13 motif is important for both elicitor activity and transglutaminases activity. Compared with wild type protein, both the

ability to elicit parsley defense and transglutaminases activity of the mutants were significantly reduced (Brunner et al. 2002). Treatment of potato cells with Pep-13 showed that Pep-13 can induce the accumulation of defense-related transcripts encoding lipoxygenase, 4-coumarate: CoA ligase and pathogenesis-related protein1. Increased transcript levels of the same genes were also detected in intact potato leaves upon infiltration of Pep-13 (Brunner et al. 2002).

4.4 T115B5 encoding protein is a secreted protein

Signal peptides have an important role in secretary and membrane-resident proteins translocation and membrane insertion. Signal peptides interact with the translocation channel machinery and direct proteins into the secretory pathway (Johnson & van Waes 1999; Johnson et al. 1999; Schatz & Dobberstein 1996). Signal peptide is also an important trait for predicting effectors. Both the cytoplasmic and apoplastic effectors need to be translocated outside the pathogen, so these proteins should contain the signal peptide. The amino acid sequence of the T115B5 protein was analyzed by the online software Signal IP v3.0. The output files showed that the T115B5 protein contains a putative 27 amino acid long signal peptide in the N-terminal of the protein sequence. This result indicates that the T115B5 protein is an extracellular protein or a membrane-resident protein. Evidence for secretion of Phytophthora transglutaminases have been reported previously: Brunner et al. (2002) detected the Pep-13 containing transglutaminase in the culture filtrate of 10 different species of *Phytophthora*. Furthermore Meijer et al. (2006) isolated two homologogous proteins from the *Phytophthora ramorum* cell wall.

4.5 T115B5 is from a gene family

We also discovered a gene family of transglutaminase in *P. cactorum*. Here both gene T511A4 and T115B5 are transglutaminases. However their gene expression profiles are different. T511A4 expresses quite highly in zoospores but very low in mycelium. T115B5 expression is the opposite. According the unpublished data (Chen et al. 2010), gene T512A5 and T512G6, from the SSH libraries also

show the best BLASTX matches are transglutaminases. Meijer et al. (2006) isolated 2 homology proteins from the *P. ramorum* cell wall. Five different transglutaminases have been described from *P. infestans* (Fabritius & Judelson 2003). A mating-induced transglutaminase (M81) of *P. infestans* and its other 4 family members (M81B, M81C, M81D and M81E) were investigated by Fabritius and Judelsen (2003). The M81 transglutaminase was the most structurally and functionally divergent from the *P. soja* enzyme with 47 % protein identity; it lacked elicitor activity, displayed mating-specific transcription, and had a novel C-terminal domain. M81C, M81D, and M81E which displayed elicitor activity and were more similar to the *P. sojae* protein (63 to 75% amino acid identity). M81C and M81D were only expressed during zoosporogenesis, while M81E mRNA accumulated only in hyphae. M81B, physically linked to M81, was an apparent pseudo gene (Fabritius & Judelson 2003).

4.6 T115B5 expression is hyphae specific

The relative expression of the gene T115B5 in 5 development stages, zoospores (ZO), 0, 0.5, 1,1.5hours after cyst germinated (GC-0, 0.5, 1, 1.5h), and 6 stages after infection, 6, 12 hours (6, 12hpi), 1, 3, 5, 7days (1, 3, 5, 7dpi), after infection, comparing with sporangium stage (SP) showed similarity to the hyphal-specific expression of gene M81E from *P. infestans*. M81E is also highly similar in sequence to the T115B5 gene (86% identity). Fabritius and Judelson (2003) investigated the stage specific patterns of expression of M81E. They discovered that the gene-specific probe signal of M81E was greatly reduced in asexual sporangia and did not reappear until after zoospores released from the sporangium and had encysted and germinated to form new hyphae (Fabritius & Judelson 2003). This description is very similar to the expression of the gene T115B5 in this study. The expression of T115B5 was very low in zoospores and new encysted cyst (0 hour after cyst germinated), but after cyst germination and new hyphae formation, the expression level was quite high, although with some temporal variations. This supports a hyphal-specific expression of T115B5.

