Development of a reporter system for the analysis of *Xylophilus ampelinus* Type III Secreted effectors

Nompumelelo Philile Praiseworth Nyembe

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Supervisor: Prof. Bongani K. Ndimba

Co-supervisor: Dr. Yolanda Petersen

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KEYWORDS

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ABSTRACT

Development of a reporter system for the analysis of *Xylophilus ampelinus* Type III secreted effectors

N. P. P. Nyembe

MSc Thesis, Department of Biotechnology, University of the Western Cape

Xylophilus ampelinus, the causal agent of bacterial blight and canker of grapevines, has long been a threat to the table grape industry in the Western Cape, leading to severe economic losses due to the reduced productivity and shortened lifespan of infected grapevines. Very little is known about the genetic makeup of the organism, especially with regard to the factors that contribute to its pathogenicity. Generally, bacterial pathogens directly inject the effector proteins into host cells via Type III secretion system (T3SS). In the attempts to identify and characterize the T3 secreted effectors, different reporter plasmid systems have been used to study the secretion and translocation mechanisms the effectors employ during pathogenicity. The aim of the study was to generate a T3 reporter plasmid system for *X. ampelinus* that will allow the identification and classification of potential pathogenicity factors as members of the Type III secretion class of effectors. First, the *avrBs1* family genes *avrBs1* and *avrA* were identified and characterized. The two avirulence genes induced HR on *Nicotiana tabacum* leaves. Due to the relatedness of the *X. ampelinus avr* sequences to those of xanthomonads, and the fact that *Xanthomonas avrBs1* has been successfully used in a number T3 effector studies, it was decided to construct an *X. ampelinus* T3 effector reporter vector based on the *avrBs1* gene. The minimal segment of the *X. ampelinus* AvrBs1 protein C-terminus, sufficient for recognition inside host cells and also responsible for HR-induction was identified and characterized using *Agrobacterium*-mediated transient expression. The $AvrBs1_{57\text{-}413}$ HR-inducing domain was cloned in-frame with the 3x FLAG epitope, into a broad-host range vector. To test the reporter vector, the full length *avrBs1* sequences of *X. ampelinus* and *Xanthomonas campestris* pv. *campestris* were cloned ahead of the 3x FLAG epitope and the constructs were transferred into *XaΔavrBs1* knockout mutant to test for protein secretion. Furthermore, the reporter construct was tested for Type III protein translocation on *Bs1* resistant pepper cultivar STAR 6657. Optimization of protein secretion and translocation

assays is however required for the improved results. This might include the application of an alternative protein tag to identify candidate *X. ampelinus* T3SS effectors.

November 2014

DECLARATION

I declare that *Development of a reporter system for the analysis of Xylophilus ampelinus Type III Secreted effectors* is my own work that it has not been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

Nompumelelo Philile Praiseworth Nyembe Date…….

Signed ……..

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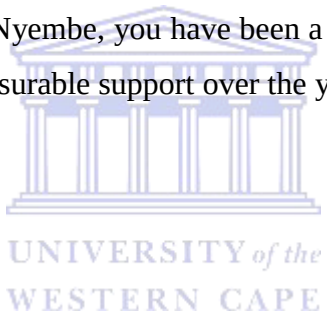

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LIST OF ABBREVIATIONS

1D	one dimensional
AAD	acidic activation domain
APS	ammonium persulphate
avr	avirulence
BLAST	basic local alignment search tool
BLASTp	protein to protein BLAST
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CBB	coomassie brilliant blue
CFU	colony-forming unit
Da	Dalton
DNA	deoxy-ribonucleic acid
dNTPs	deoxynucleotide triphosphates
DMSO	dimethyl sulphoxide
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
FD	FastDigest
Hpa	Hrp-associated
HR	hypersensitive response
hrp/ Hrp	hypersensitive response and pathogenicity
kb	kilo-base pair
kDa	kilo Dalton
LB	Luria-Bertani
LRR	leucine-rich domain
MAMPs /PAMPs	microbe/ pathogen-associated molecular patterns

MES	2-(N-Morpholino)ethanesulfonic acid
MTI	MAMP-triggered immunity
MW	molecular weight
NA	nutrient agar
NBG	nutrient broth glycerol
NBS	nucleotide binding site
NCBI	national center for biotechnology information
NLSs	nuclear-localised signals
nm	nano meter
NRPD	non-redundant protein database
OD	optical density
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PIP	plant-inducible promoter
PPRs	pathogen pattern recognition receptors
pthA	avirulence and pathogenicity
PTI	PAMP-triggered immunity
R	resistance
RNA	ribonucleic acid
SDS	sodium duodecyl sulphate
SDS-PAGE	sodium duodecyl sulphate-polyacrylamide gel electrophoresis
SOC	
T3	Type III
T3SS	Type III Secretion System
TBE	tris Borate EDTA
TBS	tris-Buffered Saline
TCA	trichloro-acetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

TTBS	TBS containing Tween20
UV	ultraviolet
V	volts
v/v	volume per volume
w/v	weight per volume
YEP	yeast tryptone
YPG	yeast peptone glucose
YPGA	yeast peptone glucose agar

SYMBOLS

Δ	=	Greek capital letter delta
β	=	Greek small letter beta
α	=	Greek small letter alpha
°C	=	Degrees Celcius
~	=	Tilde (approximate sign)
µ	=	Micro sign
′	=	Letter modifier prime
%	=	Percent

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CHAPTER 1: LITERATURE REVIEW

1.1 The importance of grapevines in the agricultural sector

Vitis vinifera (common grape vine) is a species of *Vitis*, native to the Mediterranean region, central Europe, and south-western Asia, from Morocco and Portugal north to southern Germany and east to northern Iran as well as in South Africa. There are currently between 5000 and 10,000 varieties of *Vitis vinifera* grapes though only a few are of commercial significance for wine and table grape production.

South Africa is one of the oldest and most reliable suppliers of table grapes nationally and internationally (SATI statistical booklet, 2012). In general, grapes are dried, pressed or consumed fresh as opposed to grapes that are processed to make wine, juice and/ or raisins. In South Africa, table grape is one of the most important deciduous fruit not only because of its consumption but also taking into consideration the employment opportunities, foreign exchange earnings etc. According to the SATI market value profile, in 2010 dry grapes contributed 31% which is 23 532 ha of the total area planted (75 025 ha)[2] (SATI market value chain profile, 2011), suggesting that the grapes are indeed of great importance and they contribute a lot to the country's economy. A 50% increase in gross value of the table grape production was observed over four production seasons [2006/2007 (R2 billion) to R3 billion in 2009/2010 production season] (SATI market value profile, 2011). The 2009/2010 R3 billion was 7% higher than the gross value of the previous season 2008/2009 and a 100% higher than the gross value of grapes over a decade ago, meaning the table grape industry is growing and the market value is also increasing as the demand increases.

South Africa produces a wide range of table grape varieties that are harvested over a seven month period starting in October and ending in May. The country's main table grape cultivars and their percentage contribution to total exports are Thompson Seedless (11%), Crimson Seedless (13%), Red Globe (10%), Prime Seedless (11%), and Sugraone (9%) (SATI statistical booklet, 2012). The export demand of the seedless varieties has been increasing over the five past years (SATI statistical booklet, 2013/2014). An 83% increase in demand of the Far East Africa and 80% for Russia has been observed whereas, UK and Europe remain the main export destinations accounting for 79% of the South African exports (SATI statistical booklet, 2013/2014).

Although the table grape industry shows the value increase and growth in production, there are some obstacles that halt the progress in production, one being the grapevine diseases affecting this crop.

1.2 Grapevine diseases

Grapevine is a crop that is susceptible to many diseases but the degree of susceptibility differs depending on the variety itself. However, when there is no pest management action carried out, the damage can generally be severe. The most important diseases found worldwide are bacterial blight, crown gall, downy mildew, powdery mildew, grey mould, anthracnose and black rot. Downy mildew, powdery mildew, grey mould, black rot and anthracnose are caused by fungi and they generally attack berries, reducing yield and quality. Bacterial blight and crown gall are caused by bacteria. Downey mildew is a highly destructive disease of grapevines caused by *Plasmopara viticola* (Prajongjai *et al*., 2014). Infected berries turn colour prematurely or develop spotted appearance. Anthracnose caused by *Elsinoe ampelina* reduces the quality and quantity of the fruit and the vine (Ellis and Erincik, 2008). Grey mould is generally a postharvest disease caused by *Botrytis cinerea* infecting grapes with fungal spores which turn to become more susceptible as they mature and increase their sugar content (Liu *et al*., 2010). Powdery mildew caused by *Erysiphe necator* is a major problem when not controlled. It can destroy infected clusters or reduce their quality (Gadoury *et al*., 2012). In addition to fungal diseases, Crown gall caused by *Agrobacterium vitis* is a difficult disease to control and it reduces vine vigour and growth, thus reducing crop yield (Kado, 2002).

The bacterial blight is also one of the important grapevine diseases. The disease is caused by *Xylophilus ampelinus*. The disease is prominent in *Vitis vinifera* cultivars in the Mediterranean area and in isolated locations in the Western Cape region of South Africa (Erasmus *et al*., 1974; Garau *et al*., 1988; Bradbury, 1991). Bacterial blight is a serious, chronic and destructive vascular disease affecting commercially important grapevine cultivars. Losses arise from reduced productivity and shortened life of diseased vines. Some cultivars are more susceptible than others and there are no control measures known thus far (Botha *et al*., 2001). This disease was described initially in Crete (Greece) as "Tsilik marasi" (Panagopaulos, 1969), it was then announced in Spain as "necrosis bacteriana" (Lopez *et al*., 1980) and in South Africa as "vlamsiekte" (Du Plessis, 1940). The pathogen has been isolated in the number of different countries, but only from infected grapevines showing bacterial necrosis and cankers (Grall and Manceau, 2003).

1.3 *Xylophilus ampelinus*

1.3.1 Description and phylogenetic classification

Xylophilus ampelinus was originally known as *Xanthomonas ampelina* and classified as a member of the genus *Xanthomonas* because it possesses the following features of this genus: it is an aerobic, non-spore forming Gram negative rod-shaped organism with one polar flagellum. It has oxidative carbohydrate metabolism, produces a yellow insoluble pigment and it has a mean DNA base composition similar to that of genus *Xanthomonas* (Willems *et al*., 1987). However, hybridizations between rRNA from *Xanthomonas campestris* NCPPB 528 type strain and other *Xanthomonas* species DNA showed that *Xanthomonas ampelina* is definitely not a member of the genus *Xanthomonas* (De Vos and De Ley, 1983). There are also some other additional features that differentiate *Xanthomonas ampelina* from other *Xanthomonas* species, such as the absence of xanthomonadins (Starr *et al.*, 1977), very slow growth at the optimal temperature of 24 °C, the presence of urease activity, utilization of meso-tartrate and no production of acid from glucose and sucrose (Panagopaulos, 1969). On the basis of biochemical tests and DNA-rDNA hybridisation, the bacterium was transferred from the genus *Xanthomonas* to the new genus *Xylophilus* and renamed as *Xylophilus ampelinus* (Willems *et al*., 1987).

The genus is described as a slow growing, yellow pigmented bacteria belonging to the family *Comamonadaceae* in the β-subclass of Proteobacteria (Willems *et al*., 1991a). Other members of the family *Comamonadaceae*, are *Acidovorax, Comamonas*, *Variovorax, Hyrogenophaga* genera as well as a number of phytopathogenic *Pseudomonas* species (Wen *et al*., 1999) based on the relationships determined by extensive DNA-RNA hybridization data.

The species, *Xylophilus ampelinus*, is the only member in its genus *Xylophilus.* On nutrient agar (NA), cells occur singly, in pairs or in short chains and may attain diameters of 0.2 to 0.3 and 0.6 to 0.8 mm (Willems *et al*., 1987) in about 6-10 days. Colonies appear circular, semi-translucent, slightly raised, glistening and pale to yellow. The bacterium grows well in the medium containing yeast extract, bacto-peptone, glucose and bacto-agar (YPGA) when incubated at 28°C.

1.3.2 Disease occurrence, symptoms and distribution

X. ampelinus survives in the vascular tissues of infected plants (Bradbury, 1991), and is found mainly xylem vessels (Grall and Manceau, 2003). The bacteria enter the plant through natural and artificial wounds or openings and spread to the cell sap and xylem vessels. It is believed that the environmental conditions in these parts of the plant favour bacterial growth and development (Ridé and Marcelin, 1983; Grall and Manceau, 2003). It is however, not known how the bacteria spread within the xylem system, and how the plants respond to this invasion (Chatelet *et al*., 2011). In the xylem, the bacterial cells develop as assemblages, called biofilms (Grall and Manceau, 2003). This xylem tissue invasion is also typical for other bacteria like *Xylella fastidiosa* (Davis *et al*., 1983), *Pseudomonas syzygii*; (Roberts *et al*., 1990), *Pantoea stewartii* (Pataky, 2004), and *Ralstonia solanacearum* (Hayward, 1991).

Bacterial blight of grapevine symptoms have been reported in various regions of the world. In Slovenia, the disease was localized to one vineyard (Dreo *et al*., 2005). Although the disease is reported as absent in Canary Islands, Tunisia, Argentina, Portugal, Switzerland, and in Yugoslava, these records may be unreliable (Bradbury, 1986). In Crete, Italy, Sardinia, Sicily, and Moldova the disease is still present (Bradbury, 1986; CABI/EPPO, 1999). The distribution of the disease in Spain, Greece and France is restricted and the disease is still invasive in France (CABI/EPPO, 1999; Bradbury, 1986; Manceau *et al*., 2005). In Turkey, the disease has been eradicated (CABI/EPPO, 1999). The disease is still present in the some parts of South Africa (Botha *et al*., 2001).

The life cycle of *X. ampelinus* has not yet been completely clarified. The primary infections occur mainly on shoots that are one to two years old, leaves, blossoms (Figure 1A) and grapes. The first signs of infection are linear reddish-brown streaks on the shoot, extending from the base to the shoot tip (Figure 1B). Infected plants may also show delayed bud burst where stunted shoots have been consumed by bacterial blight at early stages (Figure 1C).

Cracks appear along such shoots, become deeper and longer, forming cankers. Young shoots may develop pale yellowish-green spots on the lowest internodes. These expand upwards on the shoot, darken, crack and develop into cankers. Cracks, and later cankers, also form on more woody branches later in spring. Shoots subsequently wilt, droop and dry up. In summer, cankers are often seen on the sides of petioles, causing a characteristic one-sided necrosis of the leaf (Figure 1D).

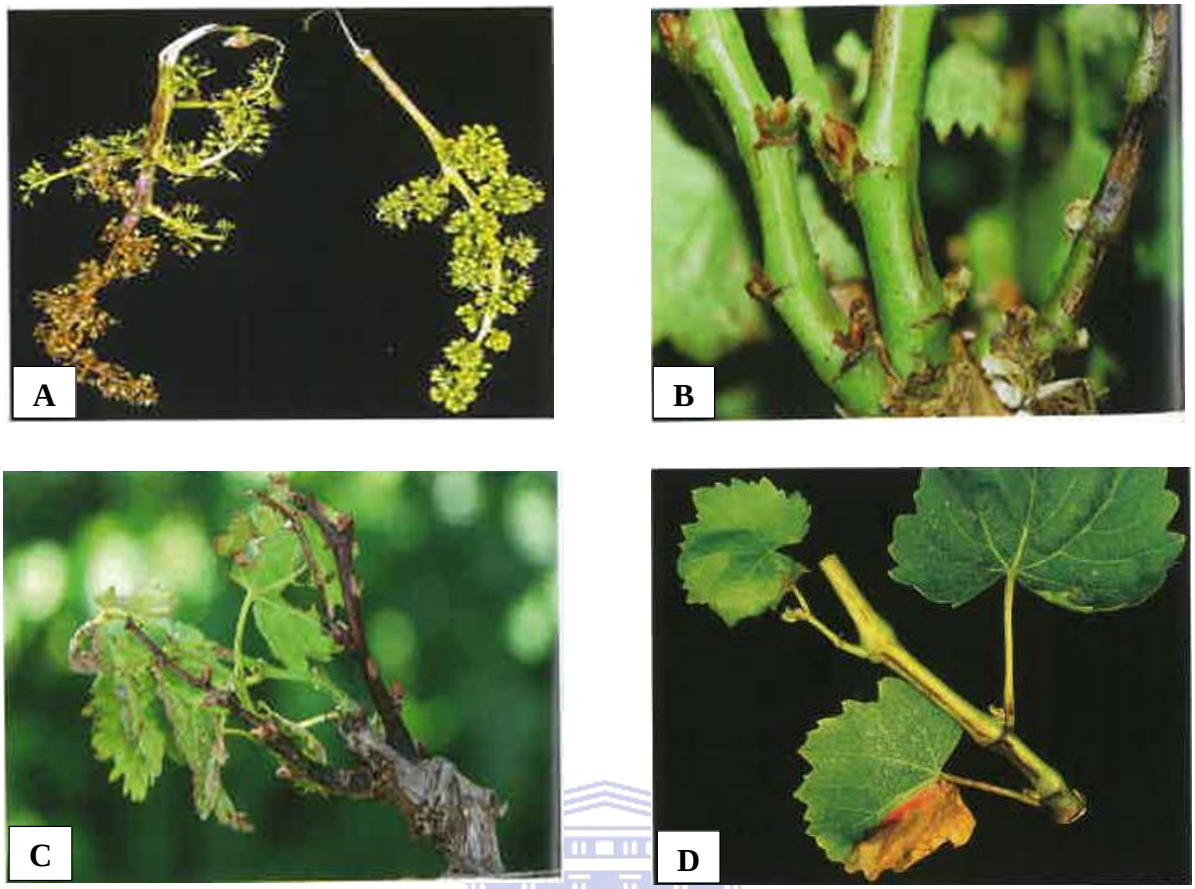

Figure 1.1: Bacterial blight symptoms. (A) Destruction of young bunch of infected vines during flowering. The dying bunch on the left is severely destructed by bacterial blight disease compared to the intact bunch on the right. (B) Infected vine showing development of small lenticular cracks on young shoots. (C) Infected vine showing delayed bud burst where stunted shoots are consumed by bacterial blight at an early stage. (D) Angular reddish-brown lesion on infected grapevine leaf, characteristic of bacterial blight when the leaves are infected through the stomatal openings. The leaf on the right shows leaf-cupping, another characteristic of bacterial blight. Anonymous, Wynboer 2012

The pathogen is transmitted with pruning tools (Ridé *et al*., 1977), therefore entering healthy tissues through pruning wounds during windy and wet seasons. The pathogen then moves to the healthy shoots in early summer because the disease is often associated with warm, moist conditions. The spreading of the disease is favoured by overhead sprinkler irrigation water and the pathogen may also be carried in irrigation water (Ridé *et al*., 1977). The bacteria survive in wood, therefore might be transmitted from nursery to nursery through infected cuttings. In the vineyards the local spread occurs in rows starting from the disease focal point.

1.3.3 Economic impact and disease control

Severe infection of susceptible cultivars can lead to serious harvest losses, with negative impact on the economy and the environment. Outbreaks of the disease happen periodically and many years may pass between symptom outbreaks in infected vineyards. In 1940, Du Plessis observed harvest losses of >70% in South Africa. Vines infected one year weakened and died back later on in the subsequent years. In France, the disease is still very active in some wine-producing areas (Manceau *et al*., 2005).

The disease was important in Spain in the past (Lopez *et al*., 1980) but it has not been observed recently. In Greece, the disease is still present in Crete, especially in Iraklion, where it occurs on the very susceptible cultivar Sultanine.

Chemicals have failed to effectively control the disease. In the past few years the control of the disease has been only obtained through viticultural practices such as destroying infected shoots, carrying out pruning in dry weather and as late as possible, and disinfecting pruning tools thoroughly during the operation. Most farmers have avoided the use of overhead sprinkler irrigation as it seemed to promote spreading of the disease. However, these efforts never stopped the disease occurrence, they only reduced the spread of the disease. Other factors that contribute to the constant recurrence of the disease include the lack of totally resistant grape cultivars, and favourable environmental conditions.

1.3.4 Bacterial detection and molecular characteristics

X. ampelinus is a slow–growing bacterium, which makes it difficult to isolate pure cultures (Panagopaulos, 1969). This bacterium has been successfully isolated from the grapevines showing the disease symptoms associated with this pathogen by a number of research groups (Serfontein *et al*., 1997). *X. ampelinus* can be isolated on non-selective YPGA and NA media. The bacteria grow best when incubated at temperatures ranging from 25 °C to 28 °C. Direct isolation, immunofluorescence, enzyme-linked immunosorbent assay (ELISA), polymerase-chain reaction (PCR), and real time PCR can be used as screening tests. A number of PCR primer pairs have been developed over the years for the detection of *X. ampelinus* (Botha *et al*., 2001; Manceau *et al*., 2005; Dreo *et al*., 2007).

1.4 Plant-pathogen interactions

In the environment where there are constant threats against the plants such as the infecting pathogens, the plants learn to fight off the infection by employing fast defense responses. The lack of immunity specialized cells in plants mean that plant defense responses only depend on the pathogen recognition by each cell.

Plant pathogens have conserved molecules called elicitors, generally referred to as pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs), which include flagellin, lipopolysaccharide, and peptidoglycan. During infection these MAMPs are recognized as non-self by the plant pathogen pattern recognition receptors (PPRs). These PPRs are localized on the surface of the plant cells. Upon MAMPs recognition by PPRs the first phase of defense responses is observed and it is termed MAMP-triggered immunity (MTI) (Jones and Dangl 2006). This immunity can protect the plant to a certain extent and it is generally sufficient for resistance against most microbes. However, the MTI is suppressed by direct transportation of bacterial effector proteins via the T3SS (He *et al*., 2006; Kim *et al*., 2008). In this case, the T3SS apparatus is believed to breach the membrane receptors or PPRs, meaning the effector proteins are introduced inside the cells where they can be rendered virulent. This results in effector-triggered susceptibility (ETS). The second line of defense often involves the plant resistance (*R*) genes. This defense model was first introduced in 1942 by Flor, and it was termed gene-for-gene interaction. It involved the recognition of the avirulence genes or bacterial effectors by the *R* gene products in the cells. This defense reaction is now termed Effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI is basically the stronger or accelerated MTI which leads to rapid programmed cell death known as hypersensitive response (HR). The plant-pathogen interactions are classified into compatible and incompatible interactions. Incompatible interactions are cultivar-specific and determined by ETI and generally induce HR. In contrast, compatible interactions are thought to lack ETI leading to susceptibility and disease development.

1.4.1 The Type III secretion system

Bacteria have a class of effectors that are injected directly to the host plant cells by the specialized Type III secretion system (T3SS). The first T3SS-associated filamentous structure was discovered in the plant pathogen *Pseudomonas syringae* (Roine *et al*., 1997). Characterization of the T3SS was initially done in the mammalian pathogen *Salmonella enterica* (Kubori *et al*., 1998). The T3SS was found to have fascinating characteristics

including the two pairs of rings that interact with the cytoplasmic and outer membrane respectively (Figure 1.2). This filamentous extension resembles a needle-like structure and it was also found and characterized in all major plant pathogens with an active T3SS (Roine *et al*., 1997; Van Gijsegem *et al*., 2000; Weber *et al*., 2005). The Hrp pili from plant pathogenic bacteria are approximately a micrometre longer than the animal pathogen Hrp pili, simply because plant pathogen Hrp pili span the thick plant cell wall that is generally an obstacle in protein transport. The needle structure, called the Hrp pilus, serves as a channel for secreted proteins or effectors from the pathogen through the host cell plasma membrane (Jin *et al*., 2001; Li *et al*., 2002).

