Construction of a tomato leaf cDNA expression library and immunoscreening for the *Pseudomonas syringae* pv. *tomato* DC3000 HrpZ1 target protein

Janina Österman
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Abbreviations

λ	(bacteriophage) lambda
aa	amino acid
ABC	ATP-binding cassette
AP	alkaline phosphatase
Avr	avirulence
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
cDNA	complementary DNA
CEL	conserved effector locus
COR	coronatine
CSPD	disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1.^{3,7}]decan)-4-yl]phenylphosphate
C-terminal	carboxy-terminal
EEL	exchangeable effector locus
ETI	effector-triggered immunity
Hop	Hrp outer protein
HR	hypersensitive response
Hrc	hypersensitive response and conserved
Hrp	hypersensitive response and pathogenicity
IPTG	isopropyl-β-D-1-thiogalactopyranoside
JA	jasmonic acid
kb	kilobase pairs
kDa	kilodalton
Mb	megabase pairs
MFP	membrane fusion protein
NBT	nitroblue-tetrazolium
NCBI	National Center for Biotechnology Information
N-terminal	amino-terminal
OMP	outer membrane protein
ORF	open reading frame
Pai	pathogenicity island
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
Pfu	plack forming units
pI	isoelectric point
PR	pathogenesis-related
PRR	pattern recognition receptor
Pst DC3000	*Pseudomonas syringae* pathovar *tomato* DC3000
PTI	PAMP-triggered immunity
PVDF	polyvinylidene fluoride
R	resistance
Rubisco	Ribulose-1,5-bisphosphate carboxylase oxygenase
SA	salicylic acid
SAR	systemic acquired resistance
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Sec	the main branch of the general secretory pathway
T3SS	type III secretion system
Tat	twin-arginine traslocation
TPS	two-partner secretion

HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty	Laitos — Institution — Department
Faculty of agriculture and forestry	HEBIOT

Tekijä — Författare — Author

Janina Österman

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Tiivistelmä — Referat — Abstract

Pseudomonas syringae pv. *tomato* DC3000 is a Gram-negative plant pathogen that causes bacterial speck disease on tomato. The virulence of this bacterium is based on the type III secretion system (T3SS). Similar systems are also used by many other plant and animal pathogens, as well as symbiotic bacteria. The T3SS enables the transfer of specific bacterial virulence proteins from the bacterial cytoplasm into the host cell. This secretion is mediated by a needle-like structure that penetrates the plant cell wall. Once inside the host cells, the effector proteins are capable of shutting down the host's immune system. However, what happens at the plant cell membrane is not well understood.

One of the first bacterial proteins that come into interaction with a host protein during *P. syringae* pv. *tomato* DC3000 infection is the HrpZ1 protein that is believed to participate in a membrane interaction, facilitating the transfer of effector proteins. Previous research has shown that HrpZ1 binds to a peptide and using an antiserum raised against this peptide in immunoblotting tests of tomato proteins separated by SDS-PAGE and isoelectric focusing, the target tomato protein of HrpZ1 has been found to be small and acidic. However, this specific protein has not yet been fully characterized. This is why the goal of the work for this thesis was to identify and characterize the target protein of HrpZ1 in tomato. For this purpose, a lambda cDNA expression library from tomato leaves was constructed, followed by immunoscreening of the library with the abovementioned antiserum, and analysing candidate clones by sequencing. Even though a good cDNA library was obtained, the immunoscreenings did not yield satisfactory results. Thus, the pursuit of the HrpZ1 target protein needs to be continued.

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HELSINGIN YLIOPISTO — HELSINGFORS UNIVERSITET — UNIVERSITY OF HELSINKI

Tiedekunta/Osasto — Fakultet/Sektion — Faculty
Agrikultur- och forstvetenskapliga fakulteten

Laitos — Institution — Department
HEBIOT

Tekijä — Författare — Author
Janina Österman

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Tillverkning av ett lambda cDNA-expressionsbibliotek från blad av tomat samt immunologisk sållning efter målproteinet för *Pseudomonas syringae* pv. *tomato* DC3000 effektorproteinet HrpZ1

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Tiivistelmä — Referat — Abstract

Pseudomonas syringae pv. *tomato* DC3000 är en Gram-negativ växtpatogen som orsakar sjukdomen bacterial speck hos tomat. Bakteriens virulens baserar sig på ett system kallat typ III sekretion (T3SS). Liknande system används också av många andra växt- och djurpatogener, såväl som av symbiotiska bakterier. Detta system möjliggör överföring av specifika bakterieproteiner som medverkar i virulensen från bakteriens cytoplasma till värdcellen. Sekretionen sker med hjälp av en struktur liknande en injektionsnål, som tränger igenom växtens cellvägg och plasmamembran. Inne i värdcellen kan dessa så kallade effektorproteiner rasera värdens immunsystem.

Ett av de första bakterieproteinerna som kommer i kontakt med ett värdprotein vid en infektion av *P. syringae* pv. *tomato* DC3000, är proteinet HrpZ1 vilket tros delta i en membraninteraktion och förebygga transporten av effektorproteiner. Tidigare forskning har visat att HrpZ1 binder till en viss peptid, och genom användning av ett antiserum mot denna peptid i immunoblot-tester med tomatproteiner som separerats med SDS-PAGE och isoelektrisk fokusering, har man kunnat konstatera att tomatproteinet som HrpZ1 binder till är litet och surt. Proteinet som HrpZ1 interagerar med har dock inte ännu helt karakteriserats. Därför var målet för denna avhandling att identifiera och karakterisera målproteinet för HrpZ1 i tomat. För ändamålet gjordes ett lambda cDNA-expressionsbibliotek från tomatblad, följt av immunologisk sållning av biblioteket med ovannämnda antiserum och analys av möjliga kandidatkloner genom sekvensering. Även om cDNA-biblioteket blev bra så gav den immunologiska sållningen inte något tillfredställande resultat. Med andra ord måste sökandet efter målproteinet för HrpZ1 ännu fortsätta.

Avainsanat — Nyckelord — Keywords
Pseudomonas syringae pv. *tomato*, HrpZ1, cDNA-expressionsbibliotek, immunologisk sållning

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1 Introduction

1.1 Tomato

Tomato (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) is a member of the *Solanaceae* family and is typically cultivated for the purpose of harvesting its fruit for human consumption. There are thousands of tomato cultivars with differing properties. In 2007, approximately 130 tonnes of tomatoes were cultivated throughout the world (FAO) (table 1), China being the largest producer. In Europe, Italy is the largest producer. In 2003, 102 million tonnes of tomatoes were consumed in the world and 82 760 tonnes in Finland alone. Tomatoes are rich in vitamin A and C, and the tomato pigment lycopene is an antioxidant believed to prevent several forms of cancer (Media Insights, Inc. 2009). The genome size of tomato is approximately 950 Mb (Arumuganathan & Earle 1991), estimated to encode for 35 000 proteins (Van der Hoeven *et al.* 2002). The genes are distributed among 12 chromosomes which are currently (2009) being sequenced as a project coordinated by the International Solanaceae Genome Project (SOL) (http://sgn.cornell.edu/solanaceae-project/).

Table 1. Tomato production quantity in 2007 (FAO). Most producing countries in the world, in Europe and production in Finland.

Country	**Production (tonnes)**
China	33 596 881
USA	14 185 180
Turkey	9 945 043
India	8 585 800
Egypt	7 550 000
Italy	6 025 613
Finland	38 171
World, total	**129 942 416**

1.2 Bacterial speck disease

Bacterial speck disease on tomato leaves and fruit is caused by the plant pathogen *Pseudomonas syringae* pv. *tomato*. Occurrence of bacterial speck has been reported worldwide during the last decade and it causes problems particularly in the humid tomato-producing areas of the United States (Goode & Sasser 1980). In Finland, bacterial speck is not a big problem since most tomatoes are cultivated in greenhouses where the conditions are more controlled than in open fields.

1.2.1 Disease development

Development of bacterial speck disease is favoured in areas with temperatures between 13ºC and 25ºC and where the relative humidity is high (over 80%) (Yunis *et al.* 1980). It is well known that rain triggers rapid growth of *P. syringae*, although such growth is rare when leaves are wet with dew (Hirano & Upper 1990). Physical wounds e.g. from hail or blowing sand, as well as human handling of the fruits, enhance the severity of the disease by providing a way for bacteria to enter the intercellular space (the apoplast) (Goode & Sasser 1980). Normally, the pathogen derives from infected seed or soil and enters the plant leaves through the stomata. The pathogen can also infect fruits, but only the skins of green fruits are infected, since the skin and flesh of red fruits and the flesh of green fruits have pH values unfavourable for the pathogen (Yunis *et al.* 1980). The spreading of the disease in a field is dependent on the amount of pathogens on individual leaves within the canopy (Hirano & Upper 1990).

1.2.2 Symptoms

Upon infection of wild-type susceptible tomato leaves by *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000), small lesions surrounded by distinct halos appear, and the leaves become chlorotic (Goode & Sasser 1980, Cohn & Martin 2005). The speck lesions on leaves usually form stripes of necrotic tissues and eventually widespread necrosis that may lead to total collapse after five to ten days from inoculation of the bacterium (Goode & Sasser 1980, Cohn & Martin 2005). Bacterial speck on fruit is formed by individual lesions of necrotic tissue ranging from almost invisible to 1 mm in

diameter. The lesions may jointly form bigger areas that can cover more than one-fourth of the fruit surface. Fruit that develop after severe defoliation are sun scalded, small and of poor quality and thereby not suitable for commercial use. (Goode & Sasser 1980)

1.2.3 Disease prevention

Pre-treatment of tomato plants by spraying with copper compounds reduces infection (Yunis *et al.* 1980). However, frequent use of copper sprays has lead to the development of plasmid-mediated resistance to copper in some *P. syringae* pv. *tomato* strains (Bender & Cooksey 1987). Nevertheless, *Pst* DC3000 does not currently possess all the genes required for copper resistance (Buell *et al*. 2003). Recently, some plant growth promoting rhizobacteria have also been indicated to induce plant resistance, hence providing suppression of bacterial speck of tomato (Ji *et al.* 2006).

1.3 *Pseudomonas syringae* pv. *tomato* DC3000

P. syringae strains are aerobic, Gram-negative rods with one or several polar flagella. They are oxidase-positive or -negative, catalase-positive and do not tolerate acid (Palleroni 1984). They are biotrophic plant pathogens that multiply on live tissues, inducing delayed symptoms (Barash & Manulis 2005). All *P. syringae* strains, except for those who are pathogenic on tobacco, elicit the hypersensitive response (HR) (see 1.4.3) in tobacco (Hirano & Upper 1990). The *P. syringae* species includes over 40 pathovars, forming groups of several strains that are all capable of infecting the same plant species. Moreover, specific strains of a pathovar can be designated a race, depending on their ability to infect different cultivars within a host species (Hirano & Upper 1990). In contrast, some strains have only an epiphytic host - the bacteria can maintain a population without causing disease - although the bacterium might also be able to infect this host (Hirano & Upper 1990). *P. syringae*, like most plant pathogenic bacteria, is a non-invasive extracellular pathogen that colonizes the intercellular space outside the plant cell wall (Hauck *et al.* 2003).

P. syringae pv. *tomato* DC3000 is a pathogen of tomato and the model plant *Arabidopsis thaliana* and has become an important model organism for the study of plant-pathogen interactions. In 2003, Buell and co-workers reported the complete

genome sequence for *Pst* DC3000. The size of the genome is 6.5 Mb, distributed among a circular chromosome and two plasmids. The genome encodes for 5763 open reading frames (ORFs) and 7% of the genome consists of mobile elements. Most virulence-related genes are found in the proximity of mobile elements. Twelve per cent of the genes in the genome are regulatory genes, probably needed for rapid adaptation that is also crucial in pathogenesis.

P. syringae pv. *tomato* can be recognized in culture by the fluorescence of a fluorescent peptide siderophore (iron(III) chelator) on King's B medium when exposed to UV light (Goode & Sasser 1980). The ability of *Pst* DC3000 to establish infection in plants is largely due to the type III secretion system (T3SS) and the virulence factors, called effector proteins, which are secreted into the host cytoplasm upon infection (see 1.5.2).