4.7 Transglutaminases are PAMPs

Transglutaminases are considered pathogen-associated molecular patterns (PAMPs) in the zigzag model of oomycete-plant interaction (Hein et al. 2009). Lots of the PAMPs are characteristic structural and enzymatic proteins that are essential for fitness. They can be recognized as non-self molecular by distinct pattern recognition receptors (PRRs) in plants, in a manner analogous to the mammalian toll- and toll-like family of receptors, and trigger innate immunity called PAMP-triggered immunity (PTI) (Schwessinger & Zipfel 2008). Only limited numbers of PAMPs have been discovered in the large numbers of bacterial, viral, fungal, and oomycete plant pathogens so far (Tor 2008; Tor et al. 2009). In *Phytophthora*, several PAMPs have been identified in addition to the transglutaminases: 1. The cellulose-binding elicitor lectin, which contributes to adhesion during infection (Mateos et al. 1997) 2. numerous small cysteine-rich (SCR) proteins (Kamoun 2006), which induces necrosis and may act as phytotoxins (Orsomando et al. 2001); 3. Elicitins, which are conserved 10 kDa proteins that elicit a wide range of defense responses in plants including hypersensitive response (HR) (Kamoun 2006); 4. The cell wall-associated necrosis-inducingprotein 1, which induces PTI responses including cell death (Fellbrich et al. 2002; Qutob et al. 2006). When PTI has been induced, a range of universal responses is normally triggered, including ion fluxes, mitogen-activated protein kinase (MAPK) cascades, production of reactive oxygen species (ROS), cell wall reinforcement and rapid induction of defense genes, largely regulated by WRKY transcription factors (Ingle et al. 2006). The PTI responses triggered by *Phytophthora* transglutaminases in parsley cells include ion fluxes, ROS generation, defense gene activation, and phytoalexin synthesis (Brunner et al. 2002; Nurnberger et al. 1994). Treatment of potato celles with Pep-13, the conserved motif from *Phytophthora* transglutaminases, led to the accumulation of defense-related transcripts encoding lipoxygenase, 4-coumarate: CoA ligase and pathogenesis-related protein 1 (Brunner et al. 2002). So, the PAMP activity of *Phytophthora* transglutaminases can be narrowed to Pep-13 motif.

Gene silencing experiments in soybean (*Glycine max*) interacting with *P. sojae* cell wall glucan elicitor (WGE), suggested that *in situ* release of active fragments from PAMPs is necessary for HR cell death. This is mediated through accumulations of the 5-deoxyisoflavones in soybean roots carrying resistance genes at the Rps 1 locus (Graham et al. 2007). Transglutaminases trigger similar responses to *P. sojae* WGE. Pep-13, the conserved motif of *Phytophthora* transglutaminases, induces nonhost defense responses in parsley (*Petroselinum crispum*) (Brunner et al. 2002), and a series of defense reactions including a salicylic acid-dependent HR-like cell death in potato (*Solanum tuberosum*) (Halim et al. 2004). Thus it would appear that PAMPs may participate directly in the HR under certain conditions or in the presence of other elicitors or modifiers.

Membrane bound plant Pattern Recognition Receptors (PRRs) were considered to be the plant receptors recognizing the PAMPs in the PTI responses. *Phytophthora* transglutaminases as PAMPs were thought to be recognized by plant PRRs, which have extracellular domains (Hein et al. 2009). PRRs include receptor-like kinases (RLKs) (Shiu & Bleecker 2003), receptor-like proteins (RLPs) (Wang *et al.*, 2008) and polygalacturonase inhibiting proteins (PGIP) (Di Matteo et al. 2003). Intracellular plant PRRs are NBS-LRR proteins (nucleotide binding site–leucine-rich repeats) (Meyers et al. 2003). The LRR-RLK proteins, FLS2 (Flagellin Sensing 2), have been identified as receptors for the bacterial PAMP flagellin (flg22) respectively. It physical interactions with the receptors have been demonstrated (Kunze et al. 2004; Zipfel et al. 2006). The flg22 interaction was one of the best-characterized systems in the activation of innate immunity in plants (Sun et al. 2006). Therefore, PRRs were considered to be the plant receptors recognizing the *Phytophthora* transglutaminases in the PTI responses. It is not known for sure which protein from the PRRs family recognizes *Phytophthora* transglutaminases. A monomeric 100 kDa integral plasma membrane protein from parsley was purified and characterized as the receptor for *Phytophthora* transglutaminases (Nennstiel et al. 1998). *Phytophthora* transglutaminases do not induce cell death in parsley (Blume et al. 2000), however, they induce a series of defense reactions including a salicylic

acid-dependent HR-like cell death in potato (*Solanum tuberosum*) (Halim et al. 2004). Thus it would appear that PAMPs may participate directly in the HR under certain conditions or in the presence of other elicitors or modifiers. In order to successfully infect a plant host, the plant pathogen should secrete effectors to suppress key modulators of the downstream signaling cascade of PTI, or the defense responses stimulated by PTI.