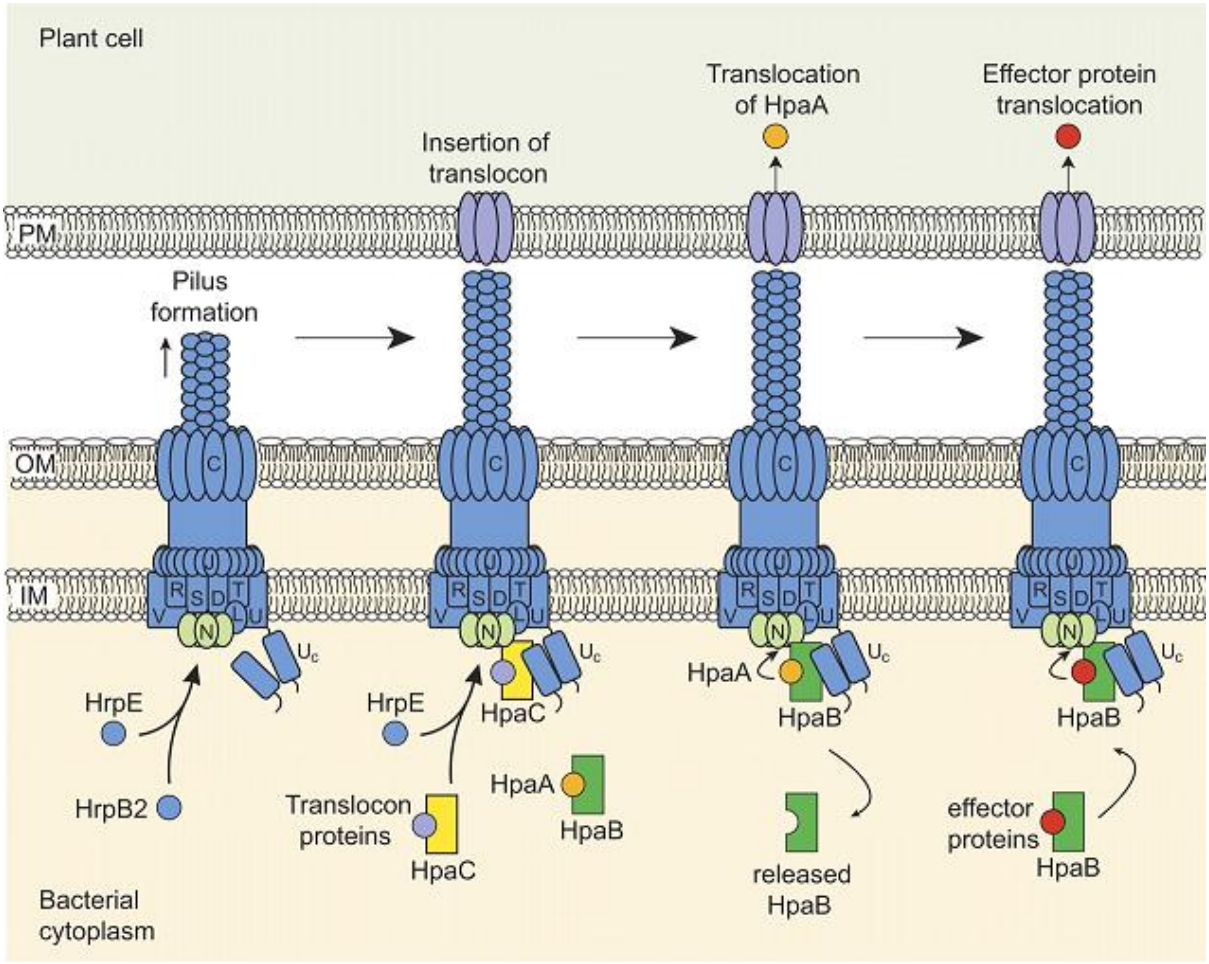

Figure 1.2: T3SS from plant pathogenic bacteria. The Hrp pilus breaches the plant cell wall membrane where it facilitates the secretion of the effector proteins. Abbreviations: PM- plasma membrane; OM- outer membrane; and IM- inner membrane (Büttner and He, 2009)

In *Xanthomonas*, the T3SS is encoded by an approximately 23-25 kb *hrp* (hypersensitive response and pathogenicity) gene cluster (Weber *et al*., 2005; Koebnik *et al*., 2006; Büttner

and Bonas, 2006; Cornelis, 2006) which was first so described Lindgren *et al*. (1986). Mutations in the *Xanthomonas* T3SS pathway renders all strains non-pathogenic suggesting that the T3SS is highly essential for pathogenicity (White *et al*., 2009; Jiang *et al*., 2013; Rossier *et al*., 2000). The T3SSs in phytopathogenic bacteria are divided into two groups, group I and II. Group I T3SS includes *Erwinia* spp., *Pantoea agglomerans* and *P. syringae* and group II T3SS includes *Xanthomonas* spp. and *R. solanacearum*.

Group I T3SSs are regulated by HrpL, a member of ECF (extra-cytoplasmic function) subfamily of alternative sigma factors (Mucyn *et al*., 2014; Xiao *et al*., 1994; Xiao and Hutcheson, 1994; Tang *et al*., 2006; Wei *et al*., 2000; Merighi *et al*., 2003). The HrpL protein recognizes and binds to the consensus sequence 5′-GGAACC-N_{15-16}-CCACNNA-3′ named the Hrp box in the promoter of Type III genes (Xiao and Hutcheson, 1994; Shen and Keen, 1993; Nissan *et al*., 2005). HrpS and HrpR proteins are positive regulatory proteins that activates *hrpL* transcription and they belong to the NtrC family of the two-component regulatory proteins (Wei *et al*., 2000). In *Erwinia* spp., a two-component signal transduction system HrpX/HrpY regulates the expression of *hrpL* which is partially controlled by HrpS (Wei *et al*., 2000; Wei and Beer, 1995). In addition to group I T3SS regulation, *P. stewartii* and *Pantoea agglomerans* pv. *gypsophilae*,phosphorylated HrpY activates transcription of *hrpS* and autoregulates the *hrpXY* operon (Merighi *et al*., 2003; Niza-Koren *et al*., 2003).

Regulation of group II T3SSs depends on a member of the AraC family transcription activators, designated HrpB in *R. solanacearum* and HrpX in *Xanthomonas* species (Cunnac *et al*., 2004; Wengelnk and Bonas; 1996; Wengelnik *et al*., 1996). Many HrpX-regulated genes of *Xanthomonas* spp. contain a *cis*-element plant-inducible promoter (PIP) box motif (TTCGC-N_{15}-TTCGC) in their promoter region (Fenselau and Bonas, 1995; Koebnik *et al*., 2006). During Type III gene regulation, HrpX binds to the PIP box motif thus regulating downstream expression of T3SS genes (Wengelnk and Bonas; 1996; Wengelnik *et al*., 1996; Huang *et al*., 2009). Type III genes that are regulated by HrpB often have a HrpII box with a consensus sequence TTGG-N_{16}-TTGG (Cunnac *et al*., 2004). The PIP and *hrp*II boxes appear to be equivalent to each other and they are generally described by consensus sequence TTCGB-N_{15} –TTCGB, where B refers to any amino acid base except adenine (Koebnik *et al*., 2006). This means that single nucleotide replacements are tolerated at all positions except for the central cytidine of each half-site, leading to 10-30% residual promoter activity (Tsuge *et al*., 2005)

In *R. solanacearum*, regulation often involves the outer membrane protein PrhA (plant regulator of *hrp* genes), membrane protein PrhR, transcription activator PrhI, PrhJ, HrpG and HrpB (Büttner and Bonas, 2006; Marenda *et al*., 1998). PrhA detects non-diffusible signals from the plant cells and transduces the signal to PrhJ which controls *hrpG* gene expression (Marenda *et al*., 1998; Fu, 2008). HrpG then activates the *hrpB* regulatory gene, which in turn induces the expression of the Type III genes. In both *Xanthomonas* spp. and *R. solanacearum* HrpX and HrpB are activated by HrpG protein which belongs to the OmpR family of the two-component signal transduction response regulators (Wengelnik *et al*., 1996; Wengelnik *et al*., 1999).

In addition to the PIP-box, there is a secretion signal carried in the first 50 amino acids of effector proteins (Guttman *et al*., 2002; Petnicki-Ocweija *et al*., 2002; Büttner *et al*., 2004). Type III-dependent secretion signal is declared to be located within the first 15-20 amino acids of the effector protein N-terminus (Guttman *et al*., 2002; Petnicki-Ocwieja *et al*., 2002, Collmer *et al*., 2002). In addition to secretion signal, the tanslocation signal is located within the first 50-100 amino acids (Sory *et al*., 1995; Mudgett *et al*., 2000; Schechter *et al*., 2004).

However, for some T3 secreted effectors, N-terminal secretion signal is not sufficient for maximal secretion. In such cases efficient type III protein export from pathogens to host cells depends on the presence of a corresponding chaperone (Parsot *et al*., 2003; Büttner *et al*., 2004; Büttner and He, 2009). Chaperones are generally small, leucine-rich, and acidic proteins that bind to and often stabilize secreted proteins (Fieldman and Cornelis, 2003; Parsot *et al*., 2003). The importance of type III chaperones was initially declared for *Erwinia amylovora* and *P. syringae* (Gaundriault *et al*., 2002; Shan *et al*., 2004). The *hrp* gene cluster also contains the *hpa* (*hrp*-associated) genes which contribute to plant-pathogen interactions (Huguet *et al*., 1998). HpaB from *Xcv* has typical features of Type III chaperone and it has been reported responsible for translocation of five effector proteins, making it an important pathogenicity factor (Büttner *et al*., 2004; 2006).

The T3SS secretes several translocator proteins which facilitate the translocation of effector proteins across the host cell membrane. HrpF from *Xcv* is a secreted translocator essential for the effector protein translocation (Büttner *et al*., 2002). HrpF contributes to bacterial pathogenicity and effector protein translocation. During protein secretion, T3SS secrete other proteins called harpins into plant cells. Not much is known regarding their role in the process,

but they contain distinct motif domains that are probably involved in the interaction of harpins with cell wall components (Büttner and He, 2009).

1.4.2 Bacterial effectors

Effector proteins possess important functions such as invading the host tissue, suppressing the host immune system and modulating host signalling pathways to promote cell infection as well as promoting pathogen growth.

1.4.2.1 Identification of effectors

Effector research has been carried out by many research groups in past decades and it is still a very important and expanding field. Through the understanding of effectors and their targets, there is a possibility that these effectors can be used to identify strategies for crop improvement. A number of discoveries have been made in effector research dating back to the discovery of the first pathogen effector, *avrA*, by Staskawicz *et al*., in 1984. In 1996, Alfano and Collmer made the major discovery that the effectors are injected directly into the host plant cells suggesting that these effectors have intracellular targets (Alfano and Collmer, 1996). About a decade ago, the genomic sequence information was made available for several plant bacterial pathogens (da Silva *et al*., 2002; Buell *et al*., 2003). This sequence information led to the initial identification of putative effectors and pathogenicity factors through bioinformatic approaches (Büttner *et al.,* 2003). To date, next generation sequencing technologies have provided the genomic sequences of many plant pathogen species playing a huge role in improving effector discovery strategies. The study of plant pathogen effectors involve both effector discovery and target discovery strategies (Alfano, 2009; Boch and Bonas, 2010).

The classic strategy to identify a pathogen effector, is to determine if it induces the effector-triggered immunity (ETI) when expressed in a virulent pathogen. This approach was introduced by Staskawicz *et al*., (1984) in the search of effectors in *Pseudomonas* species. The effector proteins with the ability to trigger ETI and induce HR were characteristically named avirulence (Avr) proteins. With the subsequent suppression of the basal defense responses and the ability of the pathogens to directly inject the effectors using the T3SS, the plants also evolved the ability to recognize these avirulence (*avr*) genes with their corresponding *R* genes (Jones and Dangl, 2006).

1.4.2.2 Bacterial Type III effectors

a) Non-TAL effectors

Avirulence genes occur in bacteria, viruses, fungi, nematodes and insects. These genes have the ability to betray the pathogen to the host defense system causing the plant to elicit resistance response against the infecting pathogen. The avirulence genes are therefore regarded as a major key to understanding host-pathogen interaction. The *avr* genes are generally defined by the corresponding plant resistance (*R*) genes, which upon encounter, in most cases results in HR or localized cell death. The *avr* genes are delivered directly to the host cells by the T3SS for two major purposes among others i.e. to enhance virulence and invasiveness. *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) *avrBs2* gene has been proven to be involved in aggressiveness in pepper plants (Kearney and Staskawicz, 1990). The mutations in this *avr* gene and its homologs results in reduced pathogen ability to multiply in the host plant cells suggesting its importance in disease formation (Kearney and Staskawicz, 1990).

The *avr* genes have been classified as having dual functions in both virulence and in avirulence. These genes have effect on virulence only when present in appropriate strains, species and/ or pathovars. Avirulence gene products with virulence effects are targeted to specific organelles within the cell. Non-TAL effectors are structurally and functionally diverse group. Non-TAL effectors are highly conserved among *Xanthomonas* strains. They share homology among strains of this genera and most of them are associated with virulence. Most non-TAL effectors have PIP box motif (*X. oryzae* pv. *oryzae XopL* and *XopAD* effector genes) suggesting that their expression is regulated by HrpX (Jiang *et al*., 2009; Furutani *et al*., 2009; Song and Yang, 2010). The functions of some are directly linked with their functional domains or motifs (Ryan *et al*., 2011), whereas some are still of unknown importance. These effectors contribute to minor and major effects of the pathogen virulence (Furutani *et al*., 2009). Not much has been reported on non-TAL effectors especially with regards to their regulation. *Xanthomonas* outer membrane proteins (Xops) are known as non-TAL bacterial effectors that are delivered to the plant cell via Hrp T3SS (Cheong *et al.*, 2013). The major roles of these effectors involve modulation of signalling in plant defence responses (Mudgett, 2005; Metz *et al*., 2005). Among a lot other Xops, XopN is secreted in a Hrp T3SS-dependent manner, where it is translocated into the cell cytoplasm and its expression is regulated by HrpX (Furutani *et al*., 2009).

AvrBs2 was the first to be classified as non-TAL effector (Minsavage *et al*., 1990; Zhao *et al*., 2013). AvrBs2 has a significant role in virulence (Zhao *et al*.,2011). Additionally, AvrBs2 activates *Bs2* resistance protein in resistant plants thus allowing the plant to suppress delivery of T3SS effectors into plant cells (Zhao *et al*., 2011).

b) TAL effectors

TAL effectors belong to the AvrBs3 family class of *avr* genes and are found in several but not all pathogenic members of genus *Xanthomonas* (Bogdanove *et al*., 2010). The proteins from this family have a transcriptional activation domain in the carboxy (C-) terminus, which is highly conserved among family members and required for the avirulence activities of some members such as AvrBs3, AvrXa10, and AvrXa7 (Zhu *et al*., 1998; 1999) from the genus *Xanthomonas*. TAL effectors have a common N-terminus required for T3 secretion and a C-terminus containing nuclear localization signals (NLSs) as well as the acidic activation domain (AAD). TAL effectors are injected via the T3SS into plant cells. Upon their delivery to the host plant cells they enter the nucleus where they bind to the TAL effector-specific binding sequences and turn on the downstream genes. Some TAL effectors activate host genes that facilitate bacterial colonization and spreading. The plants can either choose to recognize them or be victimized, therefore the TAL effectors can be virulence factors, plant-recognized avirulence factors or both (Bogdanove *et al*., 2010).

TAL effector targets were initially identified for AvrBs3 in pepper plants (Marois *et al*., 2002). In pepper plants the AvrBs3 causes hypertrophy, a condition that results due to the enlargement of the mesophyll cells in infected plant tissue and it might also help the pathogen to spread to other healthy tissues (Kay *et al*., 2007). Although the bacteria have evolved the T3SS to overcome and suppress plant immunity, plants have developed the so called decoys (van der Hoorn and Kamourn, 2008) or mousetraps (Boch, 2009) to detect effector activity thus conferring resistance against TAL effectors. *Xa27* was the first resistance gene to recognize TAL effector AvrXa27 (Gu *et al*., 2004) in rice resistant lines following the infection with *X. oryzae* pv. *oryzae*. *Xa27* induces plant defense reaction but not cell death (Wu *et al*., 2008).

Moreover, the AvrBs3 family effectors have shown some important features regarding its DNA-binding activity. TAL effectors have the highly repetitive central domain which controls the specificity of these effectors (Hebers *et al*., 1992). This specificity helps the effectors to have an affinity for DNA (Yang *et al*., 2000; Boch *et al*., 2009) inside the plant host cell

therefore, promoting the production of the effector DNA which in turn result in complete cell disruption.

1.4.3 Bacterial effector structure and function

Bacterial effectors have a number of functions that differ among the proteins and their functions are expressed in different levels depending on the strain and the host plant. This all comes to the saying that successful growth of the pathogenic bacteria in a plant host is directly associated with their ability to interfere with plant defense responses. Bacterial interference with host immunity can be linked to the action of specific T3 secreted effectors within the infected cells (Mudgett, 2005). Therefore, an infecting pathogen requires T3SS to transfer the effectors to the plant host.

Once the effectors are translocated, they have two major roles to play inside the host cell either promoting the disease (virulence) or betraying the pathogen to the plant host defences (avirulence).

The expression of *X. campestris* pv. *vesicatoria* XopC and XopJ is regulated by both HrpG and HrpX (Noël *et al*., 2001; 2003). Both XopC and XopJ encode T3 secreted proteins and their N-terminal domains contain type III translocation signals. These proteins have been proven to contribute to pathogen's virulence (Noël *et al*., 2003). XopN is positively regulated by HrpX and it acts as an important virulence factor (Cheong *et al*., 2013). XopD is nuclear localized effector (Hotson *et al*., 2003) targeting nuclear-sumoylated proteins, where it functions in suppressing symptom production. Like TAL effectors XopD is a DNA-binding protein that alters host transcription thus promoting pathogen growth while delaying the onset of symptoms (Kim *et al*., 2008).

AvrBs3/PthA family is distinguished by the central repetitive region containing varying numbers of near-identical repeats of 34 or 35 amino acids (Van der Ackerveken *et al*., 1996). Differences between the family members in terms of their biological functions depend on the particular repetitive region (White *et al*., 2009). Therefore shuffling of specific repetitive regions can disrupt avirulence activity of these effectors (Hebers *et al*., 1992). Expression of these genes strongly rely on the NLSs since their recognition and roles are within the host plant cell (Van der Ackerveken *et al*., 1996).

The roles that some of these effectors play inside host cells are still under investigation. Some roles are purely regulated by putative structural motifs and or the functional domains within

these effectors. A variety of putative structural motifs contained in the primary structure of the T3 secreted effectors provides insights into their biochemical function (White *et al*., 2009).

1.4.4 Methods for identification of T3Es

The best starting point for identifying effectors is at the genome sequence of a pathogen. About a decade ago, genomic sequence information was made available for several plant pathogens i.e. *X. campestris* pv. *campestris* strain ATCC 33913 (da Silva *et al*., 2002); *R. solanacearum* strain GM1000 (Salanoubat *et al*., 2002); *P. syringae* pv. *tomato* strain DC3000 (Buell *et al*., 2003; Collmer *et al*., 2002). Accumulation of plant pathogen genome sequences is expected to result in identification of a large number of plant pathogen effectors over the next decade. Sequence homology among effectors has not only made it easier to classify them but it has also facilitated the identification of a number of effectors. Bioinformatic approaches have played a significant role in identification and characterization of putative effectors by comparing their sequences to known effectors or the presence of conserved motifs sequence suggesting a certain role inside host cells (Noël *et al*., 2003; Büttner *et al*., 2003).

Generally in plant pathogens, regulation of effector expression depends on specific regulatory proteins such as HrpX , HrpL that recognize conserved sequence motifs (PIP, Hrp, hrpII boxes) in effector genes. The presence of these conserved sequence motifs have helped classifying such effector genes as members of the T3 effector class (Jiang *et al*., 2009). In some cases additional screens have been employed, such as determining the involvement of T3SS translocons and chaperones, suggesting that effector gene in question is secreted and translocated in a T3-dependent manner (Jiang *et al*., 2009).

Studies show that bacterial effectors have the ability to suppress host innate immunity (Boller and He, 2009; Block *et al*., 2008; Zhou and Chai, 2008) except for TAL effectors that induce transcription of susceptibility genes in the host plant (Kay and Bonas, 2009). A classic strategy to identify effector genes has been used for over a decade. This strategy involves determining whether or not effector genes induce effector-triggered immunity (ETI) when expressed, simply referred to as *R* gene-mediated responses (Astua-Monge *et al*., 2000a; Bonas *et al*., 1989; Ronald and Staskawicz, 1988). Later in the years, improvements in T3 effector identification assays were made. Assays that are independent of *R*-gene responses have facilitated the analysis of T3 effector secretion and translocation. Assays involving avirulence reporter fusions (Greenburg and Vinatzer, 2003; Guttman *et al*., 2002) and/ or calmodulin-

dependent adenylate cyclase (Cya) activity assays (Casper-Lindley *et al*., 2002; Schetcher *et al*., 2004) were developed and tested.

The calmodulin-dependent adenylate cyclase (Cya) of *Bordetella pertussis* cyclolysin was initially applied by Casper-Lindley and colleagues as a reporter protein to study the direct translocation of *Xanthomonas* effector protein AvrBs2 into the host cells (Casper-Lindley *et al*., 2002). Adenylate cyclase activity (production of cAMP) depends on the presence of eukaryotic plant calmodulin and is only active after translocation from the prokaryotic cell into the eukaryotic plant cell. AvrBs2:cya fusion resulted in increased levels of cAMP (formed by conversion of ATP to 3',5'-cyclic AMP) in the cell cytosol when expressed in wild type *Xcv* expressing the inner membrane protein *hrcV* suggesting that translocation of effectors to the cell cytosol is T3-dependent (Casper-Lindley *et al*., 2002). The Cya translocation reporter was also employed in studying *Pseudomonas* T3SS targeting signals and novel proteins (Schechter *et al*., 2004). The AvrPto-Cya fusion helped determine that effector proteins can be translocated into plant cells regardless of their minimal secretion in culture, and three novel effector proteins were also identified.

In addition to Cya reporters, effector regions of known effectors truncated at the N-terminal domain have been used as reporters. Truncated $AvrRpt2_{81-255}$ and $AvrRpt2_{101-255}$ were applied in effector assays to identify *Pseudomonas* candidate effectors (Vinatzer *et al*., 2005). The AvrRpt2 protein has an N-terminal secretion signal domain that is distinct from the HR-inducing domain carrying the effector activity (Mudgett and Staskawicz, 1999). The effector region devoid of the N-terminal secretion signal can be delivered into plants when fused to the N-terminal domain of the putative effector (Mudgett *et al*., 2000; Guttman and Greenberg, 2001). A number of effector genes have been identified using this reporter fusion.

The HR-inducing domain of *Xcc* AvrBs1 has also been employed as a reporter to characterize *X. campestris* pv. *campestris* XC1553 and to prove that XC1553 carries the translocation signal in its N-terminal domain suggesting that is indeed a T3 secreted effector (Xu *et al*., 2008). The similar domain was employed by Jiang *et al*., (2009) to characterize genes with the PIP box (plant-inducible promoter) which mediates the HrpX gene regulation in *Xanthomonas* thus classifying them as Type III secretion class of effectors.

The HR-inducing domains of the effector proteins have been widely applied as reporter fusion proteins to identify and characterize other candidate effectors (Jiang *et al*., 2009; Van der Ackerveken *et al*., 1996; Nöel *et al*., 2003; Weber and Koebnik, 2005; Xu *et al*., 2008).