1.4 Plant defence against pathogenic bacteria

Just like animals, plants have an innate immunity that recognizes pathogen-associated molecular patterns (PAMPs, also referred to as microbe-associated molecular patterns, MAMPs). These are bacterial structural elements or proteins produced by the bacterium (Nomura *et al.* 2005). However, plants are able to use a combination of constitutive and induced defence mechanisms (Staskawicz *et al*. 1995). PAMP-triggered immunity (PTI), or the basal defence, is considered the first line of defence against multiple pathogens. Because plant pathogens naturally try to suppress basal defence by effector proteins, plants also have evolved effector-triggered immunity (ETI) (Zhou & Chai 2008), formerly known as gene-for-gene resistance, which is launched as a second line of defence when effector proteins are recognized by resistance (R) proteins - often nucleotide-binding leucine-rich repeat proteins (Boller & He 2009).

1.4.1 Basal defence

Suppression of basal defence is believed to be one of the most important ways for pathogens to overcome host defences. The basal defence is activated when a plant recognizes conserved PAMPs through pattern recognition receptors (PRRs) at the cell surface (Boller & He 2009). These receptors are often leucine-rich repeat receptor

kinases that recognize highly conserved domains of the PAMPs (Boller & He 2009). One of the best known examples of this is the recognition of bacterial flagellin by the PRR flagellin sensitive 2 (FLS2). When the basal defence is activated, several molecular events are stimulated, including production of reactive oxygen species (ROS) and nitric oxide and opening of ion channels (Zhou & Chai 2008). These events further activate signalling pathways leading to plant defence, like production of antimicrobial compounds, cell wall-associated defence (including strengthening of the cell wall in certain areas) and programmed cell death in order to restrain disease development (Abramovitch & Martin 2004). RNA metabolism and vesicle trafficking have also been suggested to be part of the plant's immune response to pathogens (Boller & He 2009).

1.4.2 Plant hormone signalling

Ethylene is an important plant hormone that is involved in several processes in plants including morphogenesis, growth and senescence. In addition ethylene acts as a signal molecule during biotic and abiotic stress conditions (Bleecker & Kende 2000). Lund and co-workers (1998) showed that ethylene is also an important signal molecule for disease development in susceptible tomato plants during *P. syringae* pv. *tomato* DC3000 infection, where it regulates necrosis in leaves during the latter stage of the host response. More recently, it has been shown that the effectors AvrPto1 and HopAB2 (formerly AvrPtoB) are responsible for enhancing ethylene production by regulating genes in ethylene biosynthesis and signalling pathways (Cohn & Martin 2005).

Jasmonic acid (JA) is another plant hormone that functions as a signal molecule associated with plant responses to herbivory, wounding and pathogenic invasion (Cohn & Martin 2005). Non-host suppression of *Pst* DC3000 requires a functioning JA signalling pathway in the plant (Nomura *et al.* 2005). However, JA has also been shown to be important for *P. syringae* pv. *tomato* DC3000 in causing disease on susceptible plants. The signalling cascade that arises from the presence of JA, including expression of proteinase inhibitor and polyphenol oxidase genes is contributing to disease development (Zhao *et al*. 2003).

Ethylene is synthesized rapidly and transiently after wounding or induction by JA, and acts with JA to induce wound response (Dong 1998). JA and ethylene also regulate another resistance response, the induced systemic resistance (ISR) that can be triggered

in roots by rhizobacteria and is independent of salicylic acid (SA) and pathogenesis-related (PR) gene expression (Dong 1998) (see 1.4.3). In conclusion, jasmonic acid and ethylene are important alternative signals in activating defence against pathogenic bacteria (Dong 1998).

1.4.3 The hypersensitive response and systemic acquired resistance

When *P. syringae* pv. *tomato* DC3000 infects a susceptible host plant, the result is an increase in the amount and spread of bacteria, which in turn eventually leads to necrosis (Lindgren *et al.* 1986). On the other hand, when infecting a non-host or a resistant plant, the bacterium elicits the hypersensitive response, first described for pseudomonads by Klement *et al*. (1964). When HR occurs, there is rapid localized cell death at the site of infection in 12 to 24 h after inoculation and multiplication of the bacteria is restricted (Bender *et al.* 1999). For an HR to occur, physiological changes take place including rapid oxidative burst, ion fluxes, cellular decompartmentalization, strengthening of the cell walls, production of antimicrobial compounds and induction of PR proteins (Staskawicz *et al*. 1995). The PR proteins are secreted into the apoplastic space where the pathogen grows and can attack proteinaceous components that are important for pathogen virulence (Zhao *et al*. 2003). This defence reaction is not only elicited by *Pst* DC3000 but is a rather common plant defence event. Following HR, plants are known to activate a SA-dependent resistance mechanism throughout the whole plant; the systemic acquired resistance (SAR), which reduces the severity of infections by any type of pathogens (Staskawicz *et al*. 1995, Dong 1998, Nomura *et al*. 2005). SAR is the result of activation of several PR genes by SA, but also the ROSs produced during HR may be involved in activation of this system (Dong 1998).

1.4.4 Plant resistance genes prevent disease

Resistance in a plant is the result of a complex interplay between bacterial effectors and plant proteins. Because part of the effectors might be recognized by the plant rendering the bacterium avirulent, some of the effector protein genes have been named avirulence (*avr*) genes (Chang *et al.* 2005). The Avr proteins are nowadays also referred to as effector proteins (Büttner & Bonas 2006). For a plant to be resistant against a certain

pathovar, the plant needs to possess an *R* gene that is compatible with a specific *avr* gene of the attacking pathovar (reviewed by Flor 1971, Scofield *et al.* 1996, Tang *et al.* 1996, Parker & Coleman 1997). Plant susceptibility is the result of interaction between the plant and the invading organism when either a compatible *R* gene is lacking from the plant or a recognizable *avr* gene is missing from the invader (Martin *et al.* 1993). The presence of compatible gene pairs often leads to the HR (Tang *et al.* 1996). The HR has been identified in interactions between plants and several kinds of pathogens like bacteria, viruses, fungi and insects (Scofield *et al.* 1996). Most *R* genes are structurally alike in that they contain a nucleotide binding site followed by leucine-rich repeats, consisting of tandem repeats of 20-30 amino acids with abundance of leucines and other hydrophobic residues (Parker & Coleman 1997, Grant *et al.* 2006).

1.4.5 Resistance against *P. syringae* pv. *tomato* in tomato

Sensitivity to different bacteria varies greatly between tomato cultivars. Some of the cultivars have developed resistance to *Pst* DC3000 (e.g. cultivar Rio Grande) while others have remained sensitive to this bacterium. The *R* gene that confers resistance against *Pst* DC3000 in tomato is the gene *Pto* on chromosome five (Pitblado *et al.* 1984). This gene differs from many other *R* gene products in that it does not contain any leucine-rich repeats (Scofield *et al.* 1996), but rather constitutes a cytoplasmic serine/threonine protein kinase (Martin *et al.* 1993). Two *Pst* DC3000 effectors, AvrPto1 and HopAB2, both interact physically with Pto (Abramovitch *et al.* 2003).

AvrPto1 is a kinase inhibitor (Zhou & Chai 2008) that suppresses cell-wall based defence in susceptible plants (Grant *et al*. 2006). This was the first effector that was demonstrated to suppress basal defence in *Arabidopsis* by suppressing papillae formation (Hauck *et al.* 2003). The *avrPto1* gene is expressed in the presence of sugars and sugar alcohols and regulated by the functions of the hypersensitive response and pathogenicity (*hrp)* genes (see 1.5.2) *hrpS*, *hrp*R and *hrpL*, which together generally regulate *hrp* genes inducing the HR (Salmeron & Staskawicz 1993). The *avrPto1* gene product AvrPto1 is translocated into the host cytosol upon infection and recognized by Pto in tomato (Scofield *et al.* 1996, Tang *et al.* 1996). Tang and co-workers (1996) showed that AvrPto1 functions as a bacterial signal molecule that interacts physically with its receptor Pto. At that time, this specific interaction supported a ligand-receptor

model for the recognition of the pathogen (Scofield *et al.* 1996). However, more recent research has suggested that the interaction between effector proteins and their compatible host resistance proteins is indirect (reviewed by Grant *et al.* 2006). In this model, the avirulence gene products can target several different plant defence machineries and the resistance proteins guard these machineries, recognizing effectors upon interaction with some defence machinery components. According to this model, the recognition then leads to a defence response and HR.

HopAB2, the other effector interacting with Pto, suppresses programmed cell death (Grant *et al.* 2006) by degrading host protein kinases through ubiquitination and proteasomal degradation mediated by its C-terminal E3 ubiquitin ligase domain (Ntoukakis *et al*. 2009). Recent studies have suggested that in addition to Pto, another protein with R protein properties, the Pseudomonas resistance and fenthion sensitivity (Prf) protein, is also required for elicitation of the HR by activation through a conformational change in Pto (Grant *et al.* 2006, Zhou & Chai 2008). Pto is highly related to another tomato kinase named Fen that also forms a complex with Prf (Ntoukakis *et al*. 2009). Ntoukakis et al. (2009) recently showed that Fen is degraded by HopAB2 through ubiquitination in sensitive plants, but in resistant cultivars containing a Pto-Prf complex HopAB2 is recognized by Pto, which inactivates the E3 ligase domain through phosphorylation.

1.5 *P. syringae* factors contributing to virulence

During infection by *P. syringae*, there are many different factors contributing to virulence. The most important of these virulence factors is the type III secretion system that enables secretion of plant defence targeting effector proteins into the host plant cells. *P. syringae* is also a talented producer of phytotoxins that influence disease development. In addition to the T3SS and toxin production, the bacterium is able to increase the severity of the disease through enzymatic scavenging of reactive oxygen species produced in the plant as a means of pathogen defence, and through production of cell wall-degrading enzymes (Buell *et al*. 2003).

1.5.1 Phytotoxins

Phytotoxins are compounds produced by plant pathogens that injure the host cells and play a role in disease development. The phytotoxins produced by *P. syringae* (reviewed by Bender *et al*. 1999) are among the most well-characterized bacterial phytotoxins. Most toxins produced by *P. syringae* lack host specificity and are not required for pathogenicity, but they act as virulence factors increasing the severity of the disease. Phytotoxins produced by *P. syringae* include syringomycin and syringopeptin, coronatine, phaseolotoxin and tabtoxin. These phytotoxins generally induce chlorosis or necrosis in the plant.

The phytotoxin coronatine (COR), which is produced by *Pst* DC3000 and four other *P. syringae* pathovars, acts as an analogue of methyl jasmonate, the activator of the JA signalling pathway (Bender *et al.* 1999, Zhao *et al*. 2003, Nomura *et al*. 2005). COR induces chlorosis in many different plants (Bender *et al.* 1999) - including tomato leaves during infection by *Pst* DC3000 - and can be observed as halos around the bacterial specks (Cohn & Martin 2005). In *Pst* DC3000, synthesis of COR is strongly induced when the bacterium is in contact with a host plant (Ma *et al.* 1991) and might be regulated by interactions with the T3SS system (Fouts *et al.* 2003). COR and T3SS effectors have been suggested to have overlapping functions involved in host defence suppression (Nomura *et al.*, 2005). Virulent strains of *P. syringae* are able to express the proteinase inhibitor and polyphenol oxidase genes in tomato leaves through COR-induced systemic signalling (Zhao *et al*. 2003). Another important part of the virulence of *Pst* DC3000 is the activation of JA responses through COR and suppression of PR genes through the action of COR and effector proteins (Zhao *et al*. 2003).

1.5.2 The type III secretion system and effector proteins

Currently six different protein secretion systems involved in microbial pathogenesis have been identified in Gram-negative bacteria. These are the type I through type VI secretion systems (figure 1), with the last one named in 2006 (Pukatzki *et al.* 2006). The type III secretion system is the most important factor for the virulence of *P. syringae* pv. *tomato*.