4.8 Transglutaminases are cell wall proteins

In addition to the transglutaminase activityand acting as PAMPs, transglutaminases in *Phytophthora* also play other roles. The T115B5 protein is predicted to be extracellular or a component of the cell wall. The T115B5 protein has serine-and alanine-rich tracts, which are typical of proteins that contribute to the structure of cell walls or adhesion in extracellular matrix (Filpula et al. 1990; Fosnaugh & Loomis 1989; Gornhardt et al. 2000; Keller & Lamb 1989; Smythe et al. 1990). In *P. ramorum*, two cell wall associated proteins were identified that have homology with transglutaminases (Meijer et al. 2006). Makarova *et al*, (1999) showed that transglutaminases may be involved in the formation of covalent cross-links between wall proteins during wall construction. Transglutaminases with different physiological roles have been described in mammals, plants, and other organisms (Chandrashekar & Mehta 2000; Ichinose et al. 1990; Serafinifracassini et al. 1995). Many of these are related to the formation of resistant structures and have protective functions. A transglutaminase immunologically related to mammalian transglutaminases catalyses cross-linking of the cell wall in the green alga *Chlamydomonas reinhardtii* (Waffenschmidt et al. 1999). Green alga is from the same kingdom as *Phytophthora*, and has cell wall structure that may be similar to *Phytophthora*. The gene expression pattern also can be explained to support this conclusion. The cell walls of hyphae, zoospores, and sexual structures of *Phytophthora sp*. are structurally and biochemically distinct (Hemmes & Bartnickigarcia 1975; Wang & Bartnickigarcia 1982). Each may require functionally distinct enzymatic activities conferred by the transglutaminase family, explaining the diverse structures and transcription patterns of the gene family (Fabritius & Judelson

2003). The function of the T115B5 protein may be cell wall strengthening, connecting the plasma membrane to the cell wall by cross-linking proteins, or in cell-cell adhesion. The *Phytophthora* transglutaminases may, like many other microbial transglutaminases, have roles as either proteases or transglutaminases (Makarova et al. 1999).

4.9 Future studies

Further study will continue on transglutaminase gene (T511B5.) An agroinfiltration system will be employed for transient expressing the T511B5 gene in *N. benthamiana* leaves. It is a well developed method to test the *Avr* function of candidate oomycete gene.

Alternatively, T511B5 protein can be produced in heterologous systems and subsequently infiltrated into strawberry leaves. Purified proteins also allow to do further characterization.

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Appendix 1-SSH sequences

>T0506C4

GACGAGGAGGAGGAGACTGATGACGAGGAAGAGGAGAGCAAGGTGTTCGAACAGTTCGACGTGG
CGCAGGTGCAGCACCGGATCTTCGCAATTGCGTCGGCCGACGACACGGCGGAACGCAACCGCTCGG
CGCTTTACACGTTGT

> E2

CAAGAGCGGCCAACCCGAGGTGGACGAGCTCATGGCCGACCTCGAAAAGAATTGCCTCGACGTCAT
GGTGCTGAACGACAAGAAAGTGCCGTGGTTCCCTCGCAAGATTAACGAGTTGGACCGCTCGGTGGC
CAACATTCTGGACGCTGGAACCGACCTGGAGTCGGACCACCCGGGCTTCAGTGACCAAGAGTACCG
CAAGCGCCGCAATATGTTTGCAGAGATCGCCCAGAACTACCGCCAGGGAGACCCAATCCCTCGTCTG
GACTACACGCAAGATGAGATTAAAACGTGGGGAGTCATCTACAAGCGCATGAAGAAAATGTGGAAG
GAACACGCGTGCGACGAGTTTAACTACATTATCCCGCTCCTC

>T0511A4 (3’RACE+SSH)