Furthermore, secretion assays have been widely applied to determine if candidate effectors are secreted in T3-dependent manner before they can be classified as members of T3SS. A number of immune tags have been successfully used in secretion assays, such as HA (hemagglutinin); FLAG, 3x FLAG and His- (Histidine) tags. The use of a C-terminal HA-tag helped in validating the C-terminus of AvrRpt2 as a reporter for T3SS-dependent translocation of putative effectors into plant cells by *Pseudomonas* species (Vinatzer *et al*., 2005). The HA-tag was also employed in the identification of harpins similar to *P. syringae* HrpK1 promoting translocation of T3 effectors (Kvitko *et al*., 2007). In other studies, His-tag has been used. Its application in identification of a novel effector protein EspI from a mammalian pathogen *Citrobacter rodentium* resulted on the detection of the protein in wild type culture supernatant suggesting that the protein requires a functional T3SS for secretion into host cells (Mundy *et al*., 2004). In addition, the FLAG epitope has also been widely used. Van der Ackerveken *et al.* (1996) made a major observation on the AvrBs3 effector, using the FLAG epitope. Fusing the FLAG-epitope to the C-terminus of AvrBs3, and replacing the serine stop codon revealed that nuclear localization of AvrBs3 and its activity are correlated, meaning that recognition of this protein occurs inside the host plant cells (Van der Ackerveken *et al*., 1996). The FLAG peptide is encoded by the amino acid sequence, DYKDDDDK. FLAG epitope is more hydrophilic than other common epitope tags and therefore less likely to denature or inactivate proteins to which it is attached. Li *et al*., used expression of a FLAG-tagged version of HrpA in order to distinguish basal or apical secretion of HrpA subunits (Li *et al*., 2002). A similar experiment was used to study the role of the *hrp* pilus in T3 protein secretion in *Pseudomonas* (Li *et al*., 2002; Jin and He, 2001; Ham *et al*., 1998). The FLAG epitope has been recently used in the study of establishing an inducing medium for type III effector secretion in *X. campestris*. The expressed effectors fused with FLAG epitope were detected using Anti-FLAG M5 polyclonal antibody and goat anti-rabbit IgG antibody (Jiang *et al*., 2013).

Putative type III effectors can therefore be identified and characterized using reporter protein fusions consisting of the HR-inducing domain of an *avr* gene fused to an immuno-detectable tag in secretion assays.

1.5 Aims of the study

The aim of this study was to generate the reporter plasmid system that will allow the identification and classification of potential effectors as members of the Type III Secretion class of effectors, in *Xylophilus ampelinus*. The first objective of the study was to identify and characterize an *avrBs1*-family avirulence gene in *X. ampelinus* using bioinformatic approaches and hypersensitive response (HR) assays. The mutant was essential in the testing of the reporter system. The second objective was to use the HR-inducing domain of the *avrBs1* gene characterized in the first objective to develop the T3 effector reporter plasmid system for *X. ampelinus*. In order for the plasmid system to work in *X. ampelinus*, an *avrBs1* gene knockout mutant also had to be created via overlap extension PCR and allelic exchange. Using the *avrBs1* knockout mutant as a recipient, the reporter system was tested through *in vitro* secretion and *in vivo* translocation assays.

The reporter plasmid system developed in this study will be used in the *X. ampelinus* Type III secreted effector research by cloning N-terminal domains of putative effectors in front of the HR-inducing domain of *avrBs1* in the reporter plasmid. The analysis and classification of the putative effectors may give better understanding of the pathogen's virulence and/ pathogenicity factors.

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CHAPTER 2: Identification and characterization of *avrBs1*-family avirulence genes from *Xylophilus ampelinus*

2.1 INTRODUCTION

Pathogenicity of many Gram negative bacterial pathogens of plants and animals depend on the specialized Type III secretion system (T3SS) which spans both bacterial and host cell membranes and is associated with the extracellular appendage referred to as the needle complex for animal pathogens or the Hrp pili for plant pathogens (Büttner and He, 2009; Cornelis and van Gijsegem, 2000; Galan and Collmer, 1999). The T3SS mediates protein secretion into the extracellular medium as well as translocation of effector proteins into the host cell (Büttner and He, 2009). Mutants with mutations in the T3SS tend to lose pathogenicity, indicating that the functions of the effector proteins inside the host cell are essential for its pathogenicity (Büttner and Bonas, 2002, Galan and Collmer, 1999).

In plant pathogens, a number of effectors have been identified as the products of avirulence (*avr*) genes that betray the pathogen to the resistant plant surveillance system (Collmer, 1998). Generally, recognition of *avr* gene products by corresponding plant resistance (*R*) gene products lead to the specific induction of a strong defense response called effector-triggered immunity (ETI) that often results in the hypersensitive response (HR) (White *et al*., 2000; Noël *et al*., 2003), a rapid localized cell death at the infection site associated with arrest of bacterial growth (Klement, 1982; Staskawicz, 2001). A number of genes involved in the HR and pathogenicity (*hrp*) in many Gram negative phytopathogenic bacteria have been identified and characterized. The *hrp* genes have also been demonstrated to be required for the pathogens to cause disease in susceptible host plants and to induce HR in resistant host and non-host plants (Lindgren, 1997). Generally, *hrp* genes encode components of the T3SS (Büttner and Bonas, 2006; Cornelis, 2006). The T3SS of the plant pathogenic bacteria translocates effector proteins directly into the plant cells (Büttner and Bonas, 2006). Effectors can act to activate or suppress plant defense signal transduction and in addition to that, many avirulence proteins of plant pathogenic bacteria have been shown to be translocated effectors (Alfano and Collmer, 2004; Grant *et al*., 2006; Mudgett, 2005).

Genome sequence information has now been made available for a number of plant pathogens including members of the genera *Xanthomonas* (Cunnac *et al*., 2013; da Silva *et al*., 2002) *R. solanacearum* (Salanoubat *et al*., 2002; Xu *et al*., 2011); and *Pseudomonas* (Baltrus *et al*., 2011; Buell *et al*., 2003) . This sequence information led to the initial identification of candidate

effectors and pathogenicity factors through bioinformatic approaches (Baltrus *et al*., 2011; Büttner *et al.*, 2003). Effector gene candidates have been discovered as a result of homology to known effectors or due to the presence of conserved sequence motifs that suggest a certain role inside host cells (Noël *et al*., 2003). Identification of the complete repertoire of pathogenicity factors of plant pathogens and their biological functions is the prerequisite to understanding the pathogen-plant interactions.

Agrobacterium-mediated transient expression is based on the expression of non-integrated T-DNA and it provides a valuable procedure for readily assessing genetic information (Jones *et al*., 2005; 2009). Moreover, this technique has led to a number a studies in areas such as gene-for-gene interactions, regulation and expression of multiple genes simultaneously and gene silencing (Yang *et al*., 2000). *Agrobacterium*-mediated transient expression assay has been widely applied to many plant systems (Cheng *et al*., 2009; Li *et al*., 2009; Sparkes *et al*., 2006). *Agrobacterium*-mediated transient expression of many avirulence proteins from *P. syringae* pathovars and *Xanthomonas* species resulted in induction of HR in resistant plants (Bonas and van der Ackerveken, 1997; Kjemtrup *et al*., 2000; Escolar *et al*., 2001). This indicates the usefulness of transient expression in the identification of potential pathogenicity factors.

Xylophilus ampelinus, the causal agent of bacterial blight of grapevines, is a Gram negative plant pathogen with unknown virulence factors. Very little is known about the genetics of the pathogen, especially with regard to its pathogenicity. Transposon mutagenesis is a genetic tool employed to study gene or protein function. The technique gained recognition after it was applied and proved to have potential for studying virulence genes in plant pathogens (reviewed by Mills, 1985). A similar tool was applied in the characterization of pathogenicity and virulence factors in *X. ampelinus* (Y. Petersen, personal communication). Transposon mutant sequence analysis revealed the presence of *avrBs1* and *avrA*-like avirulence gene homologs thus motivating gene function analysis. Therefore, identifying effector proteins of *X. ampelinus* is the first step to understanding the pathogenicity factors of this pathogen. For this objective the *avrBs1*-family genes of *X. ampelinus* were characterized using bioinformatic sequence analysis and *Agrobacterium*-mediated transient expression, to better understand the roles these genes play during pathogenicity.

2.2 MATERIALS AND EXPERIMENTAL PROCEDURES

Chemicals used in this study are listed in APPENDIX A. Preparation of stock buffers and growth medium is discussed in APPENDIX B.

2.2.1 Bacterial strains, plasmids and plant growth conditions

The bacterial strains used in this study were kept as glycerol stocks at -70 °C. They were continuously revived when needed and kept frozen. All *X. ampelinus* strains were grown on YPGA agar plates at 28 °C (Grall and Manceau, 2003) for five to ten days depending on the experiment to be pursued. *Agrobacterium* strains were grown on YEP agar plates or in broth at 28 °C for 48 hours. YEP medium was always supplemented with 50 µg/ml rifampicin, unless there was a plasmid involved then additional antibiotics were added depending on plasmid requirements. *Escherichia coli* JM109 was grown on LB agar plates or broth at 37 °C for 16 hours. The medium was supplemented with appropriate antibiotics depending on the plasmid maintenance requirements. The characteristics of the bacterial cultures and plasmids used, and those generated in this study, are listed in Table 2.1.

Tobacco plants (*Nicotiana tabacum*) were grown from seeds in the glasshouse under controlled environmental conditions. Seedlings were grown for up to five weeks and leaves were then used for transient expression studies.

Table 2.1: Plant lines, strains and plasmids used in this study

Bacterial strain or plasmid	**Relevant characteristics**	**Reference or source**
***Xylophilus ampelinus* VS20**	Wild type *Xylophilus ampelinus*	ARC-PPRI[a] Culture collection
Agrobacterium tumefaciens C58C1	Rif^r	Dirk Stephan, US[b]
Agrobacterium tumefaciens EHA105	Rif^r	Lab strain
Escherichia coli JM109	*endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, laqIqZΔM15].*	Lab strain
Plasmids		
p442	Vector containing 35S Cauliflower Mosaic Virus(CaMV) promoter; Amp^r	Dirk Stephan, US
pCB301	Mini Binary vector series, *RK2 oriV*, similar to *nptIII*, plasmid *RK2 TrfA* region, T-DNA right border, *MCS*, T-DNA left border; Kan^r	Dirk Stephan, US

pCB3ΔB	Binary vector pCB301 with *Bam*HI restriction site removed; Kan^r	This study
pCB3CaMV	pCB301 with 35S CaMV promoter and terminator; Kan^r	This study
pCB3CaMV_*avrBs1*	pCB3CaMV carrying full length *avrBs1* gene; Kan^r	This study
pCB3CaMV_*avrA*	pCB3CaMV carrying full length *avrA* gene; Kan^r	This study

[a]ARC PPRI: Agricultural Research Council, Plant Protection Research Institute

[b]US: University of Stellenbosch

2.2.2 Polymerase chain reaction (PCR) primers used in this study

All primers used in this study are listed in Table 2.2 below. Most of them were designed for this study using both CLC genomics workbench Version 6 (CLC Bio, Århus, Denmark) and NCBI Primer BLAST (Altschul *et al*., 1990). All primers were ordered from IDT and resuspended in sterile water to make 100 µM stock solutions.

To prepare working solutions, the stock solution was diluted 1:10 in nuclease free sterile water resulting in 10 µM primer working solutions. For all PCR reactions a final concentration of 0.2 µM Melting temperatures (Tm) provided on each product specification sheet were used to determine the annealing temperatures of each primer set.

Table 2.2. Polymerase chain reaction primers

Primer	Sequence	Description	Reference
S3	GGTGTTAGGCCGAGTAGTGAG	Forward primer for detection of *X. ampelinus* Ribosomal ITS sequence	Botha *et al*., 2001
S4	GGTCTTTCACCTGACGCGTTA	Reverse primer for detection of *X. ampelinus* Ribosomal ITS sequence. Product length = 277 bp	Botha *et al*., 2001
YP269	ATGCTAAACCCAAAATCCGATTCC	Forward primer specific for *X. ampelinus avrA* Open reading frame (ORF)	This study
YP272	TTATTTCCGGGCATCGTTGATTTTCAAT	Reverse primer specific for *X. ampelinus avrA* ORF. YP269+272 product length = 1224 bp	This study
YP273	ATGGATATAAACCATATCGAATTTGC	Forward primer specific for *X. ampelinus avrBs1* ORF	This study

YP270	TCATTTCTCGAATATGACTTCC TGT	Reverse primer specific for *X. ampelinus avrBs1* ORF. YP273+270 product length = 1239 bp	This study
YP276	ACGTTCTACGAGCTTTGCCA	Forward primer specific for binary vector pCB301 *nptII* region	This study
YP277	AGATTGTCGTTTCCCGCCTT	Reverse primer specific for binary vector pCB301 *nptII* region; YP276+277 product length = 705 bp	This study

2.2.3 Sequence analysis

X. ampelinus sequence analysis and construction of plasmid and restriction enzyme maps were done using CLC Genomics Workbench version 6.0 and nucleotide sequence comparisons were done using BLAST (Altschul, 1990). ORF nucleotide sequences were translated to protein sequences in reading frame one and sequence comparisons were done using the BLASTp function (Altschul, 1997) on the NCBI website (http://www.ncbi.nlm.nih.gov).

2.2.4 *X. ampelinus* HR assay on tobacco leaves

Given that hypersensitive response (HR) is induced when non-host or resistant plants are invaded by a certain pathogen, therefore HR assay was conducted using the wild type *X. ampelinus* cells. Wild type cells were revived from glycerol stock into YPGA plates and grown at 28 °C for six to eight days. The cells were aseptically transferred onto fresh YPGA plates and incubated for five days at 28 °C. The cells were carefully transferred to 1x PBS buffer and adjusted to OD_{600}nm of ~0.3-0.35. Approximately 20 µL of cell suspension was infiltrated into plant leaves using 1 ml needleless syringe. 1x PBS buffer was infiltrated into plant leaves to serve as a negative control.

2.2.5 *X. ampelinus* genomic DNA extraction

Total genomic DNA of wild type *X. ampelinus* was extracted by the CTAB genomic DNA isolation procedure (Ausubel *et al*., 1989). Cells were grown for eight days on YPGA (7g/L yeast extract, 7g/L Bacto-peptone, 7g/L glucose and 15g/L bacto-agar) at 28 °C. After eight days, the bacteria were subcultured onto fresh YPGA plates by transferring a loop-full of culture onto a new plate and kept at 28 °C for five days. The bacterial growth was transferred into sterile tubes and centrifuged at 13000 rpm for one minute at room temperature.

Supernatants were discarded and the cell pellets were resuspended in 567 µL of TE buffer and gently vortexed. Thirty microlitres of 10% (w/v) SDS and three microlitres of 20 mg/ml proteinase K was added, mixed and incubated for one hour. One hundred microlitres of 5 M NaCl was added and mixed, followed by 80 µL of CTAB/NaCl and the samples were then incubated at 65 °C for one hour. An equal volume of chloroform/isoamylalcohol (24:1) solution was added and the samples were centrifuged at 13000 rpm for ten minutes. The top aqueous phase was transferred to a clean tube and the DNA was precipitated by adding 0.6 volumes of isopropanol and centrifugation at 13000 rpm for 20 minutes. The pellet was washed with 70% ethanol, air-dried and resuspended in TE buffer.

2.2.6 Preparation of competent cells

2.2.6.1 Preparation and transformation of chemical competent E. coli JM109

E. coli JM109 cells from a glycerol stock was streaked onto LB agar and incubated at 37 °C overnight. A single colony forming unit (CFU) was used to inoculate a five millilitre LB broth starter culture and incubated overnight on a shaking platform at 37 °C. This culture was used to inoculate a larger volume of LB which was then incubated at 37 °C until the OD_{600nm} reached 0.6. The cells were precipitated by centrifugation at 5000 rpm for ten minutes at four degrees Celsius. Pellets were slowly dissolved in ice cold 100 mM $MgCl_2$, chilled on ice for 20 minutes and harvested at 4000 rpm for ten minutes at 4°C. After supernatant was removed, the cell pellet was gently dissolved in two millilitres of ice cold 100 mM $CaCl_2$ with 15% (v/v) glycerol. One hundred microliter aliquots of cells were flash frozen with cold ethanol and stored at -70 °C.

For transformation, 100 µL of competent cells were thawed on ice. Five microlitres of ligation mixture was added to the cells, gently mixed and incubated for five minutes on ice. The mixture of cells and DNA was heat-shocked at 42 °C for 45 seconds and then quickly returned onto ice. The reaction was kept on ice for two minutes, before adding 900 µL of ice cold LB medium and incubation at 37 °C for one hour with shaking at 150 rpm. Transformants were recovered following overnight incubation at 37 °C on LB-agar plates supplemented with the appropriate antibiotics.

2.2.6.2 Preparation and transformation of electro-competent A. tumefaciens strain C58C1

A. tumefaciens strain C58C1 from glycerol stock was revived by culturing on YEP agar plates (10 g/L yeast extract; 10 g/L Bacto-peptone; 5 g/L NaCl; 15 g/L Bacto-agar) supplemented with 50 µg/ml of rifampicin. Plates were incubated at 28 °C for 48 hours. The starter culture was prepared by transferring a single CFU from a fresh-grown plate into five millilitres of YEP medium (with 50 µg/mL rifampicin) and the culture was grown for 48 hours at 28 ˚C on a shaking platform. The starter culture was transferred into 250 ml LB broth medium supplemented with 50 µg/mL rifampicin. The culture was kept at 28 ˚C for four to six hours until the OD_{600nm} was between 0.5 and 1.0. The cells were kept on ice for 30 minutes and harvested by centrifugation at 5000xg for ten minutes at 4 °C. Pellets were washed twice with cold sterile distilled water and centrifugation at 4000xg in between. The cells were then washed four times with ice cold sterile 10% (v/v) glycerol. Electro-competent cells were finally resuspended in one millilitre of 10% (v/v) glycerol and dispensed into sterile microcentrifuge tubes. Electro-competent cells were used directly for electroporation or stored at -70 ˚C for future use.

For electroporation, competent *A. tumefaciens* cells were thawed on ice. Two microlitres plasmid was added to 40 µL of electrocompetent cells, gently mixed and kept on ice for ten minutes. The cell-plasmid DNA mixture was transferred into cold 0.2 cm electroporation cuvettes and pulsed once with 1.8 kV, at 25 µF capacitance and 200 Ω resistance. One millilitre of cold YEP medium was added quickly and the cell suspension incubated at 28 °C for two hours shaking at 140 rpm before being plated onto YEP agar plates supplemented with 50 µg/ml each of rifampicin and kanamycin. Plates were incubated at 28 °C for 48 hours and transformants were verified by colony PCR using specific primers.

2.2.7 Construction of pCB3CaMV binary vector for transient expression

E. coli harbouring the plasmids, p442 or pCB301, were cultured on LB-agar plates supplemented with ampicillin 100 µg/ml or 50 µg/ml kanamycin. The plates were grown overnight at 37 °C. Single CFUs were inoculated into five millilitres LB broth supplemented with the appropriate antibiotic and grown overnight at 37 °C on a shaking platform. The plasmids were isolated using the Qiagen miniprep kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. The plasmid, p442, was digested with FastDigest restriction

enzymes *Hind*III and *Eco*RI (Thermo Scientific, Inqaba Biotec, South Africa) according to the manufacturer's instructions in order to release a 1 kb DNA fragment containing the Cauliflower mosaic virus (CaMV) 35S promoter and terminator (refer to figure 2.1 for complete vector manipulation process). The reactions were incubated at 37 °C for 30 minutes. The restriction digest was then electrophoresed on a 1 % agarose gel stained with 0.2 µg/ml ethidium bromide prior to setting. The *Hind*III/ *Eco*RI fragment containing the CaMV 35S promoter and terminator was cut from the gel after visualization on the ultraviolet (UV) light transilluminator (Chromato-Vue Transluminator MT-36, UVP Inc. San Gabriel, USA) and purified using the QiaexII gel extraction kit (Qiagen GmbH, Germany) according to the manufacturer's instructions.

Before the *Hind*III/ *Eco*RI fragment could be cloned into the binary vector, pCB301, *Bam*HI restriction site in the multiple cloning site (MCS) of this vector needed to be removed, since the aim was to clone the *avrBs1* and *avrA* ORFs at the *Bam*HI site situated between the CaMV 35S promoter and terminator. To achieve this, pCB301 was digested with 10 units of FastDigest *Bam*HI (Thermo Scientific) in a total reaction volume of 20 µL at 37 °C for one hour, electrophoresed on a one percent agarose gel, excised and purified using the QiaexII gel purification kit. To permanently remove the restriction site, 1 µg/ml of purified linearized pCB301 was treated with 10 U of S1 nuclease enzyme (Thermo Scientific, Inqaba, South Africa) according to the manufacturer's instructions, in a total reaction volume of 30 µL to remove the 3′- and 5′- overhangs created by sticky-end restriction with *Bam*HI. Thereafter, the reaction was electrophoresed on a one percent agarose gel, excised and purified using the QiaexII gel purification kit. The plasmid was re-circularized using five units of T4 DNA ligase (Thermo Scientific, Inqaba, South Africa) according to the manufacturer's instructions. The ligation mixture was incubated at 22 °C for 30 minutes and five microlitres of the reaction was used to transform *E. coli* JM109 competent cells by heat-shock method (Section 2.2.6.1.). The resultant plasmid, named pCB3ΔB was propagated and purified as described previously.

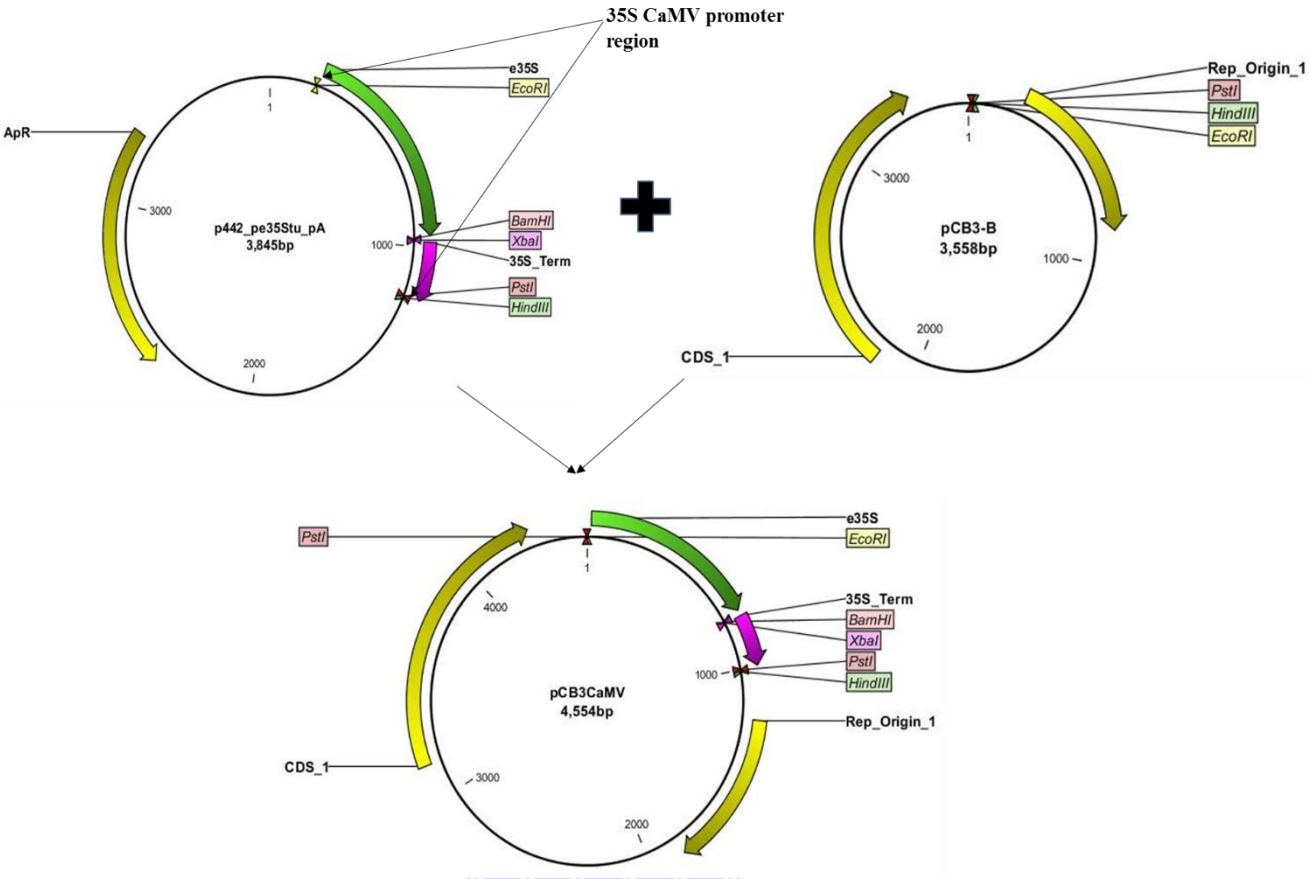

Figure 2.1. Transient expression vector construction. The 35S CaMV promoter and terminator region was excised from vector p442 and cloned between the *Hind*III and *Eco*RI restriction sites on binary vector pCB3ΔB, from which the original *BamH*I site had been removed.