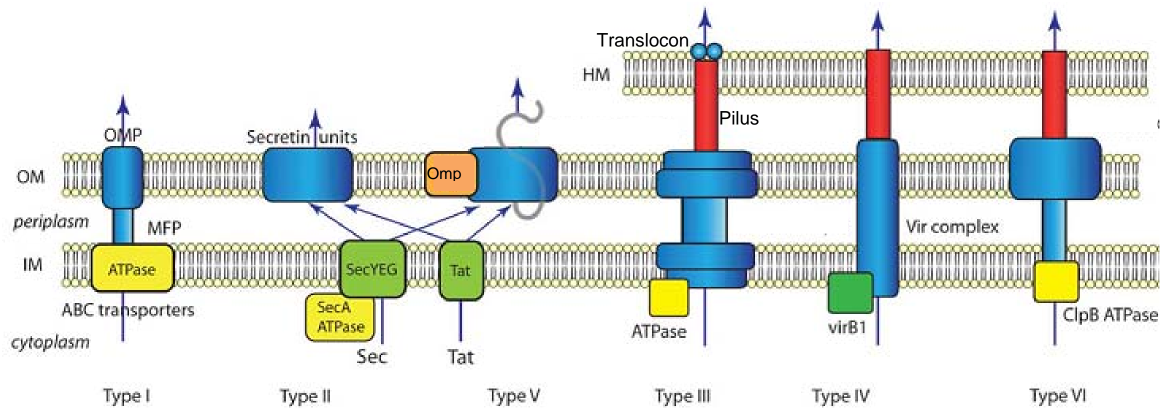


Figure 1. Simplified overview of the type I through type VI secretion machineries (Adapted from Tseng *et al*. 2009). OM = outer membrane, IM = inner membrane, HM = host membrane, OMP = outer membrane protein, MFP = membrane fusion protein.

The type III secretion system (T3SS) is a *sec*-independent protein secretion system that enables Gram-negative bacteria - both plant and animal pathogens - to inject virulence proteins from the bacterial cytoplasm into a eukaryotic host cell, passing both bacterial and host membranes and for plants also the host cell wall (Hueck 1998, He *et al.* 2004b). However, not only pathogenic bacteria use this system but it has also been found in several symbiotic *Rhizobium* strains where it plays a role in nodule formation (Viprey *et al.* 1998, Marie *et al.* 2001). While the genes encoding the actual secretion apparatus are well conserved between different pathovars, the secreted proteins differ a lot (Hueck 1998). Using the T3SS, different bacteria cause different diseases in their host plants: Fire blight of apple trees, pear trees and rosebushes as well as soft rot caused by different *Erwinia* spp., bacterial spot disease of pepper and tomato caused by *Xanthomonas campestris*, bacterial wilt caused by *Ralstonia Solanacearum* on over 200 different plants, and various diseases caused on the host plants of *Pseudomonas syringae* pathovars (Hueck 1998).

Bacteria use the T3SS to transfer so called effector proteins into the host cytoplasm. This ability of transferring effector proteins was first demonstrated for the *Yersinia enterocolitica* YopE cytotoxin (Rosqvist *et al*. 1994). In plants these effector proteins play a role either in causing the disease or in eliciting the genotype-specific HR when the corresponding resistance gene is present (Chang *et al.* 2005, Gopalan *et al.* 1996, Scofield *et al.* 1996) (see 1.4.4). For this reason, the T3SS of *P. syringae* is often called the hypersensitive response and pathogenicity (Hrp) system (Collmer *et al.*

2002). The set of effector proteins secreted by a certain pathovar also determines the host specificity at both pathovar-species and race-cultivar level (Fouts *et al.* 2003). Recent studies indicate that the number of functional effector proteins in *P. syringae* pv. *tomato* DC3000 would be 29 (Chang *et al.* 2005), although different studies have proposed varying effector numbers. Schechter *et al.* (2004) claimed that the total amount of effectors in *P. syringae* would be 40. Nevertheless, the host cellular targets of most effector proteins are still unknown (Büttner & Bonas 2006). However, currently a large number of effectors with plant defence suppressing activity have been identified in *P. syringae* (reviewed by Grant *et al.* 2006). A list of all currently known effectors can be found at www.pseudomonas-syringae.org.

The T3SS, and hence the ability of *Pseudomonas syringae* to induce HR in resistant plants as well as pathogenesis in susceptible plants, is dependent on a cluster of genes called *hrp* genes (Lindgren *et al.* 1986, He *et al.* 2004b). Part of these genes are called *hrc* genes (for hypersensitive response and conserved) because they are conserved among animal pathogens. The *hrp-hrc* genes are localized in the middle of a pathogenicity island (Pai) in the chromosomal DNA (Hueck 1998, Alfano *et al.* 2000), flanked by a unique exchangeable effector locus (EEL) and a conserved effector locus (CEL) (Alfano *et al.* 2000) (figure 2). The genes within the EEL contribute to the parasitic fitness of the pathogen, the *hrp* (*hrp-hrc*) gene cluster is responsible for the effector protein secretion machinery, while the CEL locus is not involved in effector secretion but is required for pathogenicity of *Pst* DC3000 by its strong contribution to parasitic fitness (Alfano *et al.* 2000).

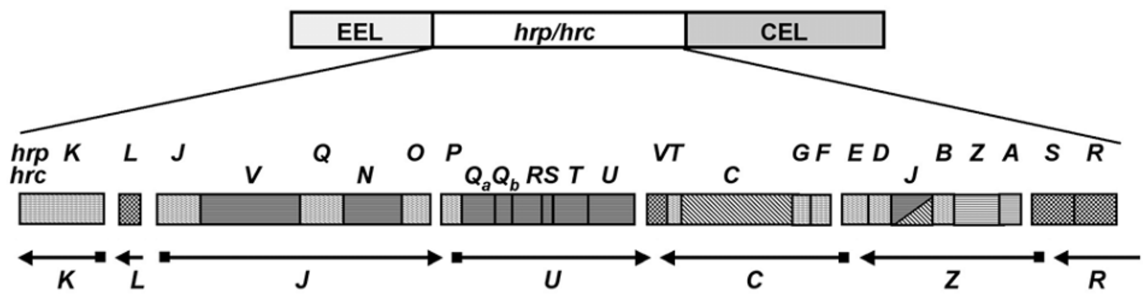


Figure 2. Representation of the *P. syringae* pv. *tomato* DC3000 pathogenicity island (adapted from Fouts *et al.* 2003). The organization of the hrp and hrc genes of the hrp-hrc cluster is marked above the boxes representing the genes. Names of the operons are indicated below the boxes, with arrows indicating the direction of transcription.

The *P. syringae* type III secretion machinery is composed of a needle-like complex: a series of basal rings that span both bacterial membranes, a hollow extracellular filamentous appendage called Hrp pilus that is anchored in the bacterial cell wall, and perhaps a translocon complex that is inserted into the host plasma membrane (He *et al.* 2004b, Tseng *et al.* 2009, Büttner & Bonas 2006). The whole machinery is composed of about 20 different components, mostly situated in the inner membrane (Hueck 1998, Büttner & Bonas 2006). A cytoplasmic ATPase that associates with the secretion machinery is required for translocation of T3SS proteins across the plant cell wall (Hueck 1998, Tseng *et al.* 2009). The Hrp pilus of *Pst* DC3000 is built up by HrpA1 subunits, and produces a 6-8 nm thick pilus that is constructed when the *hrp* genes are induced on hrp-inducing medium (Roine *et al.* 1997a). HrpA1 alone was also shown to be sufficient for formation of the filament structure (Roine *et al.* 1997b), which is assembled by adding HrpA1 subunits to the distal end of the growing pilus, suggesting that it functions as a conduit (Li *et al.* 2002).

When the T3SS is built up, Hrp pilus and translocon proteins are first secreted into the extracellular milieu, after which the effector proteins can be translocated into the host plant cytoplasm (Büttner & Bonas 2006). Many of the effectors are efficiently translocated due to chaperones that protect the effectors from premature folding or degradation, while others do not seem to have any interacting chaperones (Galán & Collmer 1999, Büttner & Bonas 2006). Some effectors interact with chaperones that do not directly target them to the secretion apparatus but rather seem to function as anti-association factors that prevent the effectors from premature interaction with proteins

involved in their secretion (Woestyn *et al.* 1996). It is still not absolutely clear how effector proteins are selected for type III secretion, but there seems to an uncharacterized signal in the N-termius of these proteins (Petnicki-Ocwieja *et al.* 2002, Schechter *et al.* 2004, Büttner & Bonas 2006). Some effectors have also been proposed to have the translocation signal in their mRNA (Anderson & Schneewind 1997).

There are two classes of type III secreted proteins: effectors that are transported into the host cell and proteins that stay outside the host cell cytoplasm - so called harpins or helper proteins. Harpins are *hrp*-encoded, hydrophilic, heat-stable proteins that are glycine-rich and devoid of cysteine and elicit the HR when purified and applied to nonhost tobacco plants (He *et al.* 1993, Preston *et al.* 1995, Collmer *et al.* 2002). Harpins are likely to be helper proteins that have functions in translocation of real effectors through host barriers (Collmer *et al.* 2002). Together, all proteins secreted or translocated by the T3SS - the effectors and helper proteins - are called Hrp outer proteins (Hops) (Collmer *et al.* 2002).

T3SS effectors are involved in suppressing PRR signaling and PTI-associated immune responses by targeting PRRs or the downstream signalling cascades (Boller & He 2009). Some effectors have been shown to suppress cell death of the host, meaning that the plant may recognize the effector but the HR is not elicited because the effector dominantly suppresses HR signalled programmed cell death if it is beneficial for the bacterium (Abramovitch & Martin 2004). Type III effector proteins may also suppress or limit cell-wall defence responses (Hauck *et al.* 2003) or activate jasmonate signalling together with the phytotoxin coronatine, which further leads to suppression of salicylic acid-dependent defences (Zhao *et al.* 2003, He *et al.* 2004a). Work by Cohn and Martin (2005) indicated that expression of over 300 genes in susceptible tomato plants, mostly involved in biosynthesis of hormones that are associated with pathogen attack and wounding, are altered by the impact of the hrp regulon within 24 h after inoculation of *Pst* DC3000.

1.5.3 Other secretion systems

The type III secretion system is not the only protein secretion system applied by bacterial pathogens. Also *P. syringae* has other secretion systems, although these are not as important for its virulence as the T3SS.

The type I secretion system (T1SS), also called the ABC transporter system (Fath & Kolter 1993), is a single step secretion pathway. The model system for this type of secretion is the secretion of the *Escherichia coli* haemolytic toxin (HlyA), which was first described by Felmlee and co-workers (1985). The type I secretion system requires three accessory proteins that form a transport channel for the proteins to be secreted: an inner membrane ATP-binding cassette (ABC) protein that provides the energy required for the secretion, a pore forming outer membrane protein (OMP), and a membrane fusion protein (MFP) that links the two other components together (Finlay & Falkow 1997, Hueck 1998). The secretion signal is located in the C-terminal region of the secreted protein, so no N-terminal signal or amino-terminal processing of the protein is needed (Finlay & Falkow 1997, Hueck 1998). In *P. syringae* pv. *tomato*, some virulence factors have been found to adapt this system (Tseng *et al.* 2009).

The type II secretion system (T2SS) involves two different machineries to transport proteins from the cytoplasm past the outer membrane (Finlay & Falkow 1997, Hueck 1998, Johnson *et al.*, 2006). This system is thought to be the most abundant secretion pathway among Gram-negative bacteria (Finlay & Falkow 1997, Hueck 1998). The T2SS was first observed in *Klebsiella pneuomoniae*, for the transport of the extracellular lipoprotein pullulanase (d'Enfert *et al.* 1987). It is also required for virulence of the plant pathogens *Ralstonia solanacearum*, *Pectobacterium atrosepticum* and *Xanthomonas campestris* pv. *campestris* (Tseng *et al.* 2009). In *Pst* DC3000, genes predicting components of this pathway have been identified (Buell *et al.* 2003). Export through this system requires a cytoplasmic protein that has an ATP-binding site, a Walker A box, which is in contact with the cytoplasmic membrane through several membrane proteins that reach into the periplasmic region (Johnson *et al.* 2006). The protein to be secreted is targeted by an amino-terminal signal peptide to either the main branch of the general secretory pathway (Sec) or the twin-arginine translocation (Tat) machinery, which export the protein from the cytoplasm to the periplasm in an unfolded or folded form, respectively (Voulhoux *et al.* 2001, Tseng *et al.* 2009). In the periplasm,

the signal peptide is cleaved by a periplasmic signal peptidase (Hueck 1998), and the folded protein is then secreted across the outer membrane though a secretion pore (Johnson *et al.* 2006).