ACTACTACTACCTTCTGGAGCTGGGCGACGCCGGTGAGATCGTCGGTGGTGAGTGGGTCTACAGCTC
GGACGACGACCACCCTGATTTCATTTGGTTCCCTAAGGCGAAGCCTGCTGCTGACACTGTGACCAGC
ATTGGTCTGAGCTACGCGGACGTGAGCATGCTCCTGGAGAAGTCTGTTGCGTGCTCGGACTCCGCTT
CTGCGTCGGGCTCGGCTGGTTCAGTTGGTTCATTGTCAACTTCGACATCGGCCTCGACCTCAGGATCT
ACCGCTGCTTCGGCTTCGGGCTCGGCAACGAGCTCTGCTTCGACCCCCACTGGTTCGTCCACGGACG
TGCCTGCTACGAGCTCGACTGCTTCCAATGCGGGCAGCAACACTGCCACCCCGACGGGCACGCCGGC
TGCTACCTACGCTAGCACTGGCTCATCTGCTGTGACGACGGCGCCTGCCTCGAGCAACCAGGGCGGA
AACCAAGGCGACGGCAATCACCACGGTAGTCGCCAGAATCAGGGTGGCAACCGGCACCAGGGTGTT
GACAGCGACCACCAACAGCAGCAGCAGGAGGAGGAAGGACGAGGCCAGCGCCAATCGCACCAGTC
CTTCTTCCACTGGTGGTAAGCTCATCCAGTTCGGT

> T0115B5

ACTCCGTCCAAGACTGGAGCTACCACGACGCCGTCGATTGTCCAGGGTGGAGCTAAGCCCAAGGAC
TGCGCCACCGGCTGGGAGGAGGAGCCGGCCACGGCCACCCGCAAGCTGCGTGACAGCGTTGTGTC
GGGCCATGTTCTCGAGGAGAAGCTCGAGAAGAAGCGTCGCCTGGAGGCTAACACGAACAAGGACA
TTGCCAAGCTTGAGGCCTACTTCGGCACCAAGATGGAGATGACCCTGAAGGACCTGCCTACCCAGG
GAGTGTTCACGCCTGCGCCATGGGCAGCCCCGT

Appendix 2-primer sequences

5'-AACAGTTCGACGTGGCGCAGGTG-3'	3`RACE primer for T506C4
5'-GCAACCGCTCGGCGCTTTACACGTT-3'	5`RACE primer for T506C4
5'-CAGGAGCTCAAGAAGCAGGAGAAG-3'	primer for T506C4 3`RACE cDNA walking
5'-CGACGTCATGGTGCTGAACGACAAG-3'	3`RACE primer for E2
5'-GGATTGGGTCTCCCTGGCGGTAGTT-3'	5`RACE primer for E2
5'-CTTGCGTGTAGTCCAGACGAGGGATTG-3'	5`RACE primer for E2(2)
5'-CCAGGGTGGaGCTAAGCCCAAGGAC-3'	3`RACE primer for T511B5
5'-AAGAAGCGTCGTCTGGAGGCTAACA-3'	5`RACE primer for T511B5
5'-GAACAATGAAGCCGATCAGGATGCGTAC-3'	5`RACE primer for T511B5(2)
5'-CTGCTACTACTTTGAACTCTGGCG-3'	primer for T115B5 3`RACE cDNA walking
5'-ACGCTGAAGCTCACTACTCCCAC-3'	Tubulin primers reverse
5'-ACGACGAGTTCTTGTTCTGCACG-3'	Tubulin primers forward
5'-CCTGGAGGCTAACACGAACAA-3'	B5 Real-time PCR primers reverse
5'-TCCATCTTGGTGCCGAAGTAG-3'	B5 Real-time PCR primers forward
5'-TGGTACGGAGTTCCTCGTGC-3'	WS41 Real-time PCR primers reverse
5'-CTCGCCGATCTCACTGGACT-3'	WS41 Real-time PCR primers forward
5'-GTATGAGCACGAAGGAGGTTGAC-3'	Tubulin Real-time PCR primers reverse
5'-ACTCGACGAAGTACGACGAGTTC-3'	Tubulin Real-time PCR primers forward
5'-GCACTGGCTCATCTGCTGTGACGAC-3'	3`RACE primer for T511A4
5'-CAACAGCAGCAGCAGGAGGAGGAAG-3'	5`RACE primer for T511A4