Vector pCB3ΔB was digested with FastDigest *Eco*RI and *Hind*III restriction enzymes, electrophoresed and gel purified as described previously. This 3.5 kb linearized vector DNA was ligated with the one kilobase pair *Eco*RI and *Hind*III DNA fragment from vector p442 carrying the CaMV 35S promoter and terminator to create the vector, pCB3CaMV. Five microliters of the ligation reaction was used to transform 100 µL of *E. coli* JM109 chemical competent cells using a heat-shock method. Transformed cells were grown on LB-agar plates containing 50 µg/ml kanamycin at 37 °C for overnight. Resulting transformants were PCR-verified using primer pair YP276 and YP277. A PCR-positive colony was transferred and grown in a liquid LB broth supplemented with 50 µg/ml kanamycin and the plasmid DNA extracted after overnight incubation at 37 °C. This plasmid vector was named pCB3CaMV.

2.2.8 Amplification and cloning of *X. ampelinus avrBs1* and *avrA* ORFs

Genomic DNA of wild type *X. ampelinus* was used as template for PCR with primers YP270 and YP273, and YP269 and YP272 to amplify the *avrBs1* and *avrA* ORFs, respectively. The proofreading polymerase, Phusion High fidelity DNA polymerase (Thermo Scientific, Inqaba Biotech, South Africa) was used for amplification according to manufacturer's instructions. Reactions typically contained 20 ng of a template DNA, 200 µM of dNTPs, 200 nM of each primer and one unit of Phusion polymerase.

The PCR programme for amplification of *avrA* was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 54 °C for 15 seconds, 72 °C for 45 seconds) and a final extension at 72 °C for five minutes. The PCR programme for amplification of *avrBs1* was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 52 °C for 15 seconds, 72 °C for 45 seconds) and a final extension at 72 °C for five minutes.

PCR products were resolved in ethidium-containing 0.8% agarose gels in Tris/Borate/EDTA (TBE) buffer electrophoresed at 100 volts for one hour. The DNA bands were visualized under UV light and photographed using the Ingenius Bio-Imaging system (Syngene, Vacutec, South Africa). The PCR products were excised from the gel using a sterile blade, and the DNA was purified using the QiaexII gel extraction kit.

The binary vector, pCB3CaMV, was digested with 10 U of FastDigest *Bam*HI enzyme in a 20 µL reaction volume. The linear vector band was excised from the gel and purified using the QiaexII kit. The vector was treated with S1 nuclease, to remove the 3′ and 5′ overhangs. The blunt-ended vector was then purified using QiaexII kit and ligated together (as previously described) with blunt-ended PCR-amplified *avrA* and *avrBs1* in separate reactions. PCR verification of transformants was done using primers specific for the *avr* inserts. Positive transformants were cultured into fresh five millilitres of LB medium supplemented with kanamycin and incubated at 37 °C for overnight. Cultured bacterial cells were harvested and plasmids were extracted with the Qiagen miniprep kit and stored at -20 °C. The plasmids were digested with *Pst*I and separated by gel electrophoresis to determine insert orientation. Two constructs with the *avr* genes in the correct orientation were selected and named pCB3CaMV_*avrBs1* and pCB3CaMV_*avrA*, respectively. The constructs were digested with *Pst*I and separated by gel electrophoresis to check insert orientation. These two constructs were used to transform *A. tumefaciens* strain C58C1 as describe in section 2.2.6.2.

2.2.9 *Agrobacterium*-mediated transient expression

A. tumefaciens strain C58C1 carrying pCB3ΔB, pCB3CaMV, pCB3CaMV_*avrBs1*, pCB3CaMV_*avrA* and untransformed *A. tumefaciens* C58C1 was grown in five millilitres of YEP broth containing 50 µg/ml each of rifampicin and kanamycin and 50 µg/ml of rifampicin only for the untransformed C58C1, at 28 °C for 48 hours. Thereafter, one millilitre of culture was transferred into fresh 50 ml YEP broth supplemented with appropriate antibiotics, 10 mM MES buffer pH 5.6 and 150 µM acetosyringone (Refer to APPENDIX B). Cell cultures were grown at 28 °C for 16 hours with constant shaking at 180 rpm to an OD_{600nm} value of approximately one. The cells were harvested by centrifugation at 3000xg for 10 minutes. The cell pellets were washed with 50 ml of the induction medium containing 10 mM $MgCl_2$ and 10 mM MES buffer in sterile distilled water. The cell suspension was centrifuged again at 4000xg for 10 minutes and the pellets were resuspended in 50 ml of infiltration medium containing 10 mM $MgCl_2$; 10 mM MES, pH 5.6 and 150 µM acetosyringone according to Annamalai and Rao (2006). Bacterial suspensions were kept at room temperature for two to three hours without shaking before plant inoculation. Bacterial suspensions were inoculated into six to eight weeks old tobacco plant leaves using a needleless syringe. After infiltration, plants were kept at room temperature in a 16 hour light and eight hour dark cycle. Leaves were photographed after a two to three days post-inoculation period.

2.3 RESULTS

2.3.1 *X. ampelinus* induces HR in *N. tabacum*

X. ampelinus induced strong HR on *N. tabacum* plants at room temperature. After incubation for 48 hours the brown-translucent lesions developed at inoculation sites. The lesions were observed just after 24 hours and they were pronounced at 48 hours incubation period. Generally, pathogenic bacteria can elicit HR when introduced into non-host plant as opposed to susceptibility and disease formation when introduced into host plants. The ability of *X. ampelinus* to elicit HR in non-host (figure 2.2) inspired the continuous search for effector proteins responsible for HR induction.

Figure 2.2: *X. ampelinus* HR assay on tobacco. Right leaf panel was inoculated with 1x PBS buffer and the left leaf panel was inoculated with wild type *X. ampelinus (wtXa)*. Brown translucent lesion on the left leaf panel represents HR-induction by wild type *X. ampelinus*.

2.3.2 Sequence analysis of wild type *X. ampelinus* avirulence open reading frames (ORFs)

Analysis of transposon mutants revealed mutant *XaTn5-742* had an insertion in a gene encoding an *avr* protein with similarity to AvrA from *P. syringae* pv. *glycinea* and *avrBs1* present in members of the genus *Xanthomonas* (Y. Petersen, personal communication). Further sequencing revealed other coding sequences with greater similarities to *avrBs1* family avirulence gene from *Xanthomonas* and *Acidovorax* species.

In my study, I analysed the sequence of the identified *avrBs1* and *avrA* homologs using CLC Bio version 6.0.The ORFS (*avrBs1* and *avrA*-like) were 1239 and 1224 bp, encoding 413 and

408 aa proteins respectively. The NCBI BLASTp function was then used to determine the percentage identity of the *X. ampelinus* protein sequences with those in the Genbank database. *X. ampelinus* AvrBs1 and AvrA-like proteins share up to 58% and 63% sequence homology with avirulence proteins from *Xanthomonas, Pseudomonas* and *Acidovorax* species (refer to Table 2.3 and 2.4).

Table 2.3: X. ampelinus AvrA alignemt with NCBI protein sequences

Hit accession number	**Protein description**	**Percentage Identity**
WP_006449061	*X. gardneri* ATCC 1986 putative T3 effector belonging to *avrBs1* class	63.81%
P11437	*P. syringae* avirulence A (AvrA) protein	61.94%
WP_010209602	*P. syringae* pv. *tomato* avirulence protein	59.83%
WP_005734998	*P. syringae* pv. *aesculi* str. 0893_23 avirulence protein AvrBs1	58.97%
WP_010215138	*P. amygdali* avirulence protein	58.97%

Table 2.4: X. ampelinus AvrBs1 alignment with NCBI protein sequences

Hit accession number	**Protein description**	**Percentage Identity**
ACS12852	*X. campestris* pv. *campestris* AvrBs1 protein	75.50%
WP_012438467	*X. campestris* pv. *campestris* str. B100 AvrBs1 protein	58.24%
WP_011037255	*X. campestris* pv. *campestris* str. ATCC 33913 avirulence protein	58.24%
P19520	*X. campestris* full uncharacterized 50 kDa protein encoded by ORF2 in *avrBs1* region	57.95%
WP_006449061	*X. gardneri* ATCC 19865 AvrBs1 protein	54.24%
WP_005734998	*P. syringae* pv. *aesculi* str. 0893_23 avirulence protein AvrBs1	53.70%

P11437	*P. syringae* avirulence A (AvrA) protein	53.59%
WP_010209602	*P. syringae* pv. *tomato* avirulence protein	53.07%
WP_011795287	*Acidovorax citruli* avirulence protein AvrBs1	41.53%

2.3.3 Cloning and transient expression of *X. ampelinus avrA* and *avrBs1* homologs in tobacco plants

The evidence that a*vrBs1* and *avrA* gene homologs from *Xanthomonas* and *Pseudomonas* strains are recognized inside plant cells and elicit HR when expressed, inspired the similar assay for *X. ampelinus* CDs. To determine if coding sequences of *avrBs1* and *avrA* elicit hypersensitive response (HR) in a non-host both genes were isolated using PCR primers specific to each CD and cloned into a binary vector pCB3CaMV under the control of 35S promoter for gene expression *in planta*. The constructs were verified by colony PCR (Figure 2.3, 2.4 and 2.5). The *avrBs1* and *avrA* homolog constructs transiently expressed by *A. tumefaciens* strain C58C1 induced HR on tobacco plants within 48 hours post inoculation (Figure 2.6).

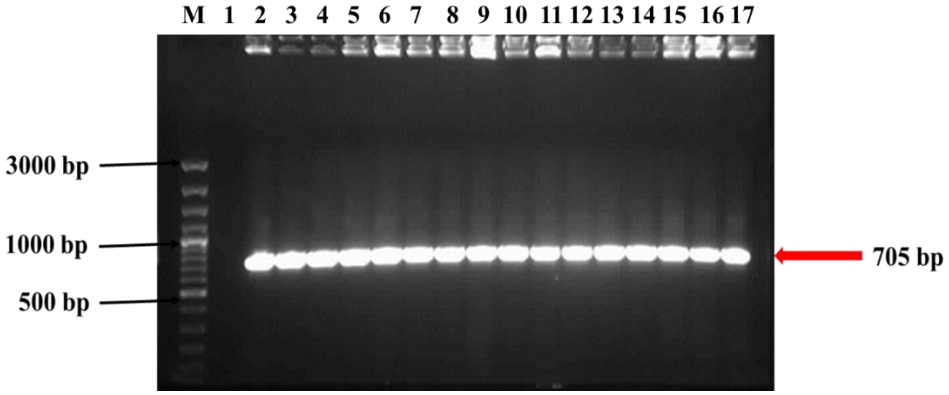

Figure 2.3: Verification of pCB3CaMV construct. Colony PCR screen was performed using primers specific to the *npt*III region (YP276 and YP277) of the binary vector. Lane M- 100 bp Plus DNA marker (Thermo Scientific, Inqaba, South Africa), lane 1- negative control (no DNA), lane 2 to 17- *Agrobacterium*-pCB3CaMV transformants.

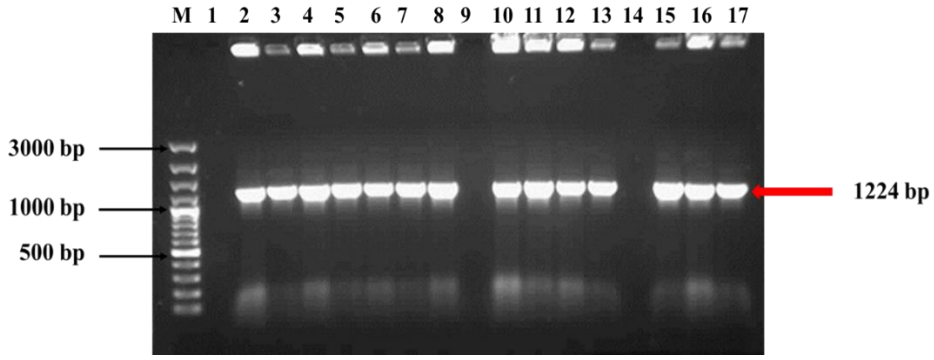

Figure 2.4: Verification of pCB3CaMV_*avrA* construct. Primer pair (YP269 and YP272) specific for *avrA* CD was used in colony screening experiments. Lane M- 100 bp Plus DNA marker, lane 1- negative control, lane 2 to 17- *Agrobacterium*-pCB3CaMV_*avrA* transformants.

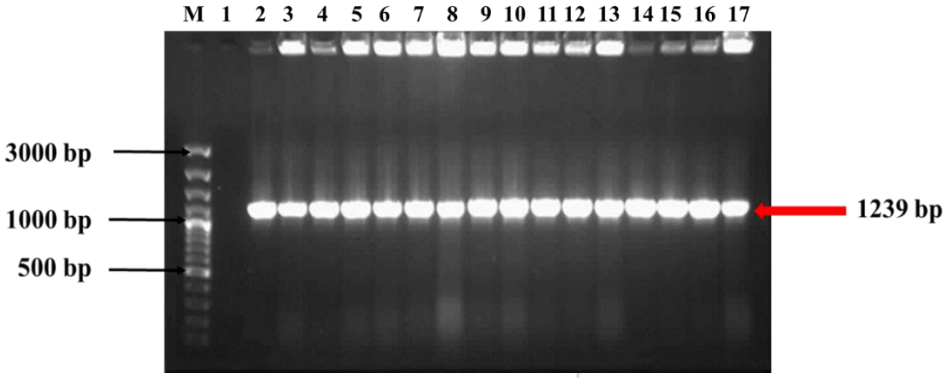

Figure 2.5: Verification of pCB3CaMV_*avrBs1* construct. Primer pair (YP270 and YP273) specific for 1239 bp *avrBs1* ORF. Lane M- 100 bp Plus DNA marker, lane 1- negative control, lane 2 to 17- *Agrobacterium*- pCB3CaMV_*avrBs1* transformants screened for the presence of *avrBs1* homolog construct.

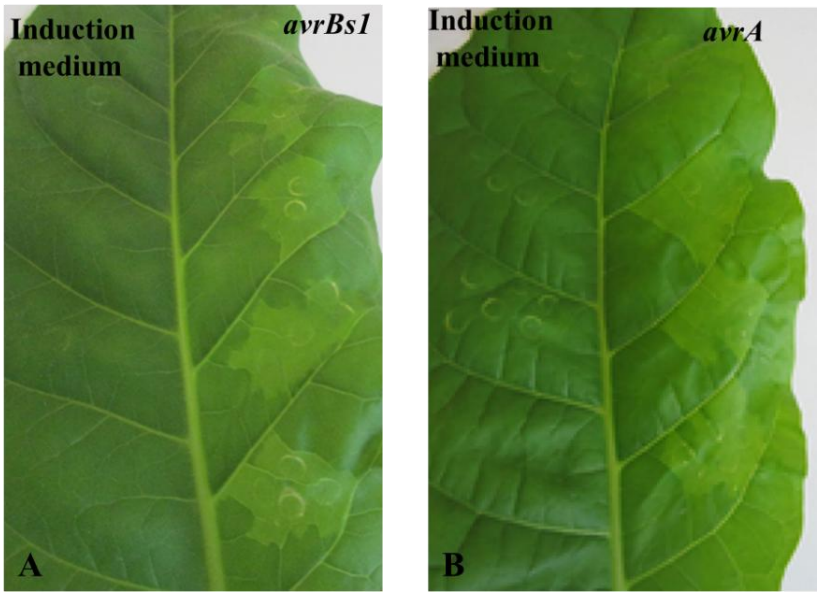

Figure 2.6. *Agrobacterium*-mediated transient expression of *avrBs1* and *avrA* in tobacco plants. Both *avrBs1* and *avrA* were expressed *in planta* under the control of the 35S promoter using *A. tumefaciens*. Expression of *avrBs1* (A) and *avrA* (B) resulted in HR on tobacco plant leaves. Right panel of both leaves shows HR lesions formed after inoculation with *X. ampelinus avrA* and *avrBs1* homologs. The left panel of both leaves were inoculated with induction medium and served as the negative control.

2.4 DISCUSSION

Out in the field, the only form of protection that plants can use against pathogens is through genetic resistance. However, genetic resistance is based on single dominant or semi-dominant genes (Tai *et al*., 1999). In general, resistance (*R*) genes confer race-specific resistance and their effectiveness depends on the interaction with complementary pathogen avirulence genes. There are however some limitations to this, i.e. the plant can have the ability to express resistance genes but pathogens keep on changing to evade host defense responses, and emergence of strain that do not express complementary gene renders resistance gene ineffective.

X. ampelinus is known to be virulent in *V. vinifera* species, but there is not much evidence about discovery of resistant plant cultivars. In this study, we tested the ability of *X. ampelinus* to elicit hypersensitive response (HR) in a non-host plant. The positive result obtained, proposed the possibility that the pathogen might be carrying active effector genes. Assuming that *X. ampelinus* employs a T3SS to transfer effector proteins, the best attempt was to discover more about these avirulence genes contributing to its pathogenicity.

Initial analysis of *X. ampelinus* transposon mutants (Y. Petersen, personal communication) revealed a mutant, XaTn5-742, which had an insertion site in a gene encoding an avirulence protein with similarities to a *P. syringae* pv. *glycinea* AvrA protein and an AvrBs1 protein present in members of genus *Xanthomonas*. Sequence analysis showed that a 1224 bp *avrA* homolog which shares up to 63% sequence identity with a number of avirulence proteins from *Xanthomonas* and *Pseudomonas* species including *X. gardneri* ATCC 19865, *P syringae* pv. *tomato* and *P. syringae* pv. *aesculi* to name a few. The protein shares 61% sequence homology with *P. syringae* pv. *glycinea* avirulence A (AvrA) protein. AvrA protein was the first to be cloned and characterized and has been widely applied in many studies involving plant pathogen interactions (Staskawicz *et al*., 1984; Lorang *et al*., 1994). *X. ampelinus* AvrA is not a complete protein but forms a part of full length *avrA* and the sequence homology is observed towards the N-terminal domain of a 907 aa *Pseudomonas* AvrA protein.

The *X. ampelinus avrBs1* ORF is 1239 bp in length and encodes a protein of 413 amino acids. It shares great homology with AvrBs1 proteins from many members of the genera *Xanthomonas*, *Pseudomonas* and *Acidovorax* including including the species *X. campestris* pv. *campestris* (*Xcc*) str. B100, *X. gardneri* str. ATCC 19865, *P. syringae* pv. *asceculi* str. 0893_23, *P. syringae* pv. *tomato* and *A. citrulli*. Discovery of this coding region encoding

AvrBs1 protein was intriguing and further characterization was required to better understand protein function. AvrBs1 protein was first identified in *X. campestris* pv. *vesicatoria* (*Xcv*)and characterized by Ronald and Staskawicz in 1988, since then the protein has been applied in many functionality studies including identification of T3 secreted effectors (Jiang *et al*., 2009; Xu *et al*., 2008).

The fact that *X. ampelinus* has AvrA and AvrBs1 homologs suggests that the pathogen utilizes T3SS in one way or the other during pathogenicity. The AvrA protein from *P. syringae* pv. *glycinea*, the causal agent of bacterial blight of soybean, led to compatibility interaction in susceptible soybean cultivars whereas in resistant cultivars it conferred resistance and induced HR (Staskawicz *et al*., 1984). The protein has been widely used in studying other putative effectors. The AvrBs1 protein from *Xcv*, the causal agent of foliage and fruit spot disease of tomato and pepper plants, induces HR when introduced into pepper plants expressing the *Bs1* resistance gene (Minsavage *et al*., 1990). This is one of the reasons *avrBs1* has been widely applied in effector research.

Therefore, we tested if *X. ampelinus avrBs1* and *avrA* homologs elicit HR in the non-host, tobacco. Cloning the full length gene coding regions individually into the binary vector under the control of Cauliflower Mosaic Virus (CaMV) promoter endorsed constitutive expression of genes through *Agrobacterium*-mediated transient expression. The wound response facilitated by acetosyringone only improved the process. The results obtained showed that *X. ampelinus avrBs1* and *avrA* were functional as seen from their ability to elicit HR in non-host tobacco plants. These results suggested that *avrBs1* and *avrA* homologs could be applied in a number of studies into *X. ampelinus* effector research, including identification of putative effectors and investigation into the compatibility and incompatibility interactions of this organism and its plant host.

Finally this study provides the basis of the T3 effector search for *X. ampelinus*. Due to the relatedness of the *X. ampelinus avr* sequences to those of xanthomonads, and the fact that *Xanthomonas avrBs1* has been successfully used in a number T3 effector studies (Jiang *et al*., 2009; Xu *et al*., 2008), it was decided to construct an *X. ampelinus* T3 effector reporter vector based on the *avrBs1* gene.

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CHAPTER 3: Development of a Type III effector reporter plasmid system using the HR-inducing domain of AvrBs1

3.1 INTRODUCTION

Plants are constantly under attack by infecting pathogens, as a result plants have evolved a specialized complex surveillance system which includes mediation by plant resistance (*R*) genes. The system can recognize and actively respond to the presence of many different plant pathogens. Generally during infection, bacterial plant pathogens inject proteins directly into plant cells via a specialized Type III secretion system (T3SS) (Collmer *et al*., 2000; Casper-Lindley *et al*., 2002). Seemingly, pathogen secreted effector proteins alter the plant cell function to create a more favourable environment for pathogen growth and development (Kearney and Staskawicz, 1990; Swarup *et al*., 1991; Kim *et al*., 2008). *R* genes monitor plant cells for presence of pathogen-secreted effector proteins and upon detection, activate rapid and strong plant defense responses (Ellis and Dodds, 2003). These plant defense responses often result in the hypersensitive response (HR), a rapid localized programmed cell death that severely reduce the ability of a pathogen to grow and cause disease (Dangl and Jones, 2001; Bonas and Lahaye, 2002).

Historically, all pathogen effector proteins that were recognized by *R* genes were termed avirulence (*avr*) genes (White *et al*., 2000). This was due to a fact that the presence of the effector gene in a pathogen prevents it from successfully infecting a host plant with the corresponding *R* gene. However, plants generally carry a number of different *R* genes as much as pathogens harbour a diverse range of effector genes, but interaction between an effector and *R* gene is quite specific. If neither of the genes is present, then no *R* gene-mediated defense response occurs during infection and the disease progresses (Wichmann and Bergelson, 2004).

Suppression of the plant's immune system by pathogen effectors can give fundamental insights to innate immune system components, which can lead to innovative strategies for crop improvement by producing more resistant plant varieties (Alfano, 2009). However, the study of effector activity and its targets is also of great importance in understanding pathogen virulence factors. Availability of genome sequences of the major phytopathogenic bacteria has facilitated the identification of T3SS effector genes in a number of bacteria (Baltrus *et al*., 2011; Büttner *et al.,* 2003; Cunnac *et al*., 2004; Linderberg *et al*., 2006; Vinatzer and Greenberg, 2007). Candidate genes encoding effectors can be identified by bioinformatic analysis of genome sequence based on homologies to known T3SS effectors in other bacterial pathogens, the presence of sequence patterns associated with *hrp* (HR and pathogenicity)

promoters and T3SS-targeting domains, genes with similar regulation by *hrp* regulatory genes, gene products exhibiting distinctive motifs, or genes flanking the *hrp* gene cluster (Alfano and Collmer, 2004; Büttner *et al*., 2003; Cunnac *et al*., 2004; Lindeberg *et al*., 2006; Vinatzer and Greenberg, 2007).