The type IV secretion system (T4SS) (reviewed by Tseng *et al.* 2009) has a common ancestral history with the bacterial conjugation machinery (Henderson *et al.* 2004). The T4SS differs from the other secretion systems, because in addition to transportation of proteins it also transports nucleic acids. This secretion system complex can span both membranes of the bacterium and is found in many bacteria. In the plant pathogen *Agrobacterium tumefaciens*, the causative agent of crown gall tumour formation, the T4SS is involved in T-DNA transfer through the VirB system, which has been a model system for T4SS studies (Tseng *et al*. 2009). However, this system is not found in all bacteria, and the T4SS of different bacteria only have the same function; the proteins involved can differ widely. No functional type IV secretion system has been identified in *Pst* DC3000 (Buell *et al*. 2003).

Protein secretion via the type V secretion system (T5SS), also called the autotransporter secretion system, is a two-step process. The T5SS was first described for the gonococcal immunoglobulin A1 protease (reviewed by Klauser *et al.* 1993), and mostly play a role in the virulence of animal or human pathogens (Tseng *et al.* 2009). This secretion system has been divided into three sub-classes (Henderson *et al.* 2004, Tseng *et al.* 2009) depending on how the protein is secreted across the outer membrane. The most abundant subclass consists of the autotransporters that mediate their own transport through the outer membrane by pore formation.

The most recently characterized secretion system of Gram-negative bacteria is the type VI secretion system (T6SS). Like in the T3SS and T4SS, proteins of this system can be transported directly into the host cell cytoplasm (Tseng *et al.* 2009). This secretion system seems to be quite general in bacteria, because more than 25% of the sequenced bacterial genomes contain genes needed for this system (Tseng *et al.* 2009). The T6SS is required for virulence in animal and human pathogens as well as in plant pathogens, but has also been found in nitrogen-fixing plant mutualists and even non-symbiotic bacteria where it is believed to play a role in environmental adaptation (Tseng *et al.* 2009). Genes required for the T6SS have also been found in *P. syringae* (Records & Gross 2007) although its function in the virulence is still unknown.

1.6 HrpZ1

HrpZ1 is a harpin of *Pst* DC3000 and one of the first proteins to be secreted through the Hrp system. In the host cell, HrpZ1 interacts with a yet unknown host protein (Li *et al*. 2005). HrpZ1 binds to lipid bilayer membranes and forms ion-conducting pores and could therefore function to release nutrients from host plant cells (Lee *et al.* 2001). Since HrpZ1 travels through the elongating Hrp pilus and is secreted from the tip of the Hrp pilus only, it could also act as a mediator in a pilus-plant membrane interaction that enables translocation of effectors into the plant (Li *et al.* 2002). The *hrpZ1* gene is part of the *hrpZ* operon (Figure 2) and the downstream genes - *hrpB*, *hrcJ*, *hrpD* and *hrpF* - show varying homology to *Yersinia* spp. virulence genes that are secreted through the T3SS (Preston *et al.* 1995). The *hrpZ1* gene encodes a 370 aa long harpin protein, which is glycine-rich, heat stable and does not have any hydrophobic signal sequence or significant secondary structure (Preston *et al.* 1995).

Li and co-workers (2005) showed that HrpZ1 of *P. syringae* pv. *phaseolicola* binds to a peptide with the consensus sequence W(L)ARWLL(G/L), thus indicating interaction of HrpZ1 with an other protein. An antiserum was raised against a peptide with the sequence WARWLLLTPA, and this antiserum recognized small (13-20 kDa) proteins in protein extracts of bean, tomato, parsley and *A. thaliana*. For tomato, the estimated size of the recognized protein is 17 kDa. When the protein extracts were analyzed on denaturing isoelectric focusing gels, the recognized proteins of bean and tomato where found to be acidic with a pI of about 4.0. Although the antiserum recognized both denatured and native proteins, the isolated $HrpZ_{Pph}$ recognized only the native protein. The peptide-binding site of the *P. syringae* pv. *phaseolicola* HrpZ was shown to be in the middle region of the protein. Interestingly, this peptide-binding region shows homology to a region in the HrpN harpin of *Erwinia* spp. In *Pst* DC3000, there is an additional 24-aa sequence inside the region corresponding to the site that counts for binding peptides in $HrpZ_{Pph}$. This 24-aa stretch shows homology to part of PopA1 of *P. solanacearum*, another tomato pathogen, and therefore horizontal transfer and common ancestry has been proposed for these two proteins (Preston *et al.* 1995).

1.7 Methodological background

1.7.1 cDNA library

A cDNA (complementary DNA) library is a library containing DNA for genes that are expressed in a eukaryotic organism or in a specific tissue. The library is generated from isolated mRNA, which is converted into DNA (cDNA); the first strand is synthesized with reverse transcriptase and the second strand with DNA polymerase. The cDNA therefore does not contain regulatory regions or introns and can be expressed in bacterial cells. The synthesized double-stranded cDNA fragments are cloned into a suitable vector to be used for further research.

1.7.2 Autoradiography

Autoradiography is a method for detecting the site of a radiolabelled entity with the help of a photographic emulsion that is sensitive to radiation. Protected from light, the radioactive source is placed towards a radiosensitive photographic film that contains silverhalogenid crystals, usually silver bromide. The energy from the decay of the radioisotope hits the photographic emulsion and produces electrons. These electrons are trapped in electron traps formed by chemical impurities in the emulsion, which creates a negative charge that attract silver ions. When the photographic film is developed in a strongly alkaline liquid (called the developer), the metallic silver that is formed can be visualized. After the development, the film has to be put in fixer that releases the undeveloped silverhalogenid crystals from the emulsion, stops the development and makes the arisen picture permanent.

1.7.3 The phosphorus isotope ^{33}P

^{33}P is an isotope of phosphorus (P) which can be used for radiolabelling. It is a beta-active form of phosphorus with a half-life of 25.4 days. The beta emission energy of ^{33}P is much lower than that of the more widely used ^{32}P-isotope, and therefore ^{33}P is safer to

handle. The decay energy of the beta emission is 0.25 MeV (megaelectron volts). The decay scheme of ^{33}P is presented in appendix 1.

1.7.4 Liquid scintillation counting

Scintillation counting is an efficient method for measuring radioactivity in samples. Liquid scintillation counting is especially useful for quantification of disintegrations of radioactive samples that emit low energy beta particles. In this system, the radioactive sample is in direct contact with the liquid detector, i.e. the scintillation liquid, by resuspension of the sample to be measured in a vial with a liquid scintillation medium, a "cocktail", that contains an aromatic solvent and a solute. This vial is placed in a lightproof chamber in the liquid scintillation counter. The energy of the beta particles emitted from the sample is transferred to the medium, causing excitations in the cocktail and emission of photons. The photons are detected by two photomultiplier tubes that convert the scintillations (the number of photons produced) into electrical pulses that can be read. (Aalto *et al.* 1994)

1.7.5 Phage lambda in molecular applications

Bacteriophage lambda (λ) is a phage infecting *E. coli*. Upon infection the phage injects its DNA into the bacterium, which leads to either a lytic cycle or lysogeny. If the phage enters the lytic cycle, the bacteriophage DNA is replicated and new phage particles released after lysis of the host cell. If lysogeny occurs, the virus DNA becomes integrated into the *E. coli* chromosome as a prophage and will be maintained there until the cell is stressed or the host cell DNA is damaged in some way (Snyder & Champness 1997, Glick & Pasternak 2003). At this point, induction events and production of the integrase and excisase proteins will lead to excision of the phage DNA from the host chromosome and production of new phage particles (Snyder & Champness 1997). The bacteriophage lambda genome is a near 50 kb linear double-stranded DNA molecule with single-stranded complementary extensions at both ends, called cohesive (cos) ends (Snyder & Champness 1997, Glick & Pasternak 2003). Following infection, the cos sites of the injected DNA base pair to form a circular DNA molecule. From this molecule, the DNA is replicated to form a long linear concatemer with cos sites

approximately every 50 kb, from which genome length pieces are packaged into new phage heads with the help of an enzyme in the phage head that recognizes and cuts off the double-stranded cos sequence as one is encountered in the concatemer (Snyder & Champness 1997, Glick & Pasternak 2003). If less than 38 kb is packaged the resulting phage is noninfective, and 52 kb is the maximum amount of DNA that can fit into a head (Glick & Pasternak 2003).

Of the 50 kb lambda genome, approximately 20 kb is needed for the integration-excision events (Glick & Pasternak 2003) and can be replaced with cloned DNA. Lambda cloning vectors containing the proper origin of replication and the cos sites, and in which up to 20 kb foreign DNA can be cloned have been constructed. The Uni-ZAP XR vector used in this work can accommodate DNA inserts from 0 to 10 kb in length (Stratagene). During *in vitro* packaging, the λ vector containing inserted DNAs are mixed with extracts of λ-infected cells containing heads and tails of the phage (Snyder & Champness 1997). The DNA with the cos sites will thus be taken up by the heads and the lambda particles self-assemble into infectious λ particles *in vitro* (Snyder & Champness 1997). The cloned DNA can then be amplified and maintained by growing the bacteriophage on *E. coli*.

The bacteriophage λ is well suited for generation and screening of cDNA libraries, since the plaques that form on *E. coli* plates, indicating that a phage has infected a cell, can be lifted onto a matrix and screened with DNA probes or antibodies. Moreover, it is easy to store libraries in the stable lambda phage heads.

1.7.6 SDS-PAGE

The method of sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was originally described by Laemmli in 1970. Briefly, proteins are first treated with SDS and then separated according to their size by electrophoresis in a polyacrylamide gel. Usually, the proteins are, after separation, stained with a protein colouring agent (e.g. Coomassie blue) to make them visible, or transferred to a membrane for further studies.

1.7.7 Immunoblotting

The concept of Western blotting was introduced by Burnette (1981) as a common name for the immunoblotting procedure. It is a method for electrophoretic transfer of proteins from SDS-PAGE gels to membranes - usually polyvinylidene fluoride (PVDF) or nitrocellulose - and the subsequent immunological detection procedure. A specific primary antibody is used, followed by a secondary antibody conjugated to a detectable agent, currently mostly alkaline phosphatase (AP), which can be detected by adding a substrate for the enzyme and using a suitable detection method for the enzymatic activity.

5-bromo-4-chloro-3-indolyl-phosphate (BCIP) is used in conjunction with nitroblue-tetrazolium (NBT) for the colorimetric detection of alkaline phosphatase activity. When alkaline phosphatase reacts with this substrate, a dark purple precipitate appears at the site of AP activity, usually on a membrane where it can be easily detected. Detection using AP and BCIP/NBT was the first standard methodology described when biotinylated nucleotides were developed as labels for nucleic acids in hybridization analyses (Leary *et al.* 1983). This kind of colorimetric detection functions well with nitrocellulose and PVDF membranes (Düring 1993).

Disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1.^{3,7}]-decan)-4-yl]phenylphosphate (CSPD) is an alternative to BCIP/NBT in AP detection. This is a derivative of the 1,2-dioxetane disodium 3-[4-methoxyspiro(1,2-dioxetane-3,2'-tricyclo[3.3.1.1.^{3,7}]decan)-4-yl]phenylphosphate (AMPPD), where a chlorine atom has been added to the adamantyl group (Martin *et al.* 1991). Alkaline phosphatase can activate AMPPD to chemiluminescence at 470 nm, enabling detection of 10^{-20} moles of AP on membranes (Bronstein *et al.* 1989). When AMPPD is cleaved by the enzyme at alkaline pH, the compound decomposes and eventually chemiluminescent light is emitted as a result of a two step reaction (Bronstein *et al.* 1989). CSPD gives much faster reactions than AMPPD (Martin *et al.* 1991) and the sensitivity of this method compared to the BCIP/NBT colorimetric detection should be about 10 to 20 times better (Düring 1993). The chemiluminescent signal emitted by CSPD is in the green light range and can be detected on x-ray films (Düring 1993).

Immunoscreening resembles the immunodetection part of immunoblotting. Clones containing protein coding DNA are induced to produce these proteins, after which cells

are lysed and protein products attached to a membrane. Detection of target proteins is performed using the same technique as in the immunoblotting part of western blotting: washing, attachment of primary and secondary antibodies and detection of these with a suitable detection method. The clone containing the DNA encoding the target protein can then be isolated by comparison.

2 Aims of the study

This project was initiated by doc. Suvi Taira and conducted at the division of general microbiology at the University of Helsinki. Tomato appears to have more disease-related genes and more complex signaling pathways than the common model plant *Arabidopsis thaliana*. Therefore the construction of a cDNA expression library from tomato could be useful in the pursuit of target proteins for effectors of the model pathogen *Pseudomonas syringae* pv. *tomato* DC3000, which infects both of these plants. Identification of the target proteins for effectors would also provide valuable information about the signalling pathways the proteins are involved in.

The specific aims of this study are:

- To construct a cDNA expression library from tomato leaves, to be used in identifying targets of effector proteins of *P. syringae* pv. *tomato* DC3000
- Screening the cDNA expression library for the target protein of HrpZ1, using an existing antiserum raised against a peptide recognized by HrpZ1.
- To gather information about the protein identified by immunoscreening through sequencing of the corresponding cDNA and comparing the sequence to existing sequence data, as well as determining the amino acid sequence size and the pI of the protein.

3 Materials and methods

3.1 RNA isolation

RNA was isolated from six to eight weeks old tomato (*Solanum lycopersicum* cultivar Agriset) plants that had been cultivated indoors; first four weeks in +22ºC under a 400 W daylight lamp (Idman) with 12 h light and 12 h darkness, and the rest of the time in +22ºC by the window with natural light conditions. The total RNA isolation was done with RNeasy Plant Mini Kit (Qiagen), using approximately 0.9 g of leaf material in 20 vials. The tomato leaves were carefully crushed in a mortar surrounded by liquid nitrogen. The material was kept frozen also during weighing, until RLT buffer (Qiagen) was added. Centrifugations were performed at 16 060 ×g (Heraeus Biofuge pico, Thermo Scientific) and the samples were treated with DNase during the isolation (RNase free DNase set, Qiagen). The RNAs of vials 1 to 14 was eluted in two times 40 µl of RNase-free water each. The two aliquots were then combined for each vial. Elution from vials 15 through 17 was done with 40 µl plus 20 µl water and for vials 18 through 20 the same 40 µl was used twice. Concentrations of the total RNA samples were determined as 1:70 dilutions in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) with a Biophotometer Plus spectrophotometer (Eppendorf). Samples with a concentration less than 600 ng/µl were combined and precipitated with 1/10 the sample volume of sterile and RNase-free 4M lithium chloride, and 2.5 times the sample volume of absolute ethanol. Precipitation was performed over night in -70ºC, after which the samples were centrifuged (13 000 ×g) for 45 min at +4ºC (Heraeus Fresco 17, Thermo Scientific) and washed with 70% ethanol that was left to evaporate before the RNA was resuspended in 50 to 80 µl of RNase-free water. After combining and precipitating samples to obtain concentrations of at least 600 ng/µl, the 12 RNA samples were analyzed using an Agilent 2100 Bioanalyzer (Agilent Technologies) and a RNA 6000 Nano LabChip (Agilent Technologies) to check the quality of the isolated RNA.

From the total RNA, mRNA was isolated using Poly(A) Purist™ MAG Kit (Ambion). After adjusting the concentration of all samples to 600 ng/µl with nuclease-free water, the samples were combined and then divided into two tubes to get approximately 210 and 420 µg mRNA respectively in the tubes. Following this, the kit

protocol was followed to acquire the mRNAs from the total RNA samples. In the end, the concentrated samples were combined and the concentration measured with Biophotometer plus (Eppendorf) as a 1:60 dilution in TE buffer. The mRNA was then stored in -70ºC.

3.2 cDNA expression library construction

3.2.1 Synthesis of first and second strands

A cDNA expression library was constructed from the isolated mRNA using ZAP-cDNA Synthesis Kit and ZAP-cDNA Gigapack III Gold Cloning Kit purchased from Stratagene. The first strand of the cDNA was synthesized with reverse transcriptase, using the isolated mRNA as template and a 50-base oligonucleotide primer containing a "GAGA" sequence, an *XhoI* restriction enzyme recognition site and an 18-base poly(dT) sequence. Because the isolated mRNA was used as such, without treating it with methylmercury hydroxide to open the secondary structures, the first-strand synthesis protocol was slightly modified: only 0.5 µl of RNase Block Ribonuclease Inhibitor was first added into the first-strand synthesis reaction mixture; thereafter the mixture was incubated at 65ºC for seven minutes and finally put on ice for ten minutes for the primer annealing and an additional 0.5 µl of RNase Block Ribonuclease Inhibitor was added after two minutes on ice. A radioactive label, α-^{33}P deoxyadenosine 5'-triphosphate (dATP [α-^{33}P], Perkin Elmer) was used in the synthesis to enable monitoring of the synthesis steps. The second strand of the cDNA was synthesized using RNase H and DNA polymerase.

After synthesis of the second strand, samples from both the first-strand synthesis reaction and the second-strand synthesis were run on a 1% alkaline agarose gel (100 to 125 mA) and transferred to a nylon membrane, followed by autoradiography in order to visualize the synthesis products. The transfer of the cDNA synthesis products onto the Hybond-N nylon transfer membrane (Amersham) was performed over night by capillary blotting using 0.4 M sodium hydroxide as transfer buffer. The membrane was left to air dry before it was put against a Super RX x-ray film (Fujifilm) in a cassette with screens that was then left in -70ºC for 26 h before the film was developed. Development was performed by keeping the film in developer solution (Kodak) for two minutes, then

rinsing in water for one minute and finally fixing in Fix solution (Kodak) for one minute. After the second strand synthesis, the termini of the double-stranded cDNA were blunted and ligated to *Eco*RI adapters, which were then phosphorylated to enable ligation to Uni-ZAP XR vector arms (Stratagene). The cDNAs were then digested with *Xho*I to release the *Eco*RI adapter from the 3' end of the cDNA and size fractionated using a drip column with Sepharose CL-2B gel filtration medium (Stratagene), as described in the kit manual.

3.2.2 Size fractionation and ligation to vector arms

During size fractionation, fraction collection was started immediately after the STE buffer (Stratagene) was added following loading of the sample. This was done to be sure that all the large fragments eluting first would be captured. Because the Geiger counter did not detect any radioactivity in most of the fractions, fractions were collected until all of the loading dye had eluted. The fractions were then analyzed with a 1450 Microbeta Trilux liquid scintillation counter (Wallac) using 4 µl of each fraction together with 1.5 ml of OptiPhase 'HiSafe' 2 scintillation cocktail (Wallac). Based on the results from this analysis, fractions most probably containing the largest cDNAs were selected for ligation to vector arms. Before ligation, after concentrating and pooling the fractions, the concentration of the cDNA sample was determined using an ethidium bromide plate assay where 0.5 µl of the cDNA sample and known standards was analyzed, as described in the ZAP-cDNA synthesis Kit manual's appendix 5. All of the cDNA was then ligated to Uni-ZAP XR vector arms, so that 2 µl of cDNA was used for the first ligation, and 1.5 µl of cDNA for three more ligations. These three later ligations were then combined. The cDNAs were ligated in a sense orientation (*Eco*RI-*Xho*I) with respect to the *lacZ* promoter of the pBluescript vector (figure 3).

3.2.3 Packaging of cDNA into phage and construction of a primary library

The cDNA ligated to the vector arms was packaged into lambda phage using the Gigapack III Gold packaging extract (Stratagene). This was done in two phases, so that five packaging reactions were first performed using 1 µl of cDNA from the first ligation. Following this test, four more packagings using 2 µl per packaging of cDNA

from the combined second round ligations were prepared. One packaging reaction from both packaging batches and a ligation control were titered and the efficiency of the Gigapack III packaging extract tested according to the Stratagene protocol. However, differing from the instructions, the host bacteria were pelleted at 4300 ×g for 15 min (Megafuge 1.0, Heraeus Instruments). The background was also determined by blue-white colour selection of the same two cDNA samples as used for titering.

The packaged cDNA was amplified according to Stratagene's instruction manual, from phage plated on 24 large 150 mm plates and suspended into SM buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgSO_4$). The final suspension containing the amplified primary library was titered as a dilution series (10^{-2} to 10^{-8}) to obtain a plate containing a suitable amount of plaques for determining the titer. From this library, bacteriophage were plated onto ten 150 mm plates to form a total of one million plaques to be used for immunoscreening.

3.3 Immunoscreening of the cDNA library

3.3.1 Optimization of the screening

In order to find the best method for immunoscreening of the cDNA library, tests were performed using 100 mm plates. The plates were prepared according to the *pico*Blue Immunoscreening Kit instruction manual (Stratagene), sections "Plaque screening" and "Antibody and alkaline phosphatase incubations". Expression of the cDNAs from the lac promoter was first induced with isopropyl-β-D-1-thiogalactopyranoside (IPTG) by placing a Protran nitrocellulose transfer membrane (Whatman) that had been soaked in 1 mM IPTG and dried, onto the plate and incubated like this for 4 h. After this first incubation, the nitrocellulose membrane was removed from the plate and replaced with a second similarly prepared membrane and incubated in the same way to make a replica. The membranes were then washed before the primary antibody was introduced and again washed before and after application of the secondary antibody. For the tests, a polyclonal antibody against Ribulose-1,5-bisphosphate carboxylase oxygenase (Rubisco) raised in rabbit (Agrisera) was used in 1:1000 dilution as primary antibody, and a monoclonal anti-rabbit IgG-alkaline phosphatase conjugate (Sigma) in 1:10000 dilution as secondary antibody.

To verify that the primary antibody was functioning well, SDS-PAGE and immunoblotting were first done with a protein preparation from the same plant material. The protein preparation protocol was adapted from a plasma membrane isolation protocol (Nieminen 2001). Fresh tomato leaves were first ground in liquid nitrogen and weighed. For each gram of plant material, 2 ml of homogenisation medium (50 mM MOPS-KOH pH 7.5, 5 mM EDTA, 0.33 M sucrose, 0.6% (w/v) PVP, 5 mM ascorbic acid, 3 mM DTT) was then added together with the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) that was added to a final concentration of 1 mM. The homogenate was then filtered through a Miracloth polyester filter, centrifuged at 7000 ×g for 15 min at +4ºC and the supernatant recovered as the protein preparation. Part of the protein preparation was run in SDS-PAGE and immunoblotting performed, using polyvinylidene fluoride (PVDF) membrane (Whatman). TBS with 0.05% (v/v) Tween was used as the blocking agent, and the antibody dilution was 1:1000. The primary antibody incubation was performed over night and the secondary antibody incubation for 1 h. Detection was performed with BCIP/NBT substrate (Promega).

In the immunoscreening, the primary antibody was allowed to bind to the membrane for two hours instead of one hour, and the final membrane washing step of the membranes was done with the colour detection solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) for five minutes. Detection of sites of attachment of the antibodies was performed in two ways: one of the replicas was exposed to a BCIP/NBT (Promega) colour development reaction, and the other one to a light producing CSPD (Roche) reaction and detection on an x-ray film. The BCIP/NBT reaction was performed by submersing the membrane in readily prepared BCIP/NBT solution after the final wash, and keeping the membrane in the solution with gentle rocking until colour appeared. The colour reaction was then terminated by rinsing the membrane in cold water. For the CSPD reaction, the CSPD substrate was diluted 1:100 in detection buffer and 800 µl of the diluted substrate was spread evenly on the membrane which was then wrapped tightly in plastic film and kept in darkness in +37ºC for 15 min before placing it against an x-ray film to be developed.