Appendix 3-RACE obtained sequences

>T506C4, SSH+3' RACE

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TACAAGAAGTTCTTCACTATCACGCACGTTGACTCGTTCCAGGCTGCGCAGGAGGAGGCTGCCAAGC
CGAAGAAGATCGTAAAGAAGGAGAAGAAGAAGGCGGAAGCTGCTGAGACTGAAACTGCGCAGGA
AGTGAAGGACGAGGAGGCTGGCGAGAAGAAAAACTCCAAGAAATCTAAGAAACGCAAAGCTAAGA
AGGAAGCCGAGGTGGAGGCTGACGTTGATGCTAAGAAGAGCGAGAATACGAAGGAGGTAAAAACC
GAGACTCCGTCTCCCAAGAAGAAGGCCAAAAAACAGAAGGTTGCTGCTGTGGTTAAGACTGAGGAC
GTCGAGGTGAAGAACTCACCTGCTGAGACCGAGAAGAGCAAAACGCAAAAAAATCAAAAGAAAAA
GCAGGAGCTCAAGAAGCAGGAGAAGCCTGTCGAGAAGAAACCTGCTGCGCCTGTGAAGAAGTCTCC
GGTTAAGGAACAGCTG

>T511A4, SSH+3'RACE

ACTACTACTACCTTCTGGAGCTGGGCGACGCCGGTGAGATCGTCGGTGGTGAGTGGGTCTACAGCTC
GGACGACGACCACCCTGATTTCATTTGGTTCCCTAAGGCGAAGCCTGCTGCTGACACTGTGACCAGC
ATTGGTCTGAGCTACGCGGACGTGAGCATGCTCCTGGAGAAGTCTGTTGCGTGCTCGGACTCCGCTT
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ACCGCTGCTTCGGCTTCGGGCTCGGCAACGAGCTCTGCTTCGACCCCCACTGGTTCGTCCACGGACG
TGCCTGCTACGAGCTCGACTGCTTCCAATGCGGGCAGCAACACTGCCACCCCGACGGGCACGCCGGC
TGCTACCTACGCTAGCACTGGCTCATCTGCTGTGACGACGGCGCCTGCCTCGAGCAACCAGGGCGGA
AACCAAGGCGACGGCAATCACCACGGTAGTCGCCAGAATCAGGGTGGCAACCGGCACCAGGGTGTT
GACAGCGACCACCAACAGCAGCAGCAGGAGGAGGAAGGACGAGGCCAGCGCCAATCGCACCAGTC
CTTCTTCCACTGGTGGTAAGCTCATCCAGTTCGGTACGCTGTAAGCTCGAGATCCAAGGTTATTGCCG
GTGGCAAAAAATCTAACGCGTTTTTAGCTTAATCAAAATCAAGAGTGAACCTGGCATAattGTTTATG
AACTGGGGTAGCAAAAAAAAAAAAAAAAAAAAAAAA

>E2

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CGCGCCCCGCCAAGGGCCACAACATGAACTACAGCTTCTTTATTGACTTCGAGGGCAAGAGCGGCCA
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GACGAGTTTAACTACATTATCCCGCTCCTCGAGAGTAACTGCGGCTATGCTGAAGACAACATCCCGC
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>T115B5

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CGGCTTCGACCACGGCCGCGCCTGCCGCGACTACGGCTACCCCCTCGAACAATGGCGGAAACCAGG
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CAACAGCAGCGACAGCAGCAGTTCGAGCACAGCGACCACCCGCGCCAGTGGTACCAGTCCTTGTTTC

ACTGGTGG<u>TAA</u>ACGGAGTTGATGGAGCAGTGCCTTAAGTGATGAAACGTGAGAGAGAATCACTTGT
AGAAAAAAAAAAAAAAAAAAAAAAAAA

Appendix 4-List of Kits and medium

List of Kits:	
	SMART II™ RACE cDNA Amplification Kit
	QIAGEN RNeasy Mini Kit
	TURBO DNase Kit
	PureLink™ Check Gel Extraction Kit
	RNA 6000 Nano Kit
	Invitrogen TOPO TA Cloning kits
	EcoRI
	QIAprep Spin Miniprep kit
	Big DyeTM Terminator Cycle Sequencing Ready Reaction kit
	ExoSAP-IT
	SuperScriptTM III Reverse transcriptase kit
	Power SYBR® Green RT-PCR Reagents Kit
LB medium:	1.0% Tryptone
	0.5% Yeast extract
	1.0%NaCl
	Aoutoclave to sterilize
LB agarose medium:	LB medium with 15g/L agarose
	Cooled to about 55 °C prior to autoclavation
	Add appropriate antibiotics
SOC medium	2.0% Tryptone
	0.5% Yeast extract
	10mM NaCl
	2.5mM KCl
	10mM MgCl2
	10mM MgS
	20mM Glucosis
	Aoutoclave to sterilize