The elucidation of T3SS-dependent and *R* gene-dependent HR in plants revealed the presence of a functional translocation signal in the N-terminal region of the candidate gene product (Greenberg and Vinatzer, 2003). The functional translocation signal of the effector proteins can also be indicative of the Type III secretion function (Xu *et al*., 2008; Jiang *et al*., 2009). Therefore, effector candidates can be functionally validated as Type III effectors by translational fusion of 5′ N-terminal coding regions of candidate effector genes with a reporter. Over the years, different reporter systems have been applied in effector research. Calmodulin-dependent adenylate cyclase (Cya) of *B. pertussis* cyclolysin and/ or effector regions of known effectors truncated at the N-terminal domain have been used as reporters (Casper-Lindley *et al*., 2002; Vinatzer *et al*., 2005; Xu *et al*., 2008). The HR-inducing effector regions of Avr proteins such as AvrRpt2 ($AvrRpt2_{81-255}$ and $AvrRpt2_{101-255}$) from *P. syringae*, AvrBs3 (AvrBs3Δ2) from *X. campestris* pv. *vesicatoria* and AvrBs1 ($AvrBs1_{59-445}$) also from *X. campestris* pv. *vesicatoria* have been employed as reporters (Vinatzer *et al*., 2005; Noël *et al*., 2003; Xu *et al*., 2008).

The objective of this study was to determine the shortest *X. ampelinus* AvrBs1 protein segment responsible for *in planta* recognition and HR elicitation in resistant pepper plants through *Agrobacterium*-mediated transient expression assays in order to construct a Type III effector (T3E) reporter vector for *X. ampelinus*. The HR-inducing segment was fused to the 5′ end of 3x FLAG epitope in the multiple cloning site (MCS) region of a broad-host range vector to create a reporter protein fusion. The functionality of the reporter vector with respect to secretion and translocation was then tested in an *X. ampelinus avrBs1* knockout mutant.

3.2 MATERIALS AND EXPERIMENTAL PROCEDURES

Chemicals used for this objective are listed in APPENDIX A. Preparation of stock buffers and growth medium is discussed in APPENDIX B.

3.2.1 Bacterial strains, plasmids and plant growth conditions

The bacterial strains used in this study were kept as glycerol stocks at -70 °C. They were continuously revived when needed and kept frozen. All *X. ampelinus* strains were grown on YPGA agar plates at 28 °C (Grall and Manceau, 2003) for five to ten days depending on the experiment to be pursued. *XaΔavrBs1* mutant was grown on YPGA plates supplemented with 10 µg/ml kanamycin and when transformed with the broad host vector and its derivatives, the medium was supplemented with 5 µg/ml gentamycin. *Agrobacterium* strains were grown on YEP agar plates or in broth at 28 °C for 48 hours. YEP medium was always supplemented with 50 µg/ml rifampicin, unless there was a plasmid involved then additional antibiotics were added depending on plasmid requirements. *E. coli* JM109 was grown on LB agar plates or broth at 37 °C for 16 hours. The medium was supplemented with appropriate antibiotics depending on the plasmid maintenance requirements. The characteristics of the bacterial cultures and plasmids used, and those generated in this study, are listed in Table 3.1.

Pepper plants, STAR 6657, were grown from seeds in the glasshouse under controlled environmental conditions. Seedlings were grown for up to five weeks before leaves were inoculated. The sweet pepper hybrid, STAR 6657, was used instead of *N. tabacum* because it carries the *Bs1*, *Bs2* and *Bs3* resistance genes, and *Bs1* recognizes *avrBs1*.

Table 3.1: Plant lines, strains and plasmids used in this study

Plant line, strain or plasmid	Relevant characteristics	Reference or source
Plant lines		
Sweet pepper *STAR 6657*	Hybrid sweet pepper with resistance to Bacterial Leaf Spot (Strain 1, 2, 3); recognizes *avrBs1*	Starke Ayres
Bacterial strains		
Xylophilus ampelinus VS20	Wild type *Xylophilus ampelinus*	ARC-PPRI[a] Culture collection
XaΔavrBs1	*X. ampelinus avrBs1* knockout mutant Kan^r	This study
Agrobacterium tumefaciens C58C1	Rif^r	Dirk Stephan. US[b] 2012.

Escherichia coli JM109	*endA1, recA1, gyrA96, thi, hsdR17 (rk–, mk+), relA1, supE44, Δ(lac-proAB), [F´ traD36, proAB, laqIqZΔM15].*	Lab strain
Plasmids		
pCB301	Mini Binary vector series, *RK2 oriV*, similar to *nptIII*, plasmid *RK2 TrfA* region, T-DNA right border, *MCS*, T-DNA left border; Kan^r	Dirk Stephan. US[b]
pCB301ΔBX	Binary vector pCB301 with *Bam*HI and *Xba*I restriction sites removed; Kan^r	This study
pCB3CamV2	pCB301ΔBX carrying a 35S CaMV promoter and terminator ; Kan^r	This study
pCB3-AvrBs1$_{Xa}$	pCB3CaMV carrying full length *avrBs1* gene; Kan^r	This study
pCB3-AvrBs1$_{22-413}$	pCB3CaMV2 carrying *avrBs1* gene N-terminal deletion from amino acid 1-21; Kan^r	This study
pCB3-AvrBs1$_{44-413}$	pCB3CaMV2 carrying *avrBs1* gene N-terminal deletion from amino acid 1-43; Kan^r	This study
pCB3-AvrBs1$_{57-413}$	pCB3CaMV2 carrying *avrBs1* gene N-terminal deletion from amino acid 1-56; Kan^r	This study
pCB3-AvrBs1$_{64-413}$	pCB3CaMV2 carrying *avrBs1* gene N-terminal deletion from amino acid 1-63; Kan^r	This study
pCB3-AvrBs1$_{100-413}$	pCB3CaMV2 carrying *avrBs1* gene N-terminal deletion from amino acid 1-99; Kan^r	This study
pCB3- AvrBs1$_{Xcc}$	pCB3CaMV2 carrying full length *Xcc. avrBs1* gene; Kan^r	This study
pBBR1-MCS5	Broad-host-range vector, *MCS*, *LacZ* alpha peptide, *RK2*, *IncP*, *IncQ*, *IncW*, *ColE1* and *P15a*-based replicons; Gm^r	Kovach *et al*., 1995
pBM5flg	pBBR1-MCS5 vector with 3x FLAG epitope; Gm^r	This study
pBM5flg-*avrBs1$_{Xa}$*	pBBR1-MCS5 vector with 3x FLAG epitope and *avrBs1* full length gene inserted at the 5′ end of 3x FLAG in *Bam*HI/*Xba*I cloning sites; Gm^r	This study
pBM5flg-AvrBs1$_{57-413}$	pBBR1-MCS5 vector with 3x FLAG epitope and AvrBs1 deletion mutant consisting of amino acids 57-413 inserted at the 5′ end of 3x FLAG in a *Bam*HI/*Xba*I cloning site; Gm^r	This study
pBM5flg_*avrBs1$_{Xcc}$*	pBBR1-MCS5 vector with 3x FLAG epitope and *Xanthomonas campestris* pv. *campestris avrBs1* inserted at the 5′ end of 3x FLAG in *Bam*HI/*Xba*I cloning sites; Gm^r	This study

[a]ARC PPRI: Agricultural Research Council, Plant Protection Research Institute

[b]US: University of Stellenbosch

3.2.2 Polymerase Chain Reaction (PCR) primers used in this study

All primers used in this study are listed in Table 3.2 below. Most of them were designed for this study using both CLC genomics workbench Version 6 (CLC Bio, Århus, Denmark) and NCBI Primer BLAST (Altschul *et al*., 1990). All primers were ordered from IDT and resuspended in sterile water to make 100 µM stock solutions. To prepare working solutions refer to Chapter 2 Section 2.2.2.

Table 3.2: Polymerase chain reaction primers

Primer name	Primer sequence (5′-3′; restriction sites underlined)	Description	Reference
S3	GGTGTTAGGCCGAGTAGTGAG	Forward primer for the detection of *X. ampelinus* ribosomal DNA	Botha *et al*., 2001
S4	GGTCTTTCACCTGACGCGTTA	Reverse primer for the detection of *X. ampelinus* ribosomal DNA; S3+4 product length = 277bp	Botha *et al*., 2001
YP273	ATGGATATAAACCATATCGAATTTGC	Forward primer specific for *X. ampelinus avrBs1* ORF	This study
YP270	TCATTTCTCGAATATGACTTCCTGT	Reverse primer specific for *X. ampelinus avrBs1* ORF; YP273+270 product length = 1239 bp	This study
YP276	ACGTTCTACGAGCTTTGCCA	Forward primer specific for binary vector pCB301 (and its derivatives) *nptIII* region	This study
YP277	AGATTGTCGTTTCCCGCCTT	Reverse primer specific for binary vector pCB301 *nptIII* region; YP276+277 product length = 705 bp	This study
YP320	TTACAATTTCCATTCGCCATTCAG	Forward primer specific for *Lac Z* operon in broad host range vector pBBR1-MCS5	This study
YP321	ATGACCATGATTACGCCAAGC	Reverse primer specific for *Lac Z* operon in broad host range vector pBBR1-MCS5; YP320+321 product length = 366 bp	This study
YP343	TCTGGATCCATGACACAGCAAAATCGGGAG	Forward primer to create *avrBs1* N-terminus deletion $avrBs1_{22\text{-}413}$ for cloning into pCB3CaMV2; Primer has added ATG-Methionine	This study

		start codon and a *Bam*HI restriction site; YP343+348 product length = 1175 bp	
YP344	TCAGGATCCATGAATACTGTCAGACCAATTGA	Forward primer to create *avrBs1*$_{44\text{-}413}$ N-terminus deletion for cloning into pCB3CaMV2; Primer has added ATG-Methionine start codon and a *Bam*HI restriction site; YP344+348 product length = 1109 bp	This study
YP345	ACTGGATCCATGAAGTCTTTGCAGACCTC	Forward primer to create *avrBs1*$_{57\text{-}413}$ N-terminus deletion for cloning into pCB3CaMV2; Primer has added ATG-Methionine start codon and a *Bam*HI restriction site; YP345+349 product length = 1070 bp	This study
YP346	GATGGATCCATGCTTCAGCGAATCCAAGA	Forward primer to create *avrBs1*$_{64\text{-}413}$ N-terminus deletion for cloning into pCB3CaMV2; Primer has added ATG-Methionine start codon and a *Bam*HI restriction site; YP346+348 product length = 1050 bp	This study
YP347	TATGGATCCATGTCCTTCGCCAAAGAAGTT	Forward primer to create *avrBs1*$_{100\text{-}413}$ N-terminus deletion for cloning into pCB3CaMV2; Primer has added ATG-Methionine start codon and a *Bam*HI restriction site; YP347+348 product length = 941 bp	This study
YP348	ACATCTAGATCATTTCTCGAATATGACTTCCTG	Reverse primer to create *avrBs1* N-terminus deletions for cloning into pCB3CaMV2; primer has added *Xba*I restriction site.	This study

YP339	TACAGGATCCAAGTCTTTGCAGACCTCGGCG	Forward primer to create *avrBs1*$_{57-413}$ N-terminus deletion for cloning into pBM5flg; YP339+342; primer has added *Bam*HI site; product length = 1064 bp	This study
YP342	TAGTCTAGATTTCTCGAATATGACTTCCTG	Reverse primer to create *avrBs1* N-terminus deletions for cloning into pBM5flg; primer has added *Xba*I restriction site.	This study
YP351	AGCCGTTCGGTCTTAGCGGGAG	Forward primer specific for SOEing sequence upstream of *avrBs1* for knocking out *avrBs1* CD	This study
YP410	ACAATTCATCGATGATGGTTGGATTTCGGGATTTCACTA	Reverse primer specific for SOEing sequence upstream of *avrBs1* for knocking out *avrBs1* CD, overlapping with promoter sequence ahead of kanamycin gene; YP351+410 product length = 1202 bp	This study
YP411	TAGTGAAATCCCGAAATCCAACCATCATCGATGAATTGT	Forward primer specific for SOEing sequence of the kanamycin gene including promoter sequence on Epicentre transposon, EzTn5, overlapping with *X. ampelinus* sequence.	This study
YP399	AATCTAATTGAGGAAAATCTTAGAAAAACTCATCGA	Reverse primer specific for SOEing kanamycin gene sequence overlapping with *Xa avrBs1* deletion on *X. ampelinus* sequence. YP411+399 product length = 926 bp	This study
YP355	GATGAGTTTTTCTAACATTTTCCTCAATTAGAT	Forward primer specific for SOEing sequence downstream of *avrBs1* ORF for knocking out *avrBs1* CD overlapping with kanamycin gene	This study

YP356	CTATCAATCAAGCATTATCCCT	Reverse primer specific for SOEing sequence downstream of *avrBs1* ORF for knockout of *avrBs1* CD; YP355+356 product length = 1206 bp	This study
YP404	TGGTCTAGAGATTACAAGGATCATGATGG CGATTACAAGGATCACGACATCGACTACA AGGATGACGATGACAAGTAAGAGCTCAC	Forward 3x FLAG epitope oligo with partial *Sac*I and *Xba*I sites	This study
YP405	GTGAGCTCTTACTTGTCATCGTCATCCTTGT AGTCGATGTCGTGATCCTTGTAATCGCCAT CATGATCCTTGTAATCTCTAGACCA	Reverse complimentary 3x FLAG epitope oligo with partial *Sac*I and *Xba*I sites	This study
YP408	ATCAGGATCCATGGATATAAACCATATCG AATTTGC	Forward primer with *Bam*HI site for amplification of *X. ampelinus* full length *avrBs1* CD	This study
YP409	TAGTCTAGATCATTTCTCGAATATGACTTC CTGT	Reverse primer with *Xba*I site for amplification of *X. ampelinus* full length *avrBs1* for cloning into pCB3CaMV2. YP408+409 product length = 1239 bp	This study
YP342	TAGTCTAGATTTCTCGAATATGACTTCCTG	Reverse primer with *Xba*I site for amplification of *X. ampelinus* full length *avrBs1* without a stop codon for cloning at 5′ end of 3x FLAG epitope in pBM5flg. YP408+342 product length = 1236 bp	This study
YP416	TACAGGATCCATGACGGACTTGTGCTCG	Forward primer specific for full length *Xcc avrBs1*. Primer modified from *avrBs1*-642U (Wichmann and Bergelson, 2004). *Sac*I site replaced with a *Bam*HI site.	This study

YP417	ACATCTAGAGTGGCGGATACTTCTTCTCT	Reverse primer specific for full length *Xcc avrBs1* for cloning into pCB3CaMV2 product size 1335 bp with *Xba*I site added	This study
YP501	ACATCTAGACGCTTCTCCTGCATTTGT	Reverse primer specific for *Xcc avrBs1* without stop codon for cloning at the 5′ end of 3x FLAG in pBM5flg. Primer modified from *avrBs1*-2069L (Wichmann and Bergelson, 2004). *Sph*I site replaced with *Xba*I site; product length = 1332 bp	This study

3.2.3 Sequence analysis

X. ampelinus sequence analysis and construction of plasmid and restriction enzyme maps were done using CLC Genomics Workbench version 6.0 and nucleotide sequence comparisons were done using BLAST (Altschul, 1990). ORF nucleotide sequences were translated to protein sequences in reading frame one and sequence comparisons were done using the BLASTp function (Altschul, 1997) on the NCBI website (http://www.ncbi.nlm.nih.gov).

3.2.4 Identification of *X. ampelinus* AvrBs1 HR-inducing domain

3.2.4.1 Construction of binary vector: restriction digestion and cloning

Binary vector pCB3CaMV2 was created by cloning the Cauliflower Mosaic Virus (CaMV) 35S promoter and terminator into the *Hind*III/ *Eco*RI sites of pCB301 after the deletion of *Bam*HI and *Xba*I sites from this vector. The *Bam*HI and *Xba*I sites needed to be removed from pCB301, since the aim was to clone the *avrBs1* ORF and its deletion mutants between the *Bam*HI and *Xba*I sites situated between the CaMV 35S promoter and terminator. Restriction digestion, gel purification and removal of the 3′- and 5′ overhangs created by sticky-end restriction was done as described in Chapter 2. Briefly, once the *Bam*HI site had been removed and the plasmid recircularized, the new plasmid named pCB3ΔB was digested with *Xba*I, the 3′- and 5′ overhangs removed with SI nuclease and recircularized to produce the plasmid, pCB3ΔBX, which was now void of both *Bam*HI and *Xba*I sites.

Vector pCB3ΔBX was digested with FastDigest *Eco*RI and *Hind*III restriction enzymes, electrophoresed and gel purified as described previously. This 3.5 kb linearized vector DNA was ligated with the one kilobase pair *Eco*RI and *Hind*III DNA fragment from vector p442 carrying the CaMV 35S promoter and terminator to create the vector, pCB3CaMV2 as previously described in Section 2.2.7. After PCR-verification with primer pair YP276 and YP277, the vector was named pCB3CaMV2 and stored at -20°C.

3.2.4.2 Amplification and cloning of X. ampelinus avrBs1 N-terminal deletion mutants

Genomic DNA of wild type *X. ampelinus* was used as template for PCR with primers YP343, YP344, YP345, YP346, YP347 and reverse primer YP348 to amplify five *avrBs1* N-terminal deletion mutants $AvrBs1_{22\text{-}413}$, $AvrBs1_{44\text{-}413}$, $AvrBs1_{57\text{-}413}$, $AvrBs1_{64\text{-}413}$ and $AvrBs1_{100\text{-}413}$, respectively. All forward primers had added ATG (Methionine) start codon to mark the

beginning site of protein synthesis. The proofreading polymerase, Phusion High fidelity DNA polymerase (Thermo Scientific, Inqaba Biotech, South Africa) was used for amplification according to manufacturer's instructions. Reactions typically contained 20 ng of a template DNA, 200 µM of dNTPs, 200 nM of each primer and one unit of Phusion polymerase.

Table 3.3 below shows all PCR conditions for each primer pair. Annealing temperatures were dependent on each primer set melting temperatures. Extension times were also dependent on the length of each product. Initial denaturation was 98 °C for 30 seconds for all primer pairs and 30 cycles for denaturation, primer annealing and extension steps for each primer pair. The final extension remained the same for all reactions at 72 °C for five minutes and a 4 °C storage step was included for all reactions.

Table 3.3: PCR cycling conditions for all primer sets

Component	**YP343+ YP348**		**YP344+ YP 348**		**YP345+ YP348**		**YP346+ YP348**		**YP347+ YP348**	
	Temp	Time	Temp	Time	Temp	Time	Temp	Time	Temp	Time
Denaturation	98 °C	15 sec	98 °C	15 sec	98 °C	15 sec	98 °C	15 sec	98 °C	15 sec
Primer annealing	59°C	15 sec	57°C	15 sec	58°C	15 sec	58°C	15 sec	58°C	15 sec
Extension	72°C	30 sec	72°C	30 sec	72°C	30 sec	72°C	25 sec	72°C	20 sec

Along with five *avrBs1* N-terminal deletions, the full length *avrBs1* genes were amplified and used as positive controls. *X. ampelinus* genomic DNA was used as template for PCR with primers YP408 and YP409 to amplify *Xa avrBs1* ORF. Furthermore, *X. campestris* pv. *campestris* genomic DNA was used as template for PCR with primers YP416 and YP417 to amplify *Xcc avrBs1*. The PCR programme for amplification of *Xa avrBs1* was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 57 °C for 15 seconds, 72 °C for 45 seconds) and a final extension at 72 °C for five minutes. The PCR programme for amplification of *Xcc avrBs1* was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 60 °C for 15 seconds, 72 °C for one minute) and a final extension at 72 °C for five minutes.

PCR products were resolved in ethidium-containing 0.8% agarose gels in Tris/Borate/EDTA (TBE) buffer electrophoresed at 100 volts for one hour. The DNA bands were visualized under UV light and photographed using the Ingenius Bio-Imaging system (Syngene, Vacutec, South

Africa). The PCR products were excised from the gel using a sterile blade, and the DNA was purified using the QiaexII gel extraction kit.

The PCR products were individually digested with *Bam*HI and *Xba*I, and individually ligated between the *Bam*HI and *Xba*I sites of binary vector, pCB3CaMV2. The newly created plasmid constructs were used to transform *E. coli* JM109 competent cells as described before (Chapter 2 Section 2.2.6.1). The plasmids were extracted from cells, verified with FastDigest *Eco*RV and used to transform *A. tumefaciens* strain C5C81.

3.2.4.3 Transformation of A. tumefaciens electro-competent cells

The pCB3CaMV2-N-terminal deletion construct series (pCB3- AvrBs1$_{22-413}$, -AvrBs1$_{44-413}$, -AvrBs1$_{57-413}$, -AvrBs1$_{64-413}$ and -AvrBs1$_{100-413}$) along with full length *X. ampelinus avrBs1* and *Xcc avrBs1* as well as pCB3CaMV2 empty vector construct were used to transform *Agrobacterium* strain C58C1 (Refer to Chapter 2 Section 2.2.9). Transformed cells were cultured on YEP-agar plates with 50 µg/ml each of kanamycin and rifampicin. Cultured plates were incubated at 28 °C for 48 hours. CFUs were verified by colony PCR using primers specific for the *avrBs1* deletion mutants, *Xa avrBs1* and *Xcc avrBs1* homologs respectively.

3.2.4.4 Agrobacterium-mediated transient expression

A. tumefaciens strain C58C1 carrying binary vector constructs (pCB3-AvrBs1$_{Xa}$ and pCB3-AvrBs1$_{Xcc}$ positive controls; pCB3- AvrBs1$_{22-413}$, -AvrBs1$_{44-413}$, -AvrBs1$_{57-413}$, -AvrBs1$_{64-413}$ and -AvrBs1$_{100-413}$ N-terminus vector deletion series; and pCB3CaMV2 empty vector negative control) and untransformed *A. tumefaciens* C58C1 were grown in five millilitres of YEP broth containing 50 µg/mg each of rifampicin and kanamycin and 50 µg/ml of rifampicin only for the untransformed C58C1, at 28 °C for 48 hours. The transient expression experiment was carried out as described in Chapter 2 Section 2.2.10. Bacterial suspensions were inoculated into six to eight weeks old pepper plant leaves using a needleless syringe. After infiltration, plants were kept at room temperature in a 16 hour light and eight hour dark cycle. Leaves were photographed after a two to three days post-inoculation.

3.2.5 Preparation and transformation of *X. ampelinus* electro-competent cells

X. ampelinus from glycerol stock was revived by culturing on YPGA agar plates (7 g/L Yeasst extract; 7g/L Bacto-peptone; 7 g/L Glucose; 15 g/L Bacto-agar). Plates were incubated at 28 °C for six to eight days. After sixth or eighth day, the cells were transferred onto as many new YPGA plates as possible for full growth and incubated for five days at 28 °C. On day five, the cells were scraped off the plates and aseptically resuspended in sterile distilled water. The cells were diluted to an OD600nm of approximately one. The cells were harvested by centrifugation at 5000xg for 10 minutes at 4 °C. Pellets were washed twice with cold sterile distilled water (1/10th of initial volume) and centrifugation at 5000xg for 10 minutes. The cells were then washed five to six times with ice cold sterile 15% (v/v) glycerol. Electro-competent cells were finally resuspended in 1/100th of the initial volume of 15% (v/v) glycerol and dispensed into sterile microcentrifuge tubes. Electro-competent cells were used directly for electroporation or stored at -70 ˚C for future use.