3.3.2 Library screening

Immunoscreening of the actual cDNA library followed the same scheme as described above (see 3.3.1) for the test screenings. Here, the anti-ZBP3 antiserum raised in rabbit against a peptide sequence, obtained through phage display experiments with *Pseudomonas syringae* pv. *tomato* DC3000 HrpZ1 (Li *et al.* 2005), was used as primary antibody. Differing from the instructions, this primary antibody was incubated with the membranes over night. As secondary antibody, the same anti-rabbit IgG as in the test screenings was used in the same concentration. Detection of possible positive clones was done with the BCIP/NBT colorimetric assay. Putative positive plaques were isolated from the primary screening plates and transferred into 150 µl of SM buffer together with some microliters of chloroform, vortexed and left to diffuse into the buffer over night at +4ºC, whereupon secondary and tertiary screenings were performed in the same way. Clones giving rise to a colorimetric signal differing from other plaques after detection were considered good candidates for further research.

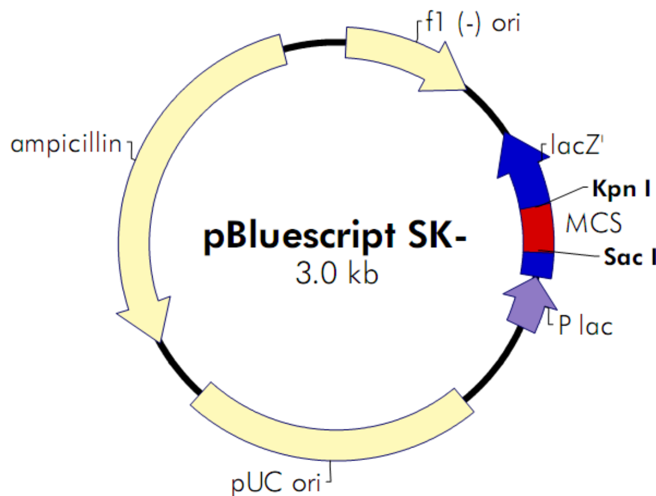
3.4 Verification of the contents of selected clones

3.4.1 *In vivo* excision of cDNA-containing plasmids and cDNA sequencing

The cDNA content of the phage clones that were most promising was excised as part of a pBluescript phagemid (figure 3A) and transferred to the *E. coli* strain SOLR with the help of the ExAssist helper phage, as described in Stratagene's ZAP-cDNA Synthesis Kit protocol. The phagemids were then isolated using QIAprep Spin Miniprep (Qiagen). *Eco*RI digestion of the phagemids was performed, followed by electrophoresis on a 1% agarose gel, to find out the approximate size of the cDNA inserts. The cDNAs of the chosen clones were then analyzed by sequencing. The clone identified with the help of the anti-Rubisco antibody was sequenced using the T7 primer and the others with either the SK primer or the T3 primer (figure 3B). Sequencing was done by the DNA sequencing service of the Institute of Biotechnology (University of Helsinki). The obtained sequences were then compared to the NCBI (National Center for Biotechnology Information) databases (http://blast.ncbi.nlm.nih.gov/) using BLAST

(Basic Local Alignment Search Tool) (Altschul *et al.* 1990) algorithms blastn and blastx. The DNA sequence data was also translated into protein sequences using the Translate tool on the ExPASy server (http://au.expasy.org/tools/dna.html). Based on the acquired sequences, peptide sequences resembling the sequence used to make the antiserum were searched for.

A.



B.

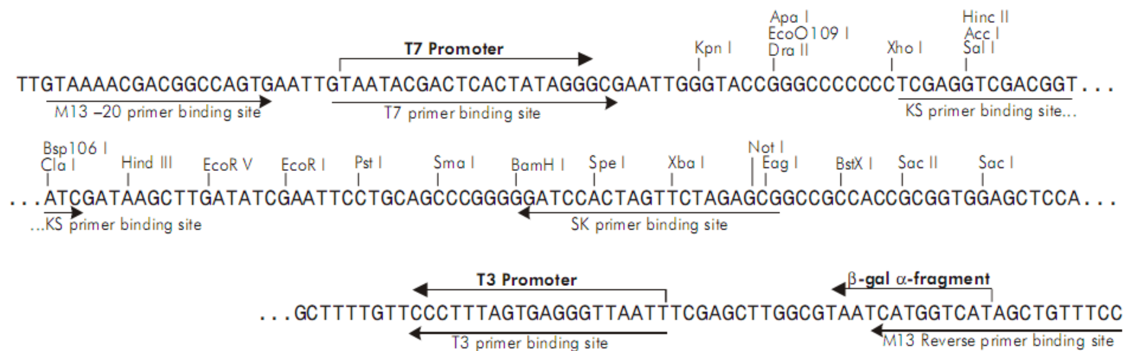


Figure 3. **A**) Illustration of the pBluescript SK- plasmid. **B**) Sequence data and primer binding sites of the pBluescript SK- multiple cloning site, base pairs 601 to 826. (Adapted from Stratagene, http://www.stratagene.com/vectors/maps/pdf/pBluescript%20II%20SK-_webpg.pdf)

3.4.2 Overproduction of proteins from selected clones

Attempts to overproduce the proteins encoded by the cDNA inserts in the SOLR strain were also done to verify recognition by the antiserum after immunoblotting. This was done by growing the cells in +37ºC to an OD_{600} of 0.5 and then adding IPTG to a concentration of 1 mM, followed by continued growth to an OD_{600} of more than 2.0. From these cells, 3 ml was then centrifuged and resuspended in 120 µl of 1 × SDS-PAGE loading buffer, boiled for 5 min and 1 µl run on a 12% SDS-PAGE. After this Coomassie blue staining and immunoblotting were performed for each sample, using the same antibodies that were used for the plaque screenings and BCIP/NBT colouring for detection of the samples.

3.5 Characterization of the insert lengths of the library

A quick characterization of the inserts in the cDNA library was performed by using PCR. One microliter (containing approximately 7.1×10^6 phage) of the primary library was used as the template and amplification performed with DyNAzyme II polymerase (Finnzymes), T7 (3'-CGGGATATCACTCAGCATA ATG-5') and RP (5'-GGAAACAGCTATGACCATG-3') primers, and with ^{33}P-dATP nucleotides added into the reaction mixture. PCR was performed by 30 cycles of 1 min denaturing at 94ºC, 1 min annealing at 48ºC and 3 min extension at 72ºC. An initial denaturation step was done for 5 min at 94ºC and a final extension for 7 min at 72ºC. PCR products were then run in a 1% agarose gel (electrode space 30 cm, ca 100 mA, 200 min) with GeneRuler DNA Ladder Mix (Bio-Rad) as a standard. The gel was photographed in UV light before the PCR products were transferred to a nylon transfer membrane as described above (section 3.2.1). An x-ray film was developed from a 24-hour autoradiography of the dried membrane. The dark areas of the film were compared to the UV gel picture with the help of the distances that the different sized fragments had travelled in the gel.

4 Results

4.1 cDNA library construction

For elution of total RNA from the RNA isolation vials, the method of first adding 40 µl of water and then an additional 20 µl seemed to be the most efficient one, because this procedure gave a well-concentrated RNA-solution without leaving much of the RNA in the vials. With the help of the RNA Nano LabChip (Agilent technologies) analysis (appendix 2), it was concluded that the isolated total RNA was of good quality and would make up a good basis for the cDNA library. This conclusion was drawn by comparing obtained results to results of previous runs done by other researchers, because the electropherogram of tomato total RNA does not resemble the typical electropherogram of a eukaryotic sample (personal communication, E. M. Turkki, Institute of Biotechnology, University of Helsinki and N. von Numers, University of Helsinki). The peaks of the ribosomal RNAs 28S and 18S were present, as well as four additional peaks that seem to be characteristic for tomato samples (appendix 2). The 28S:18S rRNA ratio of different samples ranged from 1.4 to 3.1. The peaks were distinct and the same peaks were present in all samples.

The cDNA fractions to be used for packaging into lambda phage were picked out based on the results from the liquid scintillation counting (table 2) after size fractionation. The first fraction did not contain any detectable radioactivity and thus no radioactively labeled cDNA, but the second fraction already generated some counts and was chosen to be the first fraction for further use. The fractions 8 to 11 gave rise to markedly higher counts than the rest of the fractions and therefore, together with the observation made that the dye eluted from the column around fraction number 11, it was concluded that the abovementioned fractions contained free nucleotides. To avoid the smallest fragments representing truncated cDNA products, fractions two through six were thereby chosen for packaging into lambda phage.

Table 2. Liquid scintillation counting results. CCPM = mean corrected counts per minute.

Fraction	CCPM
reference	40
1	44
2	254
3	386
4	360
5	342
6	244
7	456
8	3440
9	53013
10	55410
11	4086
12	235
13	114
14	90
15	82
16	111

From the ethidium bromide plate assay, it was concluded that the cDNA solution to be packaged into lambda phage had a concentration of about 50 to 75 ng/µl (figure 4).

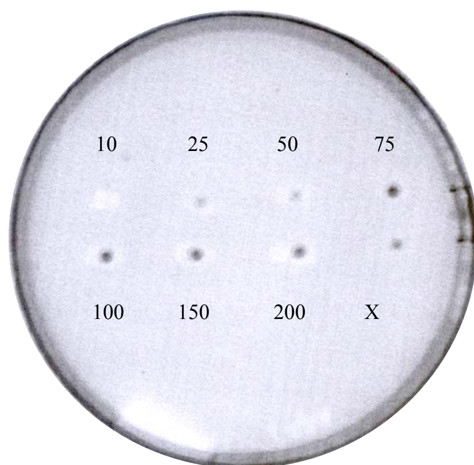
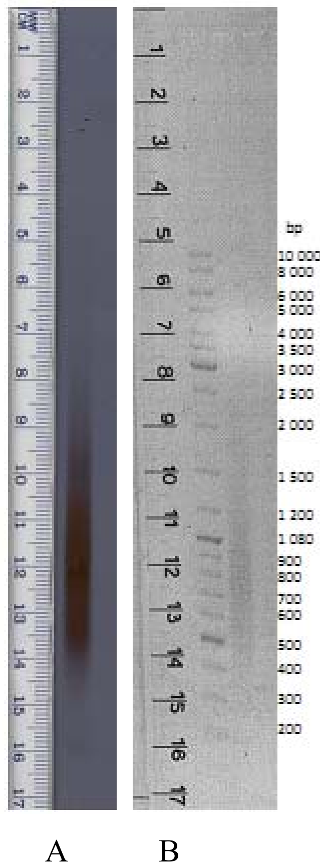


Figure 4. Ethidium bromide plate assay. The numbers indicate the DNA concentration of the standards in ng/µl. X = cDNA sample to be determined. The strength of the X spot is between those of 50 ng/µl and 75 ng/µl.

After packaging of the cDNA into lambda phage, the packaging reactions were titered, yielding the following results: the first batch of packaging reactions generated 3×10^5 pfu/ml (plack forming units per millilitre) and the second batch 3.5×10^5 pfu/ml. As expected, the titration of the ligation control did not generate any plaques. The wild type lambda DNA control generated 366 pfu from 10 µl of a 10^{-4} dilution, and thus the package extract efficiency was 1.8×10^9 pfu/μg_{DNA}. The blue-white colour selection confirmed the usability of the cDNA-containing phage in the library, with 4 plaques out of 261 being blue which makes out a background of 1.5%. The blue-white colour selection test result of the second packaging was even lower.

4.2 Quality of the cDNA library

The cDNA library was made out of 1.2×10^6 primary plaques from 3.8 ml of packaging reaction suspension. After amplification, a titer of 7.1×10^9 pfu/ml was obtained, with 98.5 % of the clones containing an insert. To determine the size range of the cDNA fragments of the library, PCR of the primary library was performed. It was concluded that the size of most cDNA fragments in the library ranged from approximately 300 to 1200 bp, although one could distinguish some fragments of sizes up to approximately 5000 bp (figure 5).