For electroporation, competent *X. ampelinus*, plasmid constructs and frozen SOC medium were thawed on ice. Two to five microlitres of plasmid construct was added to 50 µL electro-competent cells, mixed by flicking the tube, and kept on ice for 10 minutes. The cell-plasmid DNA mixture was transferred into cold 0.2 cm electroporation cuvettes and pulsed once with 1.8 kV, at 25 µF capacitance and 200 Ω resistance. One millilitre of cold SOC medium was added quickly and the cell suspension incubated at 28 °C for four hours shaking at 140 rpm. The cells were plated on YPGA supplemented with the appropriate antibiotics and incubated at 28 °C for seven to ten days. CFUs were verified by colony PCR using specific primers.

3.2.6 Generation of *X. ampelinus avrBs1*-knockout mutant

3.2.6.1 "Splicing by Overlap Extension" PCR (SOEing PCR)

Genomic DNA of wild type *X. ampelinus* was used as template for PCR with primers YP351 and YP410, and YP355 and YP356 to amplify 1.2 kb DNA fragments each at the upstream and downstream regions of the *avrBs1* CD. The amplified fragments were designated *avrBs1*_AB and *avrBs1*_CD. A kanamycin resistance cassette was amplified with primers YP411 and YP399 from a plasmid carrying the Epicentre EzTn5<Kan2> transposon cassette. The proofreading polymerase, Phusion High fidelity DNA polymerase (Thermo Scientific, Inqaba Biotech, South Africa) was used for amplification according to manufacturer's instructions. Reactions typically contained 20 ng of a template DNA, 200 µM of dNTPs, 200 nM of each primer and one unit of Phusion polymerase.

The PCR programme for amplification of *avrBs1*_AB was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 60 °C for 15 seconds, 72 °C for 45 seconds) and a final extension at 72 °C for five minutes. The PCR programme for amplification of *avrBs1*_CD 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 53 °C for 15 seconds, 72 °C for 45 seconds) and a final extension at 72 °C for five minutes. The PCR programme for amplification of the kanamycin cassette was as follows: 98 °C for 30 seconds, 30 cycles of (98 °C for 15 seconds, 60 °C for 15 seconds, 72 °C for 30 seconds) and a final extension at 72 °C for five minutes. PCR products were gel purified as mentioned previously. Approximately 20 ng of each purified PCR product was used in SOEing overlap extension PCR. A pictorial overview of the process followed to generate the construct to be used to delete the *avrBs1* CD from the *X. ampelinus* chromosome is shown in Figure 3.1.

The actual method for synthesis by overlap extension (SOE) PCR was as follows: two separate master mixes A and B were prepared on ice (Refer to Table 3.4 below for volumes and concentrations).

Table 3.4: Generic SOEing PCR

Component	**Master Mix A**	**Master Mix B**
5X Phusion buffer	5 µl	5 µl
10 MM dNTPs	0.5 µl	0.5 µl
Phusion polymerase	0.25 µl	0.25 µl
10 µM external forward primer	0 µl	1 µl
10 µM external reverse primer	0 µl	1 µl
AB_Δ*avrBs1*	20 ng/µl	0 µl
Kanamycin cassette	20 ng/µl	0 µl
CD_Δ*avrBs1*	20 ng/µl	0 µl
Nuclease free water	up to 25 µl	up to 25 µl
TOTAL	**25 µl**	**25 µl**

Master mix A containing the three DNA templates were set up to run for 15 cycles to create a single template from three sequences in the absence of primers (Refer to figure 3.1 below). The overlapping sequences were able to hybridize with their complementary sequences at the terminals of each fragment. The primary PCR reaction (A) was set as follows: 98 °C for 30 seconds, 15 cycles of (98 °C for 15 seconds, 57 °C for 30 seconds and 72 °C for 30 seconds) and a final extension at 72 ° for two minutes. The samples were put on hold at ten degrees

Celsius to add master mix B. After adding master mix B, a new program (PCR reaction B) was loaded as follows: 98 ℃ for 30 seconds, 30 cycles of (98 ℃ for 15 seconds, 55 ℃ for 15 seconds, 72 ℃ for one minute) and a final extension at 72 ℃ for ten minutes and stored at 10 ℃.

The PCR product was resolved in ethidium-containing 0.8% agarose gels in Tris/Borate/EDTA (TBE) buffer electrophoresed at 100 volts for one hour. The DNA band was visualized under UV light and photographed using the Ingenius Bio-Imaging. The PCR product was excised from the gel using a sterile blade, and the DNA was purified using the QiaexII gel extraction kit. The purified SOEing PCR product was ligated into a suicide vector, pJET1.2/Blunt (Figure 3.2) using the CloneJET™ PCR Cloning Kit (Thermo-Scientific, Inqaba, South Africa) according to the manufacturer's protocol.

Five microliters of SOEing ligation mixture was used to transform *E. coli* JM109 chemical competent cells using a heat-shock method (Section 2.2.6.1) and grown on LB-agar plates supplemented with 100 µg/ml ampicillin and 40 µg/ml kanamycin. Resulting transformants were PCR verified using primer pair YP411 and YP399. A positive CFU was selected, grown in LB broth supplemented with 40 µg/ml kanamycin, and the SOEing plasmid construct, extracted after overnight incubation at 37 °C. This plasmid construct was named pJET1.2/SOEing. Two to three microliters of pJET1.2/SOEing was used to transform electro-competent wild type *X. ampelinus* as described in Section 3.2.5.

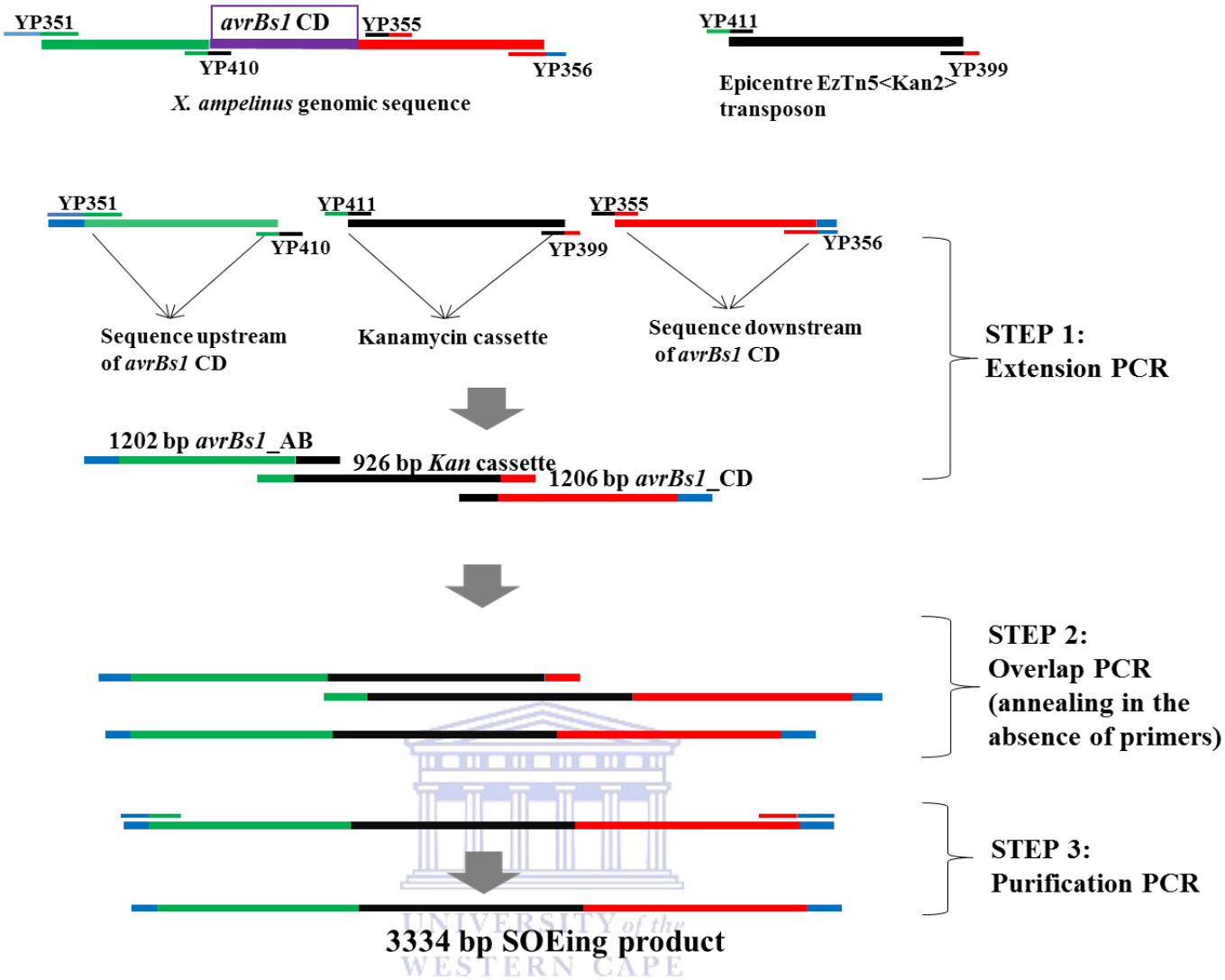

Figure 3.1. **SOEing PCR Overlap extension.** The first step shows the DNA fragments for the SOEing construct with their specific primer pairs. Individual PCR products were purified and used in overlap PCR. The purification PCR step involved the use of primer pair YP351 and YP356 to amplify a 3.33 kb SOEing product.

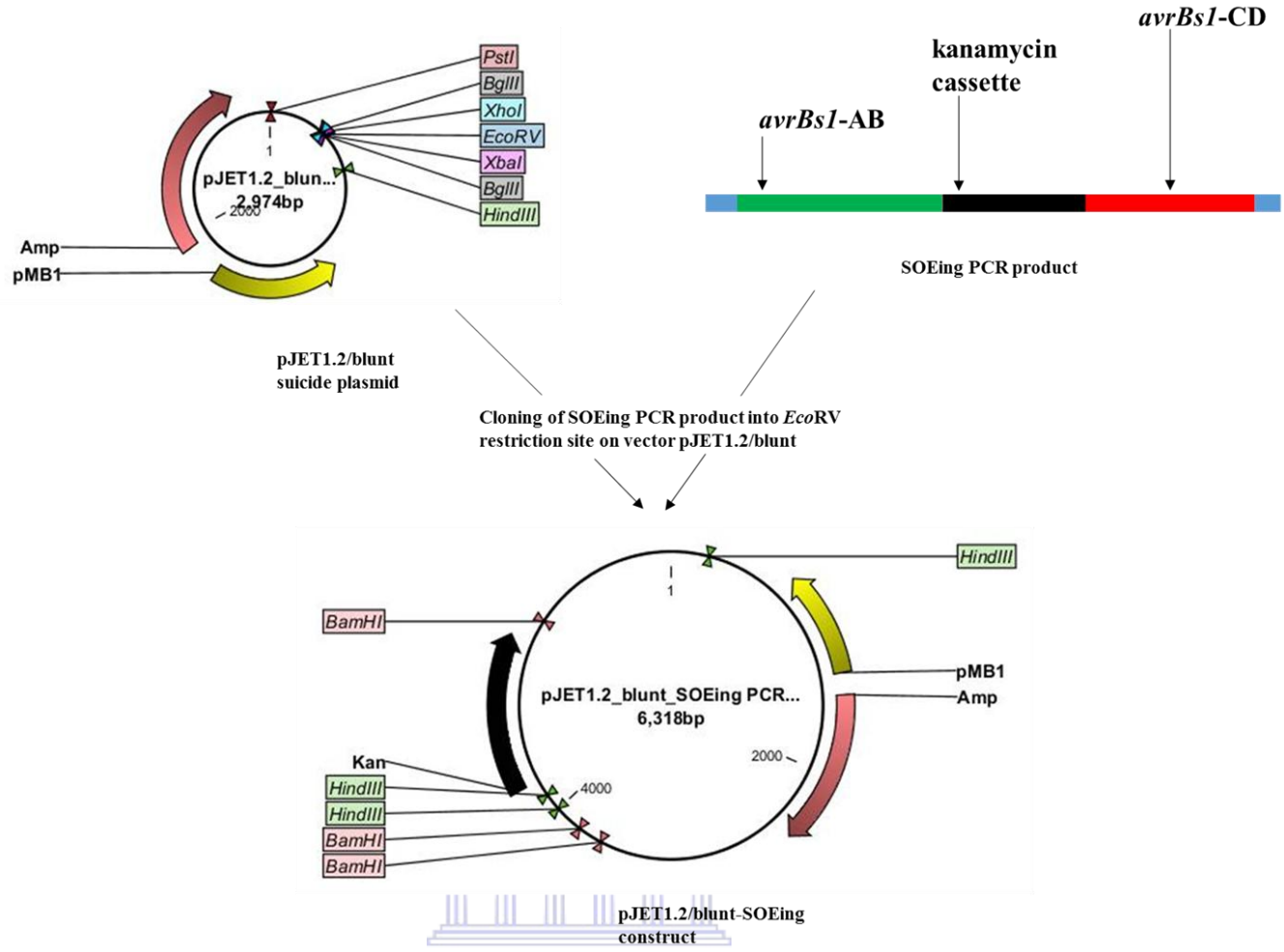

Figure 3.2: pJET1.2/SOEing vector construction. The 3.3 kb SOEing PCR product containing the regions upstream and downstream of *X. ampelinus avrBs1* as well as the kanamycin resistance cassette was ligated into the suicide vector, pJET1.2/Blunt.

3.2.6.2 Confirmation of allelic exchange

To confirm that the *avrBs1* gene on the chromosome had been replaced by the kanamycin cassette, two separate colony PCR reactions were carried out using primers specific for either the kanamycin cassette (YP399+411) or *avrBs1* (YP270+273). To confirm that the pJET1.2/SOEing construct was not present in the cells, CFUs which were PCR positive for kanamycin and negative for *avrBs1*, were sub-cultured onto YPGA plates supplemented with 30, 40 or 50 µg/ml ampicillin. The plates were incubated at 28 °C for eight to ten days, checking the growth of cells every day after the fifth day. The isolate that didn't grow on ampicillin-agar plates was named *XaΔavrBs1*, and used to make a glycerol stock with nutrient broth in 10% (v/v) glycerol and stored at -70 °C.

3.2.6.3 Testing XaΔavrBs1 mutant for HR induction on pepper

XaΔavrBs1 mutant strain was transferred onto YPGA plates with kanamycin 10 µg/ml and grown for four days along with the wild type *X. ampelinus* as a positive control. After a four-day incubation period, the cells were scraped from plates and resuspended in sterile 1x PBS (phosphate buffered saline). Cell suspensions with OD_{600nm} readings of approximately 0.3 to 0.35 were immediately used for inoculation. Approximately 20 µL of cell suspension was infiltrated into six to eight weeks old pepper, STAR 6657. After inoculation, plants were kept at room temperature in an eight hour dark and 16 hour light cycle. Leaves were photographed after 24 to 48 hours post-inoculation.

3.2.7 Development of the T3E reporter plasmid system

3.2.7.1 Linker ligation and cloning of 3x FLAG into a broad-host range vector

The 3x FLAG peptide is a synthetic peptide consisting of 22 amino acid residues with sequence DYKDHDGDYKDHDIDYKDDDDK. Two complementary oligonucleotides (YP404 and YP405) were designed based on this peptide sequence and a stop codon was included at the 3′ end. The oligos were designed to have partial *XbaI* and *SacI* restriction sites at the 5′ and 3′ ends, respectively to facilitate cloning into the broad host range vector, pBBR1-MCS5, as well as fusion to the N-terminal sequences of candidate T3Es. The two oligos were annealed using a modified linker ligation protocol (http://structure.biochem.queensu.ca/protocols/linkerligation). Briefly, oligonucleotide stock solutions of 100 µM concentration was prepared in a 1x linker buffer (refer to APPENDIX B for buffer composition). For a single linker ligation reaction, 10 µL of each oligonucleotide was mixed together to give a final concentration of 50 µM of each oligo, and annealed as follows: 95 °C for two minutes; 60 °C for 10 minutes; and storage at 4 °C.

The pBBR1-MCS5 vector was prepared by inoculating five millilitres of LB broth supplemented with 10 µg/ml gentamicin with a single colony grown on antibiotic-supplemented LB-agar plates and incubating the cultures overnight at 37 °C on a shaking platform. The plasmid was isolated using the Qiagen miniprep kit (Qiagen GmbH, Germany) according to manufacturer's instructions and stored at -20 °C. The plasmid, pBBR1-MCS5 was digested with FastDigest restriction enzymes *Xba*I and *Sac*I (Thermo Scientific, Inqaba Biotec, South Africa) according to the manufacturer's instructions in order to create compatible sites for insertion (linker ligation) of the double-stranded 3x FLAG sequence. The linearized vector was gel purified as explained in previous sections and stored at -20 °C.

The linker ligation reaction was set up as follows: 0.5 µL of the 50 µM the annealed 3x FLAG oligonucleotide reaction was added to five microliters of pBBR1-MCS5 *Sac*I/ *Xba*I linear fragment, two microliters of 10x ligation buffer and five units of T4 DNA ligase (Roche, SA) in a 20 µL total reaction volume. The ligation reaction was carried out overnight at 16 °C in a PCR machine. Five microliters of the overnight ligation mixture was used to transform 100 µL of chemical competent *E. coli* using the heat shock method and cells were plated onto LB agar containing 10 µg/ml gentamicin for incubation at 37 °C for overnight. Resulting transformants were PCR-verified using primer pair YP320 and YP321. A PCR-positive colony was selected, grown in liquid LB broth supplemented with 10 µg/ml gentamicin and the plasmid DNA extracted after overnight incubation at 37 °C. This plasmid construct was named pBM5flg and stored at -20 °C.

3.2.7.2 Construction of the T3E reporter plasmid using the HR-inducing domain of avrBs1

Genomic DNA of wild type *X. ampelinus* was used as template for PCR with primers YP339 and YP342 to amplify the AvrBs1 HR-inducing domain (amino acids 57-413). At the same time, two control constructs were also created by amplifying, full length *avrBs1*$_{Xa}$ and *avrBs1*$_{Xcc}$ CDs from wild type *X. ampelinus* and *X. campestris* pv. *campestris*, genomes, with primers YP408 and YP342, and YP416 and YP501, respectively . The proofreading polymerase, Phusion High fidelity DNA polymerase was used for amplification according to manufacturer's instructions. Reactions typically contained 20 ng of a template DNA, 200 µM of dNTPs, 200 nM of each primer and one unit of Phusion polymerase. The PCR cycling conditions for each primer pair are shown in Table 3.5. Initial denaturation was 98 °C for 30 seconds for all primer pairs and 30 cycles for denaturation, primer annealing and extension steps for each primer pair. The final extension remained the same for all reactions at 72 °C for five minutes and a 4 °C storage step was included for all reactions.

Table 3.5: PCR cycling conditions for avr amplification

Component	YP339+ YP342		YP408+ YP342		YP416+ YP501	
	Temp	Time	Temp	Time	Temp	Time
Denaturation	98 °C	15 sec	98 °C	15 sec	98 °C	15 sec
Primer annealing	60 °C	15 sec	57 °C	15 sec	60 °C	15 sec
Extension	72 °C	30 sec	72 °C	45 sec	72 °C	1min

All PCR products were gel purified as previously described, digested individually with FastDigest *Bam*HI and *Xba*I enzymes, before being gel purified again. These purified products, which all lacked a stop codon, were individually ligated between the *Bam*HI and *Xba*I sites located at the 5′ end of the 3x FLAG tag in the broad host range vector, pBM5flg, in order to give rise to a fusion protein that would be detectable via western blotting. Each of the three ligation reactions were used to transform *E. coli* JM109 competent cells as described before (Chapter 2 Section 2.2.6.1).

Resulting transformants were PCR-verified using primer pair YP320 and YP321 as well as primer pairs specific for each insert. The plasmid constructs were extracted from cells, verified with FastDigest *Pvu*II and used to transform electrocompetent mutant, *XaΔavrBs1*. The constructs were named pBM5flg_AvrBs1$_{57\text{-}413}$, pBM5flg-*avrBs1*$_{Xa}$ and pBM5flg-*avrBs1*$_{Xcc}$, respectively and stored at -20 °C. As an additional control, the empty vector, pBM5flg, was also electroporated into the mutant, *XaΔavrBs1*. PCR-verified CFUs were stored at -70 °C in 10% (v/v/) nutrient broth glycerol (NBG). These isolates were sub-cultured onto fresh YPGA agar plates with appropriate antibiotics for HR, protein secretion and translocation assays described in the following sections.

3.2.8 Testing of the T3E reporter plasmid system I: Protein Secretion assay

3.2.8.1 Protein extraction from bacterial cell cultures

The *Xa*Δ*avrBs1* mutant carrying pBM5flg, pBM5flg-*avrBs1*$_{Xa}$, -*avrBs1*$_{Xcc}$ and -AvrBs1$_{57\text{-}413}$ constructs and untransformed strain were grown separately on YPGA plates with kanamycin 10 µg/ml and gentamicin 5 µg/ml for five days at 28 °C. On day five, the cells were resuspended in 50 ml YPG broth and the absorbance (OD_{600} value) was adjusted to ~0.5. The cells were harvested by centrifuging at 5000 xg for 10 minutes. Cell pellets were washed once with 20 ml of YPG and/ or minimal medium (XAM3) per sample. The cells were pelleted at 5000 xg for 10 minutes and finally resuspended in 50 ml of YPG / or XAM3 medium (per each sample) and grown at 28 °C for 24 hours shaking at 200 rpm. Cultures were separated into cellular and supernatant fractions by 10 minutes of centrifugation at 10000 xg. The pellets were washed once with 25 ml 1xPBS buffer and finally resuspended in 25 ml 1xPBS buffer. The supernatant was filtered through a 0.22 µM-pore membrane filter to eliminate residual cells. Success of this step was confirmed by culturing 200 µL onto YPGA plates. The proteins in both the cellular and supernatant fractions were precipitated with one volume of 25% trichloroacetic acid followed by overnight incubation at 4 °C. After overnight incubation the protein precipitates were collected by centrifugation at 6000 xg for 30 minutes at 4 °C. The protein pellets were washed three times with two milliliters ice cold 90% acetone. Precipitated proteins were recovered by centrifugation at 15000 rpm for five minutes and the pellets were dried and resuspended in 100 µL PBS buffer.

3.2.8.2 Protein quantification: Bradford assay

The concentrations of all protein extracts were determined using a modified Bradford assay (Bradford, 1976). After resuspension of proteins in 1xPBS, the samples were prepared for quantification. Bovine serum albumin (BSA) standards were prepared in duplicate from 20 mg/ml BSA stock solution in 1000 µL plastic cuvettes as indicated in Table 3.6 below. Protein extracts were prepared in plastic cuvettes by mixing 2.5 µL of protein sample with 17.5 µL of 1xPBS. The Bradford reagent (Bio-RAD; Hercules, CA, USA) was diluted five times with distilled water prior to use. A volume of 980 µL of diluted Bradford reagent was added to all standards and protein extracts, mixed and incubated for five minutes at room temperature. Absorbance was measured at 595 nm on a BioDrop TOUCH UV/Visible Spectrophotometer (Integrated Scientific Solutions) using 1xPBS as a blank solution. The standards were used to

plot a standard curve from which concentrations of all protein extract samples were extrapolated. All samples were stored at -20 °C until needed.

Table 3.6: Preparation of BSA protein standards for protein quantification

BSA final concentration (µg/ml)	BSA 20 mg/ml stock solution (µL)	1xPBS Buffer (µL)
0*	0	20
10	0.5	19.5
20	1	19
30	1.5	18.5
40	2	18
50	2.5	17.5
75	3.75	16.25
100	5	15

*blank solution

3.2.8.3 SDS-PAGE and Coomassie Brilliant blue staining of gels

The protein extracts were separated on 1D SDS-PAGE, a system composed of a stacking and resolving gel. The resolving and stacking gels were prepared as described in Table 3.7. The resolving gel solution was allowed to polymerize for 15 minutes at room temperature after the addition of 1 ml isopropanol to prevent air bubbles from forming. After 15 minutes the isopropanol was removed, and the gel was washed twice with 1 ml distilled water and the stacking gel solution was then added on top of the resolving gel. The comb was carefully inserted and the gel was allowed to polymerize for 30 to 40 minutes. Each 20 µl protein sample contained 5 µl Laemmli sample buffer (4:1 Laemmli buffer to mercapto-ethanol) and 15 µl protein extract. The samples were denatured at 90 °C for five minutes. After the samples cooled down at room temperature, 10 µl of Precision Plus Protein™ Dual Color Standards and total protein samples were loaded into the wells and electrophoresed at 100 V for three to four hours or until the sample dye ran out into the buffer. The electrophoresis was carried out at low temperatures with constant cooling with ice.