A B

Figure 5. Establishment of the size range of the cDNA library. **A**) X-ray film from the autoradiography of the PCR products from the primary library after run in agarose gel, accompanied by a scale showing the distance travelled in the gel. **B**) The corresponding UV light gel picture where the standards are visible. The darkest area, and so the majority of the PCR fragments, in the autoradiography picture is in the 10.5 to 13.5 cm range, which corresponds to fragment sizes between approximately 500 and 1350 bp. Taking into account the parts deriving from the pBluescript plasmid, which make up 180 bp, most of the cDNA fragments are about 320 to 1170 bp long.

4.3 Testing of the immunoscreening procedure

The immunoscreening procedure was tested by screening the library with an anti-Rubisco antibody. Rubisco is the most abundant protein in plant leaves and therefore the library should contain several clones with DNA encoding Rubisco. For this reason a positive signal should be detectable also from a small sample. However, there were some problems with the Rubisco antibody because it did not dissolve properly. Therefore, an SDS-PAGE run and immunoblotting of Rubisco from a tomato protein preparation was performed to test the antibody. The test showed that the anti-Rubisco antibody did detect a protein when used at a concentration of 1:1000. Through this

method a band at approximately 55 kDa was detected (figure 6), corresponding to the large subunit of Rubisco. Apparently also some non-denatured proteins were detected in concentrated samples.

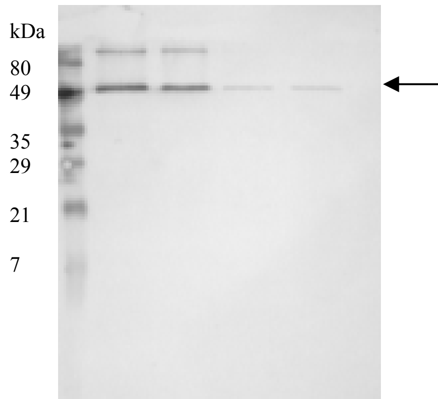


Figure 6. Immunoblot showing detected Rubisco from different concentrations of a tomato leaf protein preparation. The standards used are prestained standards, broad range (Bio-Rad). The first two lanes following the standards contain approximately 0.1 to 0.5 μg Rubisco protein as estimated from a Coomassie blue stained gel (data not shown), while the two last lanes contain 10 times less protein. The detected protein is approximately at the location of 55 kDa, which is the size of the Rubisco large subunit.

In the immunoscreening tests, neither the BCIP/NBT nor the CSPD method gave notably strong, distinct signals with the anti-Rubisco antibody. In both cases, there was quite a lot of background. This was the most important reason why the CSPD method was not chosen for further use - the high background made it difficult to discern positive signals from background, and when signals that were thought to be positive were compared to the plate that the membrane derived from they did not match the plaques. The BCIP/NBT detection generated some diffuse dark rings around spots where plaques had been (figure 7). These were expected to be positive signals. The result was confirmed by sequencing one of the clones, which turned out to contain the sequence of the Rubisco small subunit. This was why the BCIP-NBT method was chosen for the later screenings.

4.4 Screening of the library for the protein of interest

When the abovementioned screening procedure was applied to the actual immunoscreening for the target protein of HrpZ1, the method was not devoid of problems. There were many different kinds of conceivable positive signals, but none of them was clearly outstanding so that it could have been interpreted as a definite positive signal. However, there were 12 good candidates and the cDNAs of these were isolated as part of the pBluescript plasmid by *in vivo* excision with the ExAssist helper phage (Stratagene).

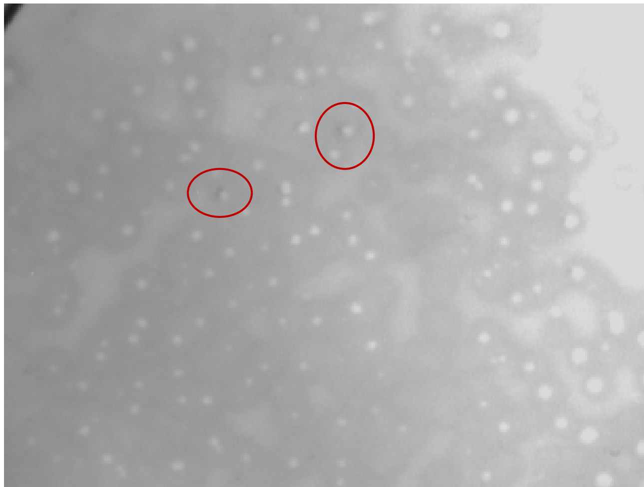


Figure 7. Positive plaques surrounded by a darker area in the anti-Rubisco immunoscreening test are marked with circles.

4.5 Selected cDNA clones

After *in vivo* excision of the plasmids from selected clones, these plasmids were isolated from the *E. coli* cells and digested with *Eco*RI before they were run on an agarose gel to get an estimation of the sizes of the cDNA fragments (figure 8). Based on the gel picture, the sizes of the selected cDNA fragments ranged from approximately 200 to 2000 bp long. The cDNA fragments were then sequenced in order to get information on what proteins these cDNAs were encoding. The lengths of the cDNA sequences from the clones that were sequenced were examined and found to range from 219 to over

1150 bp (table 3). Based on these, the average length is over 800 bp and 50% of these sequences are longer than 1000 bp (table 3). This result also seems to match quite well with the results of the library PCR (figure 5).

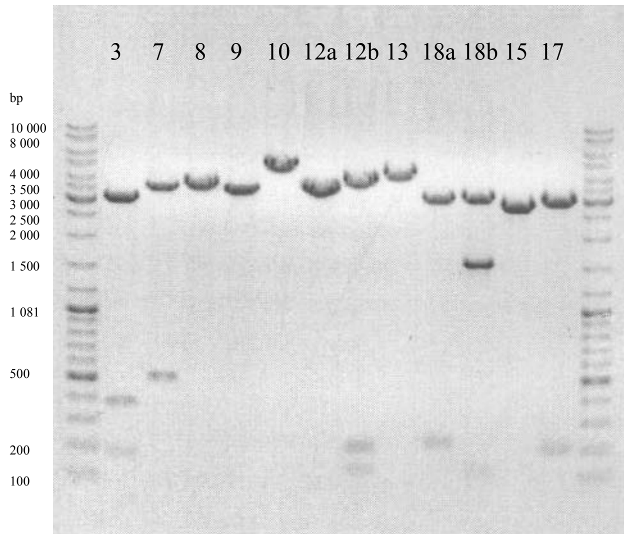


Figure 8. pBluescript phagemids containing cDNA of selected clones, run in an agarose gel after digestion with *Eco*RI (which cuts the vector once). GeneRuler DNA ladder mix (Fermentas) was used as standard. Some of the inserts contained one or several additional *Eco*RI restriction sites in the cDNA, resulting in several fragments. Clone number 15 did not contain any insert, and clone number 14 is not shown on this gel.

Table 3. Lengths of the sequenced cDNA clones. The mark > denotes that the cDNA clone was too long for the sequencer to be able to sequence the whole fragment from one primer and thus the calculated base pair number is the sequenced part.

Sequence	cDNA length (bases)
3	764
7	> 1113
8	725
9	485
10	> 1153
12a	491
12b	> 1027
13	> 1138
14	> 1144
17	219
18a	364
18b	> 1153

The cDNA sequences were compared to existing tomato sequence data using the BLAST service, and a few possible pathogenicity-related matches were observed (results not shown). The sequence data was also translated into protein sequences using the ExPasy sevice. However, it was not possible to pick out any clone that would clearly resemble the HrpZ-binding peptide. The reading frames of the sequenced cDNAs were checked for the case that the fragments would have been inserted in a way that is not in correspondence with the right reading frame. When expression of the cDNA clones was induced in *E. coli*, no specific recognition was observed when the induced samples were run on SDS-PAGE, immunoblotted and detection attempted with anti-ZBP3. Detection of Rubisco in this way was also ambiguous and unspecific bands were observed.

5 Discussion

Construction of the cDNA expression library was successful because the RNA was of good quality, synthesis of both strands as well as packaging into lambda phage succeeded and finally a titer of 7×10^9 pfu/ml was obtained from 1.2×10^6 primary plaques. This was a good result compared to previously constructed lambda cDNA libraries (Ruszczyk *et al.* 2008, Chen *et al.* 2009). According to Stratagene, amplification of 1×10^6 plaques should yield a library with a titer of about 10^9 to 10^{11} pfu/ml. The sizes of the cDNA inserts analyzed by PCR of the library (figure 5) corresponded to the liquid scintillation counts (table 2) and demonstrated that most of the fragments are from the middle fractions with "average" length from about 500 to 1300 bp. There is also a significant amount of larger fragments than these average-length fragments (figure 5), indicating successful cDNA synthesis during library generation. However, smaller cDNA fragments are also present, indicating that the library also contains a portion of truncated cDNAs.

Although the cDNA library construction was successful as well as the immunoscreening for Rubisco, the immunoscreening for the protein of interest was not. No immunoscreening signal was clearly detected, and when the cDNA sequences from the isolated putative positives were analyzed, they did not predict any protein sequences that were similar to the peptide sequence against which the antiserum was raised. Either the screening itself did not work, the expression failed or the detection method used was either not suitable or not optimized well enough. Theoretically, alkaline phosphatase detection with light producing CSPD should be much more sensitive than colorimetric BCIP/NBT detection, but in this case it was impossible to get any clear picture from the autoradiography films developed when CSPD was used as a substrate; there was remarkable background and signals that looked real were sometimes even outside the area of the filter. BCIP/NBT development, however, seemed to give positive signals when screenings for Rubisco were performed, and for this reason the BCIP/NBT substrate was chosen for detection. Nevertheless, when performing the actual screenings for the HrpZ1-binding protein, the BCIP/NBT detection did not give rise to any clearly distinguishable positive signals. To this there can be several possible explanations. There is a possibility that there was no positive clone among the plaques screened.

Explanations for this could be that for some reason, no cDNA was made out of that specific mRNA encoding the target protein, or the mRNA producing the target protein could be very rare and for that reason the corresponding cDNA did not end up in the library. If the peptide recognized by anti-ZBP3 is in the N-terminus of the target protein there is also a possibility that the target cDNA was not recognized because this cDNA was truncated. All of the C-terminal parts of the proteins are certainly present in the library, but in the case of truncated cDNAs the N-termini are not represented.

Moreover, there might have been some problem with expression - either the induction can have failed or the DNA was not expressed. Because the *E. coli* cell is not an optimal milieu for a tomato protein, one also has to consider the possibility that the protein was presented in wrong conformation, which could have caused the failure of the recognition. There is also the possibility that expression worked fine, but the target product was somehow toxic to the host SOLR bacterium or degraded for some reason. In this case, there could have been a positive signal on the filter after BCIP/NBT detection although nothing showed in the overproduction and immunoblotting test. A cloned gene that encodes a toxic protein is not synthesized until the phage infects the cell, which is destined to lysis anyway (Snyder & Champness 1997). Small fragments of a degraded protein could have been detected during immunoscreening but in a Western blot assay these small fragments would run out of the gel during size separation in SDS-PAGE. Another option is that the detection testing and optimisation should have been done even more thoroughly so that conditions where a positive signal would have been more obvious could have been obtained.

Because a good tomato cDNA expression library was constructed in this work, it can facilitate future work where such a library can be used, e.g. for finding target proteins of other effectors than HrpZ1. Having a tomato cDNA library is important because tomato is a more complex plant than the model plant *Arabidopsis thaliana*, and is therefore important for studying microbe-plant interactions. Also, defence signaling pathways seem to differ between plant species, which makes it important to study other plants than *A. thaliana* for comparison.