Table 3.7: Preparation of resolving and stacking gel solutions for two 10x10 cm gels using 1 mm spacers

Component	12% Resolving gel	Stacking gel
Distilled water	5.25 ml	4.2 ml
30% stock acrylamide soulution	6 ml	0.65 ml
4x Tris SDS resolving solution gel pH 8.8	3.75 ml	0 ml
4x Tris SDS stacking gel solution pH 6.8	0 ml	1.6 ml
10% Ammonium persulphate	150 µl	67 µl
TEMED	15 µl	6.7 µl

Protein separated by ID SDS-PAGE were routinely detected using a modified CBB R-250 staining protocol using one way step. Because of less complexity with bacterial proteins there was no need for sequential staining. After electrophoresis, the gels were dismounted from the gel plate assembly and immersed in CBB staining solution. The gels were gently rocked on a shaking platform for overnight at 30 rpm. After staining, the gels were immersed in distaining solution (5% methanol, 10% acetic acid in water) with constant shaking at room temperature until the bands were visibly distinct against a clear background. The gels were photographed using a general white light camera.

3.2.8.4 Protein secretion analysis: Western blotting

a) Transfer of protein from 1D SDS-PAGE gels onto Nitrocellulose membrane

Proteins samples separated by 1D SDS-PAGE were transferred onto 0.45 µm pore Nitrocellulose membrane (Bio-RAD, South Africa). Prior to protein transfer, electrophoresed gels were pre-equilibrated in cold transfer buffer [25 mM Tris, 192 mM glycine and 20% (v/v) methanol] for 20 minutes along with filter pads with shaking at room temperature. The gel-membrane sandwich was prepared on the gel holder cassette according to the Enduro electrophoresis system manual (Labnet International Inc). Electrophoretic transfer of proteins was performed at a constant current of 400 mA for three and half hours with constant cooling effect.

b) Immunoprobing of Nitrocellulose membrane with antibodies

After protein transfer, the membrane was incubated in the blocking solution (1% (w/v) Elite fat free instant milk in TBS buffer) at 4 °C overnight. After overnight incubation the membrane was agitated at room temperature for 1 hour before processing. The membrane was incubated with the primary antibody, Anti-FLAG M2 monoclonal antibody (Agilent Technologies Incorporation, USA) diluted 1:500 in 1% (w/v) blocking solution for the minimum of one hour. The membrane was washed three times with TTBS buffer with a minimum of three minutes per wash. The membrane was incubated with the secondary antibody, Goat anti-mouse IgG alkaline phosphate conjugate (BIO-RAD), diluted 1:3000 in 1% (w/v) blocking solution for one hour.

c) Immunodetection of proteins using Alkaline Phosphatase Conjugate Substrate kit

After secondary antibody incubation, the membrane was washed three times in TTBS buffer for five minutes per wash. FLAG-fusion proteins were detected using the Alkaline phosphatase conjugate substrate kit (BIO-RAD) according to manufacturer's instructions. The membrane was left for 30 minutes in the dark to allow colour development.

3.2.9 Testing of a T3E reporter plasmid system II: Protein Translocation Assay

*Xa*Δ*avrBs1* mutant strain carrying pBM5flg, pBM5flg-*avrBs1*$_{Xa}$, -*avrBs1*$_{Xcc}$ and -AvrBs1$_{57\text{-}413}$ constructs and untransformed strain were grown separately on YPGA plates supplemented with appropriate antibiotics for six to eight days at 28 °C. After the eighth day, bacterial cells were transferred onto new YPGA plates supplemented with appropriate antibiotics and grown at 28 °C for five days. On day five, the cells were resuspended in sterile 1xPBS buffer and the absorbance (OD_{600}) of all samples was adjusted to 0.3- 0.35. Bacterial suspensions were inoculated into six to eight weeks old Sweet pepper STAR 6657 plant leaves using a needleless syringe. After infiltration, plants were kept at room temperature in a 16 hour light and eight hour dark cycle. Leaves were photographed after two to three days post-inoculation.

3.3 RESULTS

3.3.1 Identification of *X. ampelinus* AvrBs1 HR-inducing domain

Given that *Xanthomonas* AvrBs1 protein HR-inducing domain is within its C-terminus, a similar study was used to determine *X. ampelinus* AvrBs1 HR-inducing domain through N-terminal deletion and transient expression of deletion mutants in sweet pepper. To determine AvrBs1 HR-inducing domain amino acids at the N-terminus were deleted, and these deletion mutants were cloned into a binary vector pCB3CaMV2 under the control of 35S promoter. *X. ampelinus* and *X. campestris* pv. *campestris* full length AvrBs1 proteins induced strong HR. *Agrobacterium*-mediated transient expression of AvrBs1 N-terminal deletion constructs on pepper plants revealed that $AvrBs1_{57\text{-}413}$ is the shortest protein segment that elicited HR (Figure 3.3, Table 3.7). The N-terminal deletion constructs, $AvrBs1_{64\text{-}413}$ and $AvrBs1_{100\text{-}413}$ did not induce HR (Figure 3.3, Table 3.7). Therefore *X. ampelinus* AvrBs1 amino acids 57-413, harbouring the HR-inducing domain was used in the development of a reporter plasmid system.

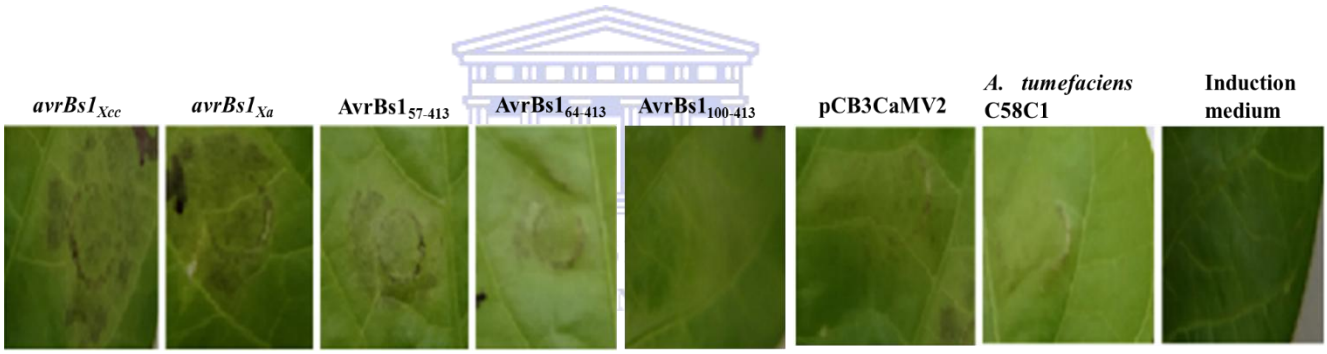

Figure 3.3: HR analysis of AvrBs1 protein N-terminus deletion mutants. HR inducing ability of AvrBs1 protein N-terminal deletions was measured by comparing the response intensity resulting from inoculating with full length genes and N-terminus deletion mutants. All DNA inserts were expressed from binary vector pCB3CaMV2 within *Agrobacterium* cells under Cauliflower Mosaic Virus (CaMV) 35S promoter control.

Table 3.8: Identification of X. ampelinus AvrBs1 protein HR-inducing domain through Agrobacterium-mediated transient expression in sweet pepper STAR 6657

Construct	Description	HR test
pCB3-*avrBs1*$_{Xcc}$	Binary vector with CaMV 35S promoter and *X. campestris* pv. *campestris* full length *avrBs1* gene	+
pCB3-*avrBs1*$_{Xa}$	Binary vector with CaMV 35S promoter and *X. ampelinus* full length *avrBs1* gene	+
pCB3-AvrBs1$_{22-413}$	Binary vector with CaMV 35S promoter and *X. ampelinus* AvrBs1 N-terminal deletion starting from amino acid 22	+
pCB3-AvrBs1$_{57-413}$	Binary vector with CaMV 35S promoter and *X. ampelinus* AvrBs1 N-terminal deletion starting from amino acid 57	+
pCB3-AvrBs1$_{64-413}$	Binary vector with CaMV 35S promoter and *X. ampelinus* AvrBs1 N-terminal deletion starting from amino acid 64	-
pCB3-AvrBs1$_{100-413}$	Binary vector with CaMV 35S promoter and *X. ampelinus* AvrBs1 N-terminal deletion starting from amino acid 100	-
pCB3CaMV2	Binary vector with CaMV 35S promoter	-

Key: - no HR; + full-blown HR

3.3.2 Generation and testing of the *XaΔavrBs1* mutant

Generation of gene knockout mutants is an important and popular practice commonly applied in determining gene function. In order for us to be able to test a T3E reporter system based on the *avrBs1* gene, a mutant *X. ampelinus* strain with deleted *avrBs1* gene was required. To generate the mutant, PCR-overlap extension was used to create a gene SOEing insert by joining *avrBs1* flanking sequences together with a kanamycin resistance cassette. The SOEing insert was cloned into a suicide plasmid pJET1.2/blunt, and the construct was verified by restriction digest using FastDigest *Hind*III and *Bam*HI restriction enzymes. The two size bands observed in each reaction (Figure 3.4) corresponds to the sizes for the plasmid and insert as generated by CLC Genomics workbench v6. To determine whether allelic exchange had occurred, *X.*

ampelinus cells transformed with pJET1.2/blunt-SOEing were PCR-verified with primers specific for the kanamycin resistance cassette and *avrBs1* CD (Figure 3.5). The HR test of the *XaΔavrBs1* mutant on pepper leaves expressing the *Bs1* resistance gene revealed the loss of the HR-inducing ability (Figure 3.7). This *XaΔavrBs1* knockout mutant was used in the subsequent testing of the T3E reporter system.

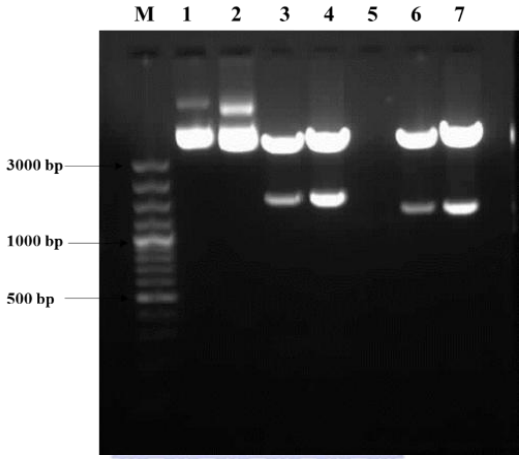

Figure 3.4: Verification of pJET1.2/SOEing construct. Lane M- 100 bp Plus DNA marker, lane 1- and 2- uncut pJET1.2/SOEing construct, lane 3- and 4- pJET1.2/SOEing construct digested with *Hind*III; lane 5- empty; lane 6- and 7- pJET1.2/SOEing digested with *Bam*HI.

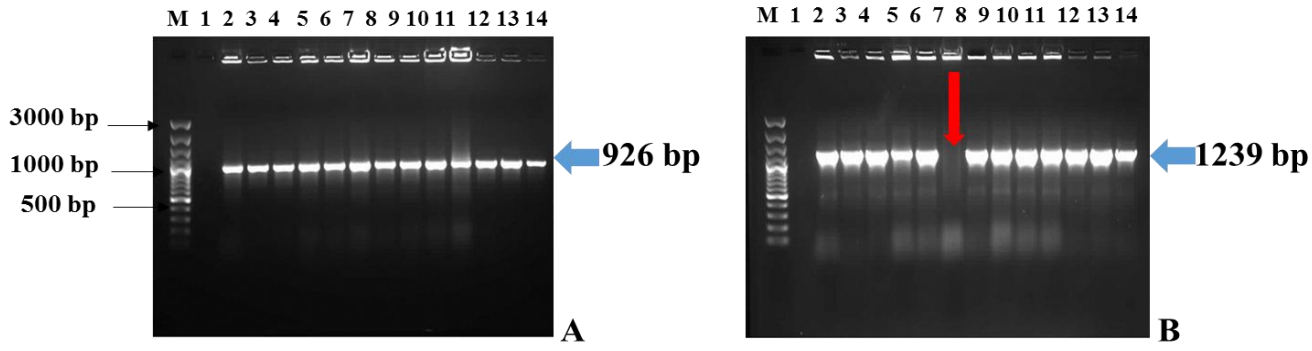

Figure 3.5: Verification of *XaΔavrBs1* knockout mutant using primers specific for the kanamycin cassette (A) and *avrBs1* CD (B). Lane M- 100bp Plus DNA marker; lane 1- negative control (no DNA), lane 2 to 14- wt *X. ampelinus*-pJET1.2/SOEing transformants.

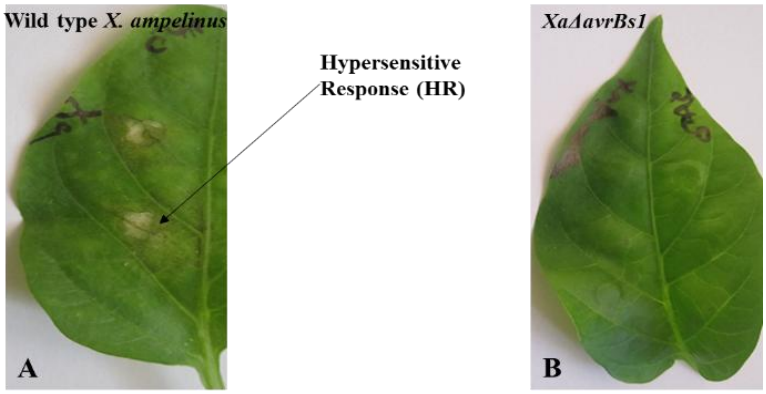

Figure 3.6: *XaΔavrBs1* knockout mutant HR assay on pepper STAR 6657. In the wild type, HR appeared with 24 hours as necrosis at the sites of inoculation (A), while the mutant did not produce an HR reaction (B).

3.3.3 Development and testing of the T3E reporter plasmid system

HR reporter plasmid systems have been widely applied in effector research (Xu *et al*., 2008; Schechter *et al*., 2004). HR-inducing domains of known effector proteins from *Xanthomonas* and *Pseudomonas* have been used to develop the reporter systems for the identification and classification of candidate effectors. In this study we determined the HR-inducing domain of the *X. ampelinus* AvrBs1 protein (Section 3.3.1) which played a huge role in the development of the reporter. To develop a reporter protein construct, the AvrBs1$_{57\text{-}413}$ HR-inducing domain was fused to the 5′ end of a 3x FLAG epitope in the broad host range vector pBM5flg. Full length *avrBs1$_{Xa}$ and avrBs1$_{Xcc}$* CDs were individually fused to the 5′ end of a 3x FLAG epitope to serve as positive controls. Transformants for each construct were screened by colony PCR and the final isolates selected for the secretion and translocation assay were again PCR-verified to ensure that *avrBs1* had been deleted from the chromosome and replaced with the kanamycin resistance cassette, and that each construct in the broad host range vector was present in the respective *XaΔavrBs1* isolates (Figure 3.7).

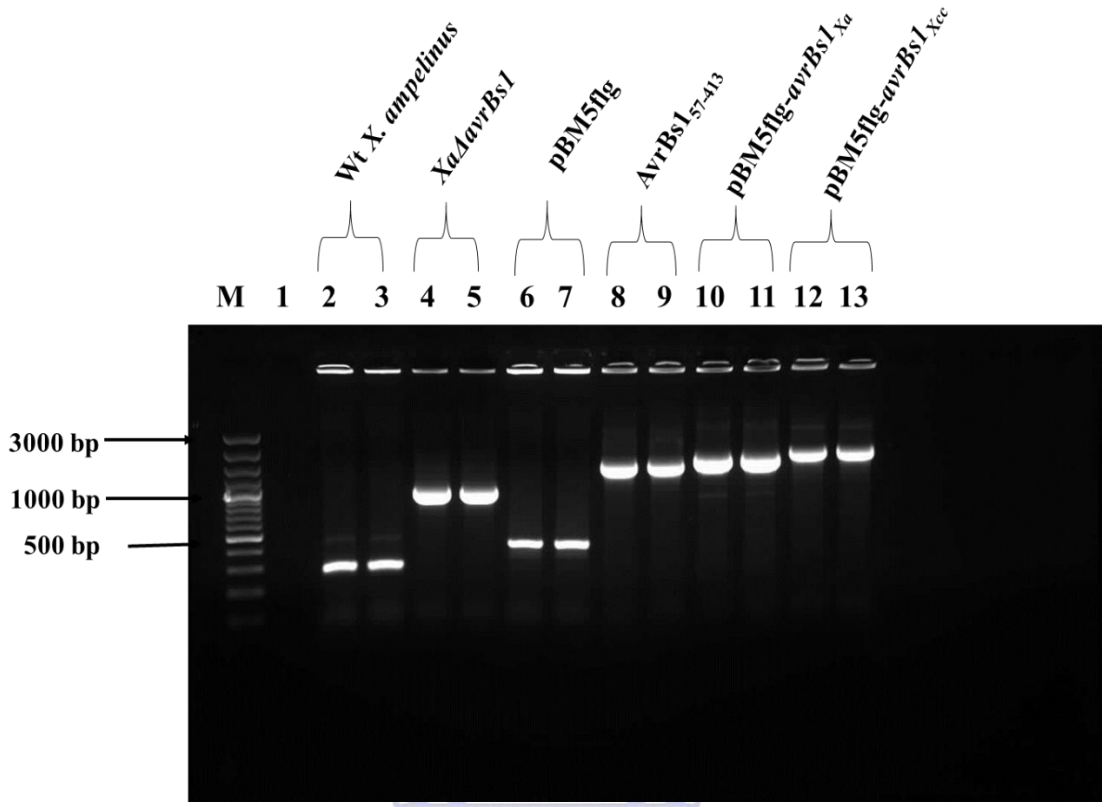

Figure 3.7: Verification of isolates to be used for secretion and translocation assays. The PCR primers specific for the following were used: *X. ampelinus* ITS sequence, *XaΔavrBs1* mutant kanamycin cassette, pBM5flg Lac Z operon, AvrBs1$_{57\text{-}413,}$ deletion mutant, pBM5flg-*avrBs1*$_{Xa}$ full length *X. ampelinus avrBs1*, pBM5flg-*avrBs1*$_{Xcc}$ full length *X. campestris* pv. *campestris avrBs1*. Lane M- 100 pb Plus DNA marker, lane 1- negative control (no DNA), lane 2 and 3- wild type *X. ampelinus* CFUs, lane 4 and 5- *XaΔavrBs1,* lane 6 and 7- *XaΔavrBs1*-pBM5flg, lane 8 and 9- *XaΔavrBs1*-pBM5flg- AvrBs1$_{57\text{-}413,}$ lane 10 and 11- *XaΔavrBs1*-pBM5flg-*avrBs1*$_{Xa}$, lane 12 and 13- *XaΔavrBs1-avrBs1*$_{Xcc}$ pBM5flg CFUs.

Protein secretion assays of the full length *avrBs1*$_{Xa}$, *avrBs1*$_{Xcc}$ and the reporter protein constructs expressed by the mutant, *XaΔavrBs1*, resulted in the expression of the avirulence protein AvrBs1 from both *X. ampelinus* and *X. campestris* pv. *campestris* as well as the reporter protein construct when grown in either YPG or minimal medium (Figure 3.8). The secretion of proteins to the medium was, however, not observed (Figure 3.9). The results obtained for the protein translocation assay in pepper STAR 6657 was not satisfactory, since the isolates, *XaΔavrBs1* and *XaΔavrBs1*-pBM5flg unexpectedly also induced HR (Figure 3.10).

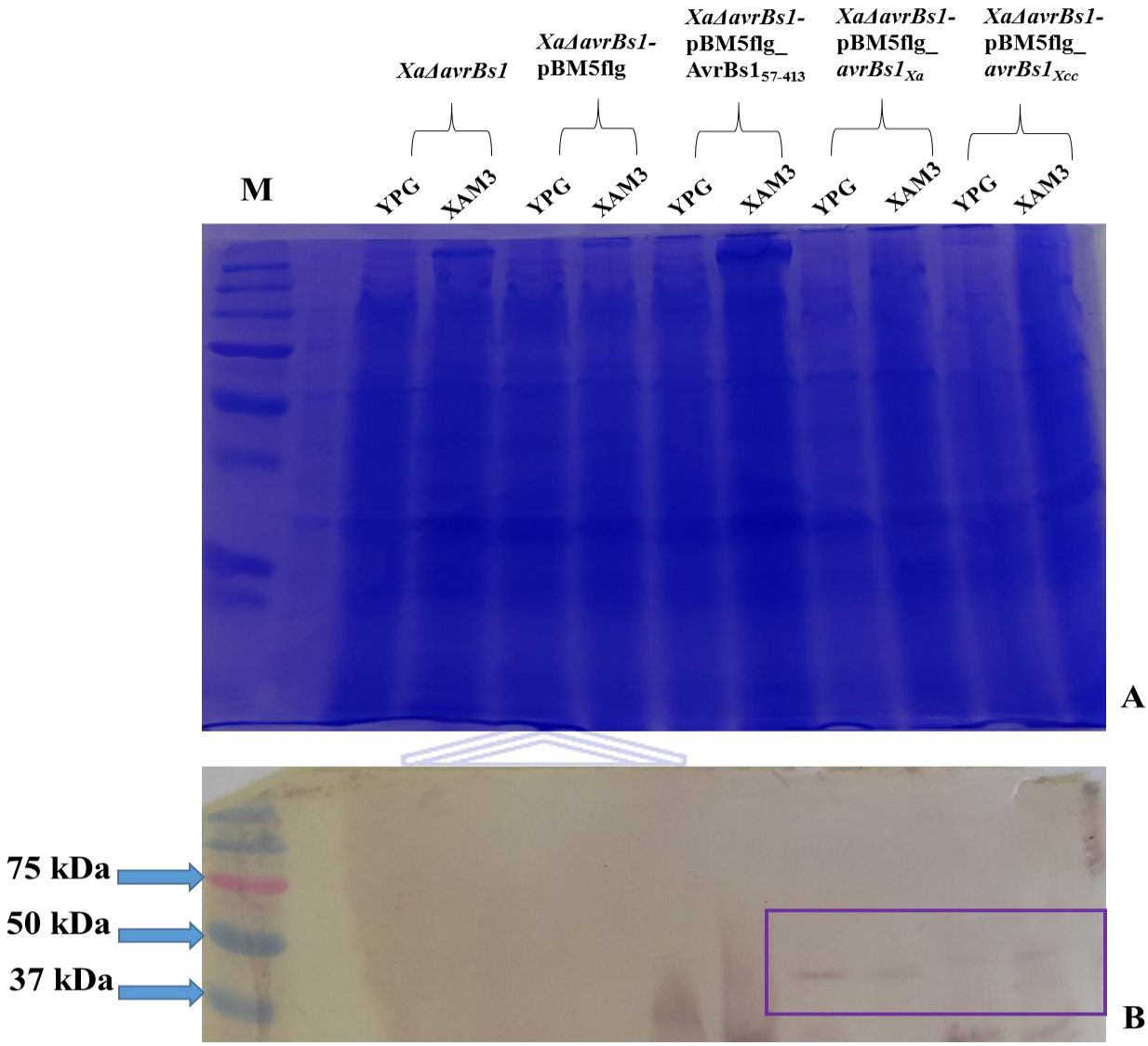

Figure 3.8: Protein secretion assay- cell pellets. Total protein extracts of the cellular fraction of *X. ampelinus* grown in YPG and XAM3 medium were analysed by 1D SDS-PAGE (A) and Western blot (B). Lane M- Precision Plus Protein™ Dual Color Standards, lane 2 and 3- *XaΔavrBs1* mutant in YPG and XAM3 medium, lane 4 and 5- *XaΔavrBs1*-pBM5flg in YPG and XAM3, lane 6 and 7- *XaΔavrBs1*-pBM5flg-AvrBs1$_{57-413}$ in YPG and XAM3, lane 8 and 9- *XaΔavrBs1*-pBM5flg-*avrBs1*$_{Xa}$ in YPG and XAM3, lane 10 and 11- *XaΔavrBs1*-pBM5flg-*avrBs1*$_{Xcc}$ in YPG and XAM3.