However, the primary objective would be to continue the research that was started here, either from the same point of view with more optimization of detection and new screenings performed or from a completely new starting point. One alternative starting point could be to do a phage display assay using cDNA epitopes and testing the affinity to HrpZ1. Positive peptides could then be localized in the tomato genome by

comparison to available genomic data in databases. Another possible approach to identify the target protein would be the use of mass spectrometry after immunological detection, either by separating HrpZ1-bound proteins from a liquid mixture with the help of magnetic beads as has been done with various kinds of samples (e.g. Guan *et al*. 2007, Careri *et al*. 2008), or from a nitrocellulose membrane (Dufresne-Martin *et al.* 2005, Luque-Garcia *et al*. 2008). Obtaining the main goal of this research would be important. Gaining more knowledge on the mechanism of the T3SS could promote development of disease resistant plants, and on the animal side it could be of great importance for medical research where the same system could be used in directing medical substrates to specific locations in the organism or cell.

NOTE ADDED AFTER REVISION:

During revision of this work, it was realized that the anti-Rubisco antibody used for the test screenings of the cDNA library in this study was directed against the large subunit (RbcL) of Rubisco only. This explains why the immunoscreenings for Rubisco were not as prominent as expected. Because first strand synthesis of the cDNA was primed with an oligonucleotide containing a poly(dT), only the mRNAs containing a poly(A) tail were turned into cDNA. The Rubisco large subunit is encoded by chloroplast DNA and thereby does not contain any poly(A) tail. In other words, only the Rubisco small subunit (RbcS), which is encoded by the nucleus, was present in the constructed cDNA library. The weak signal obtained for the small subunit when screening the library with the anti-Rubisco antibody may have been due to some minor RbcS contamination in the preparation used to raise the antibody. Therefore, the screening method may have worked just fine; there simply was nothing for the antibody to detect.

6 Conclusions

In this work a representative tomato leaf lambda cDNA expression library was constructed, and by that the most critical step of this study and an important aim was achieved successfully. Although the immunoscreening of the library was not that successful, picking out specific clones, isolating the phagemids and sequencing the cDNAs of these indicated that this method could work well for achieving the goal. This may, however, require some improvements of the detection method, on the assumption that there is a clone to detect. Nevertheless, the cDNA library constructed in this work will probably still contribute to some piece of important information on how the type III secretion system functions.

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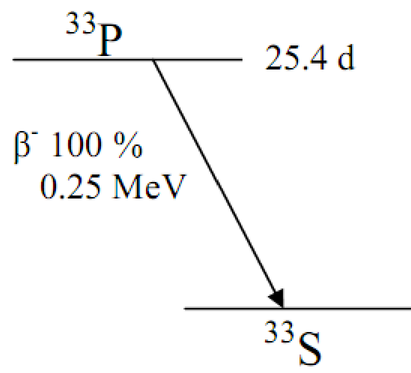
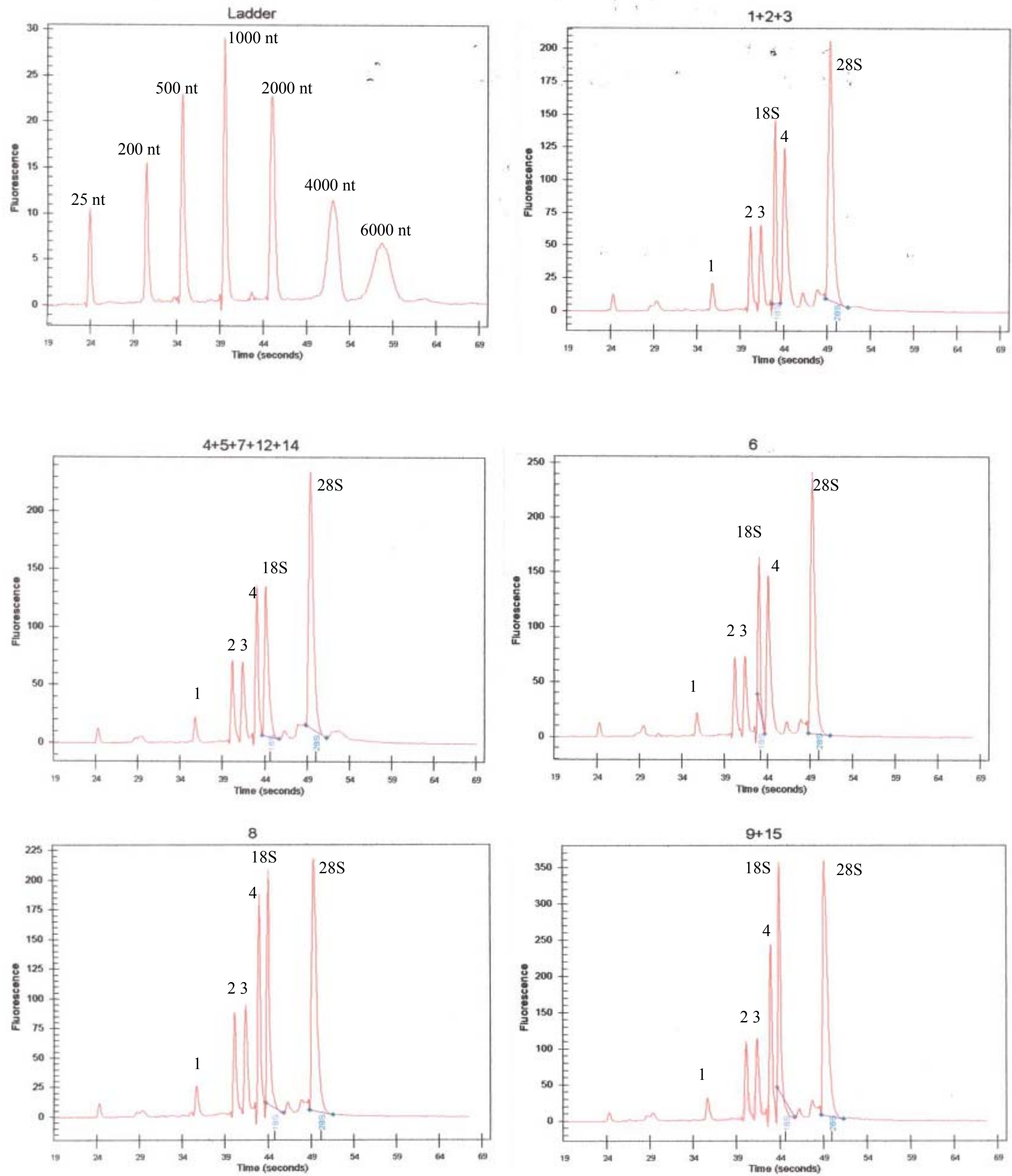
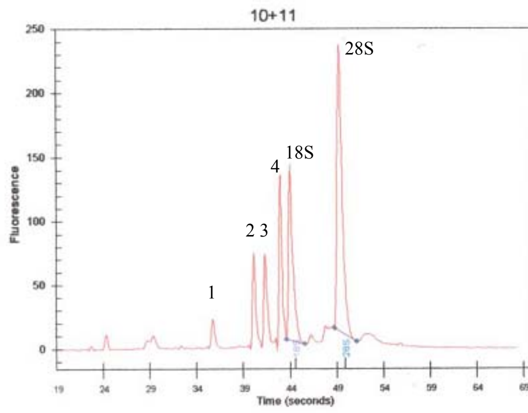
APPENDIX 1. Decay scheme of ^{33}P



APPENDIX 2. Electropherograms of RNA samples from RNA Nano LabChip analysis. The peaks characteristic for tomato samples are marked with numbers 1 to 4 and the peaks for ribosomal 18S and 28S RNAs as the computer programme assigned them.

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Fluorescence
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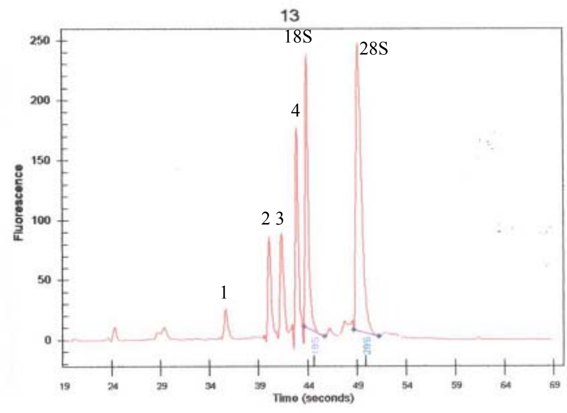
19 24 29 34 39 44 49 54 59 64 69
Time (seconds)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Fluorescence
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18S
28S
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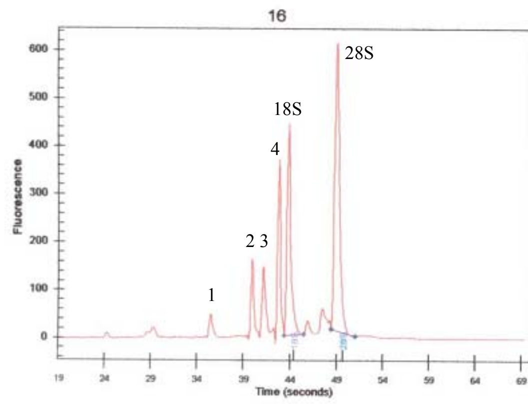
19 24 29 34 39 44 49 54 59 64 69
Time (seconds)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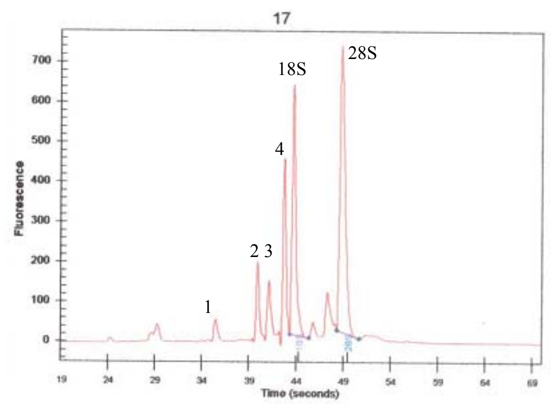
Time (seconds)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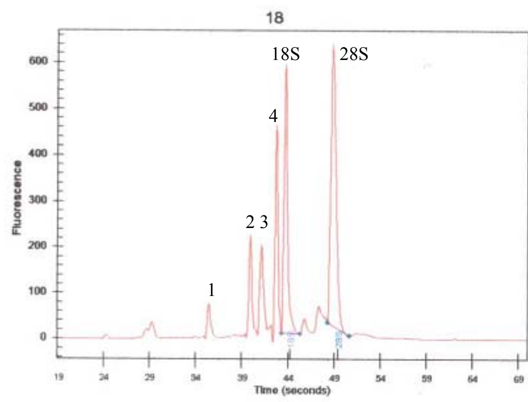
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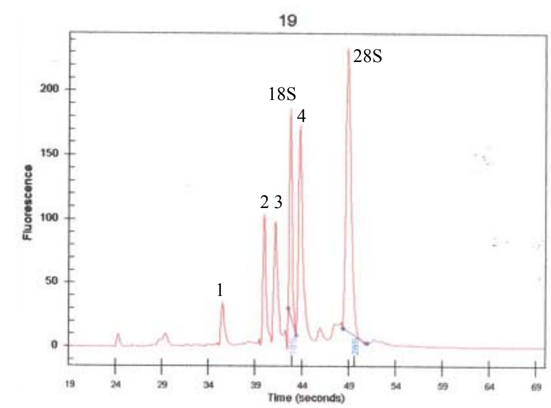
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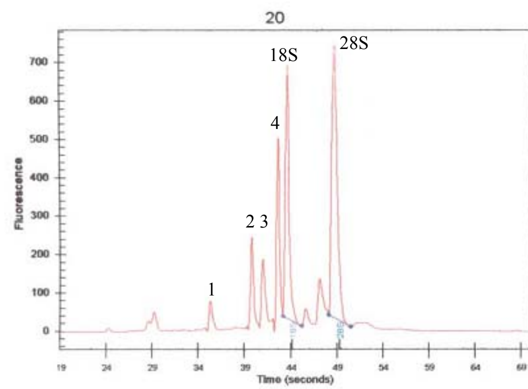
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Time (seconds)