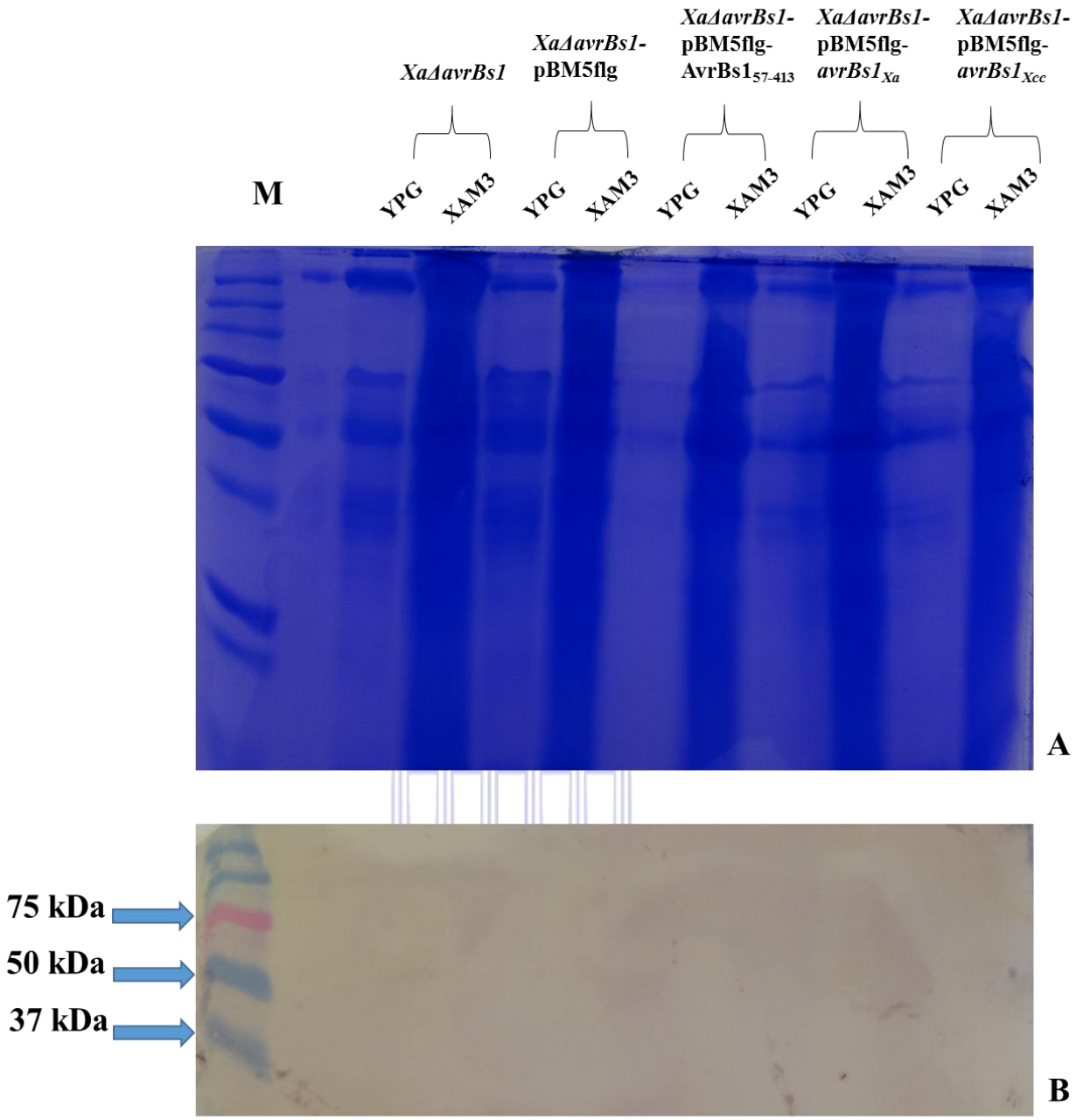

Figure 3.9: Protein secretion assay- supernatants. Total protein extracts of the supernatants of *X. ampelinus* grown in YPG and XAM3 medium were analysed by 1D SDS-PAGE (A) and Western blot (B). Lane M- Precision Plus Protein™ Dual Color Standards, lane 2 and 3- *XaΔavrBs1* mutant in YPG and XAM3 medium, lane 4 and 5- *XaΔavrBs1*-pBM5flg in YPG and XAM3, lane 6 and 7- *XaΔavrBs1*-pBM5flg-AvrBs1$_{57-413}$ in YPG and XAM3, lane 8 and 9- *XaΔavrBs1*-pBM5flg-*avrBs1*$_{Xa}$ in YPG and XAM3, lane 10 and 11- *XaΔavrBs1*-pBM5flg-*avrBs1*$_{Xcc}$ in YPG and XAM3.

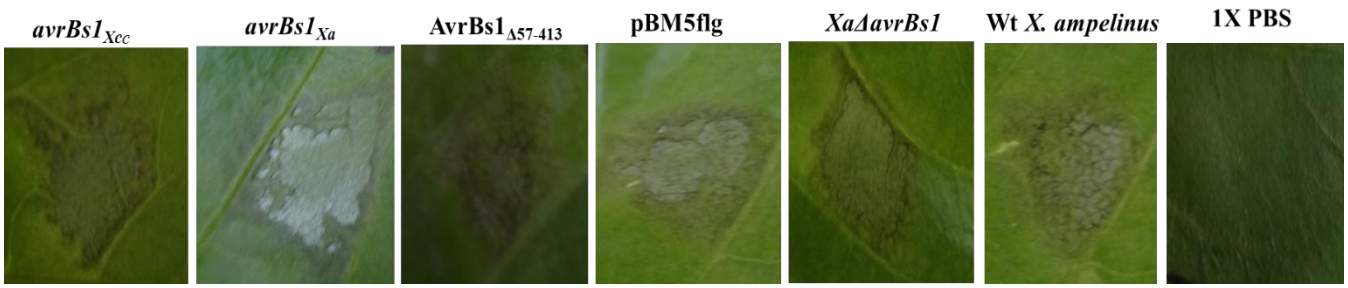

Figure 3.10. Protein translocation assay of the reporter system. Strains wild type *X. ampelinus*, untransformed mutant *XaΔavrBs1, XaΔavrBs1*-pBM5flg, *XaΔavrBs1*-pBM5flg-AvrBs1$_{57\text{-}413}$ (Type III reporter vector construct), *XaΔavrBs1*-pBM5flg-*avrBs1$_{Xa}$*, and *XaΔavrBs1*-pBM5flg-*avrBs1$_{Xcc}$* HR test on sweet pepper STAR 6657 plants.

3.4 DISCUSSION

Effector discovery has been of increasing importance lately. Many research groups have focused on identification and characterization of bacterial pathogen effector proteins to better understand the pathogenicity factors as well as plant-microbe interactions (Alfano, 2009). In this search, application of bioinformatic approaches as well as studying effector protein functional domains have gained increasing popularity. The effectors from many plant pathogenic bacteria have been identified by comparing their sequences with sequences from other known effectors or sequence motifs that suggest certain roles during pathogenicity.

Functional sequence motifs required by pathogens to cause disease or colonize the host cells have been identified. This has expanded to the identification and characterization of effector protein functional domains (Boch and Bonas, 2010). The studies suggest that in the majority of effector proteins the N-terminal domain encodes the translocation signal (Sory *et al*., 1995; Schesser *et al*., 1996; Guttman and Greenberg, 2001). Many studies have focused on the C-terminal domain of effector proteins. The studies suggest that the C-terminal domain is responsible for HR-induction and that the domain is sufficient for recognition inside host cells (Schechter *et al*., 2004; Mudgett *et al*., 2000; Guttman and Greenberg, 2001). This finding led to a range of analysis and classification of T3 secreted effectors studies. A number of research groups have applied the HR-inducing domains of effector proteins in the development of reporter plasmid systems to identify and classify candidate T3 secreted candidate effectors (Jiang *et al*., 2009; Nöel *et al*., 2003; Xu *et al*., 2008).

The availability of genomic sequences of known pathogens and genetic sequencing tools have played a major role in effector research. Sequence homologies within effector protein families have facilitated the identification and characterization of effector proteins due to the presence of conserved sequence motifs and protein domains that serve certain major functions during pathogenicity. AvrBs1 protein from *X. ampelinus* shares up to 58% identity with known T3 effector proteins from *Xanthomonas*. The sequence homology is observed towards the C-terminus (Chapter 2). *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *campestris* AvrBs1 effector proteins have been widely applied in effector identification studies (Jiang *et al*., 2009; Xu *et al*., 2008). Therefore, sequence homology between these effectors presented a challenge to identify the HR-inducing domain of AvrBs1 protein from *X. ampelinus*, so that it too could be applied to the identification of T3 secreted effectors. *Agrobacterium*-mediated transient expression of AvrBs1 protein N-terminal deletion mutants revealed that amino acids 57-413 is

the shortest segment required for *in planta* recognition and HR-induction in Sweet pepper hybrid STAR 6657 carrying *Bs1* resistance gene which recognizes *avrBs1* gene products. Characterization of *X. ampelinus* AvrBs1 HR-inducing domain suggested its recognition inside plant cells, thus making the protein a viable option as a reporter for protein translocation studies.

A broad-host range vector, pBBR1-MCS5 was used in construction of the reporter due to the relatively small size of the vector of which made it more suitable for easy transformation; (Kovach *et al*., 1995). Cloning of the DNA segments lacking the stop codons, allowed protein fusion between the *avrBs1* HR-inducing domain and the 5′ end of the 3x FLAG epitope, thus allowing easy selection of expressed proteins.

The *XaΔavrBs1* mutant, for the testing of a reporter system, was generated using PCR-overlap extension and allelic exchange where an *avrBs1* gene was replaced with a kanamycin gene for selection purposes. The knockout mutant was verified through PCR using primers specific for the kanamycin gene and primers specific for an *avrBs1* gene to confirm its complete replacement with kanamycin. Ampicillin sensitivity test confirmed assimilation of kanamycin gene in the chromosome proving that kanamycin resistance expressed by the mutant was not conferred by SOEing construct and also proving the destruction of a suicide plasmid during allelic exchange. This was all confirmed by inability of the bacteria to grow in ampicillin containing medium. *XaΔavrBs1* mutant lost total ability to elicit HR when inoculated on pepper. Even though the pepper hybrid STAR 6657 expresses two other dominant resistance genes *Bs2* and *Bs3*, *avrBs2* and *avrBs3* were not located on *X. ampelinus* chromosome (Y. Petersen, personal communication).

In vitro protein secretion assays revealed that full length *X. ampelinus* and *Xcc* AvrBs1 proteins were expressed within the bacterial cells. However, colourimetric detection of the secreted proteins in the supernatant was not successful. This does not mean that the proteins were not secreted. The result may have been due to an ineffective minimal medium composition which led to undetectable levels of protein secretion or perhaps the protein concentrating method used resulted in loss of the already low levels of secreted protein in the supernatant. If the proteins are being secreted at low levels, it might be of benefit to try a chemiluminescent detection method which has a greater sensitivity (Noël *et al*., 2003; Xiao *et al*., 2007; Li *et al*., 2014).

The results from the translocation assay in pepper leaves also did not give the expected result. In this assay, the negative control, *XaΔavrBs1* also induced HR. The assay was repeated three

times and the similar results were observed, despite the fact that when initially tested, *XaΔavrBs1* did not elicit HR.

In addition, although the hybrid pepper, STAR 6657, carries active *Bs1*, *Bs2*, and Bs3 resistance genes, X. *ampelinus* has only the *avrBs1* gene which would be recognised *in planta*. The fact that *X. ampelinus* AvrA-like protein shares some homology with AvrBs1 proteins suggest that the protein might have contributed to the response observed. Therefore deleting both *avrBs1* and *avrA* CDs from *X. ampelinus* might improve the translocation assay specificity. Another recommendation would be the application of Cya as a reporter to create the AvrBs1-Cya fusion that can facilitate and perhaps improve the successful translocation of the effectors into plant cells despite of the low concentration of secreted protein in the culture medium. This reporter has been successfully used by Schechter *et al*. (2004).

3.5 CONCLUSION

The determination of the *X. ampelinus* AvrBs1 HR-inducing domain using *Agrobacterium*-mediated transient expression led to the development of the reporter system by cloning the domain at the 5′ end of 3x FLAG in pBBR1-MCS5 broad host range vector. The testing of a reporter plasmid construct was divided into two assays *viz*. protein secretion and protein translocation. The production and expression of an ~44 kDa *X. ampelinus* AvrBs1 protein inside cells suggested that the pathogen can express the proteins. Provided the medium composition supports AvrBs1 expression and secretion, the availability of sensitive protein detection kits could also enhance the testing of secreted proteins. For the translocation assays a better gene knockout mutant void of *avrBs1*-family genes and the application of an alternative translocation reporter may facilitate the testing of the T3 effector reporter on *Bs1* resistant pepper plants

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CHAPTER 4: General discussion, conclusion and future prospects

Xylophilus ampelinus, the causal agent of bacterial blight of grapevines has long been a threat to South African table grape industry. Bacterial blight has led to some serious losses in past due to its occurrence. To date there are no successful control measures that are known and have been tested, meaning the disease is still of great importance. *X. ampelinus* is a Gram negative, slow growing rod-shaped member of *Comamonadaceae*, a β-subclass of Proteobacteria. The pathogen lives in the vascular vessels of infected vines. The symptoms are generally observed in young shoots that are 2-3 years old, leaves, and bunches.

Unfortunately there is really not much known about the genetic make-up of the pathogen especially with regards to the factors contributing to its pathogenicity. The research conducted at the ARC Infruitec-Nietvoorbij has made extensive progress in sequencing of the bacterial genome. Availability of the pathogen's genomic sequence has assisted in the attempts to discover its virulence factors. Although there are limited resources to start with since the bacteria is the only species in its genus *Xylophilus*, we managed to engage other close relatives in the attempts to learn more about the pathogen.

Dating back to the initial detection of the pathogen, it was classified as belonging to the genus *Xanthomonas* due to features such as being an aerobic, non-spore forming Gram negative rod-shaped organism with one polar flagellum. It has oxidative carbohydrate metabolism, produces a yellow insoluble pigment and it has a mean DNA base composition similar to that of genus *Xanthomonas*. Even though the bacteria was lately moved to its genus *Xylophilus*, these features were not about to go unnoticed. These features suggested that the two genera are somehow related, not only phenotypically but also at genetic level. Luckily a lot of research has focused on *Xanthomonas* and *Pseudomonas* species. These pathogens have been reported to utilize the Type III secretion system (T3SS) in attempts to evade plant innate immunity. From literature, it has been repeatedly mentioned that the T3SS facilitates pathogenicity or virulence by directly injecting effectors inside host cells. This was interesting and the big question that remained was: does *X. ampelinus* employ the T3SS like all its relatives?

Initial analysis of *X. ampelinus* transposon mutants revealed a mutant, XaTn5-742, which had an insertion site in a gene encoding an avirulence protein with similarities to major avirulence gene protein families in *P. syringae* pv. *glycinea* and some *Xanthomonas* species.

Bio-informatic analysis of the open reading frames (ORFs) in this region of the *X. ampelinus* genomic DNA sequence seemed like a great start in identifying gene coding sequences that are somehow involved in pathogenicity. The search at protein level proved what was observed in transposon mutagenesis study and returned two hits which were closely related to *Xanthomonas* and *Pseudomonas* T3 secreted effectors. The *X. ampelinus* CDs shared above 50% sequence identity with known *avrBs1*-family avirulence genes secreted as type III effectors. These two homologs were easy enough to work with based on high level of sequence identity. Avirulence genes are defined based on their ability to betray the pathogen to the host defense system causing the plant to elicit resistance response against the infecting pathogen. *Xanthomonas avrBs1* genes have been widely characterized as HR-inducing genes and secreted in a Type III dependent manner.

The first step was to characterize an avirulence gene family from *X. ampelinus* strain VS20. The full length *avrA* and *avrBs1*-like homologs induced HR when transiently expressed in tobacco plants. Even though this was not the final conclusion since the plants did not really express the corresponding *Bs1* resistance gene, it was enough to say the genes can elicit HR in non-host. Generally, effector protein secretion and translocation signals are located at the N-terminal domain whereas, the HR-inducing domain is within the C-terminal region. Through literature it has been proven that the HR-inducing domain of the effector protein is sufficient for *in planta* recognition and this region has been widely applied in reporter fusion studies. Among other effector proteins *Xanthomonas* AvrBs1 HR-inducing domain has been successfully applied as a reporter to identify and classify Type III secreted effector repertoire.

The aim of the study was to generate a reporter system for the analysis and classification of *X. ampelinus* candidate effectors as T3-secreted class of effectors. Identification and characterization of an *avrBs1*-family gene was enough motivation to determine the HR-inducing domain of the protein and apply it in reporter protein fusions. *Agrobacterium*-mediated transient expressions in sweet pepper STAR 6657 hybrid expressing the *Bs1* gene revealed that the HR-inducing domain of *X. ampelinus* AvrBs1 protein was within amino acid 57 and 413 region. *X. ampelinus* AvrBs1 HR-inducing domain was enough for *in planta* recognition and therefore it was sufficient for reporter protein fusion. The cloning of this domain into a FLAG-containing broad host range vector, pBBR1MCS5, produced a reporter fusion construct that could be used in T3 effector studies.

As mentioned before, effector secretion and translocation signals are localized at their N-terminal domains. Therefore the effector protein requires a secretion signal for its secretion in the medium and/ or translocation in plants. The C-terminus HR-reporter was tested along with full length *avrBs1* proteins for protein secretion into growth medium and translocation in plants. The expression of AvrBs1 fusion proteins was observed in the cellular fraction, indicating that there was no problem with the transcription and translation of the full length AvrBs1-3x FLAG fusion protein, however, the protein was not detected in the supernatant fraction using the growth medium, and protein precipitation and detection methods mentioned in Chapter 3. Perhaps the use of the Cya reporter can enhance effector protein secretion and translocation in combination with a more sensitive detection method base on chemilumniscence.

Although the *X. ampelinus* T3 effector reporter vector did not produce all the results expected, a number of conclusions can be made. Firstly, *X. ampelinus* AvrBs1 and AvrA-like proteins share up to 60% homology with known avirulence proteins from *Xanthomonas*, *Pseudomonas*, and *Acidovorax* species, which are secreted by the T3SS, therefore suggesting that *X. ampelinus* might be employing the T3SS to secrete and translocate effectors during pathogenicity.

We showed that *X. ampelinus* AvrBs1 and AvrA-like proteins are functional as elicitors of HR in *N. tabacum* when transiently expressed. Expression through *Agrobacterium*-mediated transient expression under the control of 35S promoter gave strong HR suggesting that the two avirulence proteins can be used as reporter system for the analysis of T3 effector repertoire. Although a reporter vector was successfully constructed, additional research is required to determine why the *XaΔavrBs1* knockout mutant, which was HR-negative when initially tested, later produced a different result when the translocation experiment was done. Further research into suitability of growth medium for optimal T3 effector secretion is also required.

Development of the T3 effector reporter system for *X. ampelinus* was progress in attempts to analyse and characterize the effector repertoire in this pathogen. The N-terminal secretion signal is a key element in T3 effector classification, which is why candidate effector N-terminal domains will be cloned at the 5′ end of AvrBs1$_{57\text{-}413}$ and a FLAG tag in a reporter system. Effectors secreted only when expressed in a strain with full T3SS activity will be then classified as Type III secreted class of effector.

In conclusion, effector discovery is a long road that at the end will give full understanding of the pathogen's pathogenicity factors. The lack of resistant cultivars is still a major problem in world's economy. However, baby steps in understanding plant pathogen effectors may lead to crop improvement. Discovering effectors, their targets and the functions they express when reaching the target could be the way to go.

APPENDIX A

General Chemicals and Suppliers

All chemicals used in this study, their suppliers and perspective catalogue numbers are listed alphabetically in the table below.

Table A: List of chemicals used in the study

Chemical name	Supplier	Catalog number
Absolute ethanol	Merck	SAAR2233540LP
Acetone	Kimix	5144 K07/07/14
Acetosyringone	Sigma	D134406
Acrylamide stock solution (30%)	BioRad	161-0158
Agarose	Lonza	50004
Ammonium acetate	Merck	101115/6
Ammonium di-Hydrogen phosphate	Merck	A464426
Ammonium persulphate (APS)	BioRad	161-0700
Ampicilin	Roche	10835242001
Anti-FLAG M2 antibody	Agilent	200472-21
Anti-mouse antibody IgG	Promega	W4028
Bacto-agar	Biolab Merck	1023407
Bacto- Casamino Acids	Difco	0230-01-1
Bacto-peptone	Kimix	211677/2156028
Boric Acid	Merck	SAAR1405200EM
Bovine Serum albumin (BSA)	Roche	10227825/711454
Bromocresol purple	Chemicaland21	115-40-2

Calcium Chloride	AnalaR BDH	10070
Coomassie Blue R250	BiorRad	161-0400
CTAB	BDH AnalaR	276654L
D(+) Xylose	Sigma	X3877
di-Sodium Hydrogen Phosphate	Sigma	71640
DMSO	Merck	802912
dNTP mix	Promega	C1141
EDTA	Merck	SAAR2236020EM
Ethidium bromide	Sigma	E8751
FastAP dephosphorylation kit	Thermo-scientific	EF0651
Fast digest *Bam*HI	Thermo-scientific	FD0054
Fast digest *Eco*RI	Fermentas	FD0274
Fast digest *Eco*RV	Fermentas	FD0304
Fast digest *Hind*III	Fermentas	FD0504
Fast digest *Pst*I	Thermo-scientific	FD0614
Fast digest *Pvu*II	Fermentas	ER0634
Fast digest *Sac*I	Thermo-scientific	FD1133
Fast digest *Xba*I	Fermentas	FD0684
Fat Free Milk powder	Clover	3284
Ferrous Sulphate	UniLAB Saarchem	53076
Generuler DNA Ladder	Thermo-scientific	SM0323
Glucose	Kimix	27642 K25/0712
Glycerol	Kimix	BASX7A2759 K09/1111

Glycine	Kimix	ZY080718 K21/1008
GoTaq Flexy DNA polymerase	Promega	M8305
Hydrochloric Acid (HCl)	AnalaR BDH	10307
Isopropanol	Kimix	4200 K16/0311
Kanamycin	Sigma	K4378
L(+) Arabinose	Sigma	A3256
Loading dye	Thermo-scientific	R 6 111
Magnesium chloride	Sigma	M8266
Magnesium sulphate	BDH AnalaR	BB101514Y
Mecarpto-ethanol	Kimix	805740
MES hydrate	Sigma	M2933
Methanol	Kimix	4145 K05/1110
Nutrient agar	Difco	213000
Nutrient broth	Difco	234000
Phusion DNA polymerase	Thermo-Scientific	F-530S
Potasium chloride	AnalaR BDH	10198
Potassium phosphate monobasic	Sigma	P9791
Potasium phosphate dibasic	Sigma	P2222
Precision Plus Protein Standards	BioRad	161-0363
Primers	IDT White-head Scientific	
Proteinase K	Macherey-Nagel	740506
QiaexII gel purification kit	Qiagen	20021
Qiagen plasmid mini-prep kit	Qiagen	27106

Rifampicin	Sigma	R 3 501
RNAse	Fermentas	EN0531
S1 Nuclease DNA blunting kit	Fermentas	EN0321
Sodium chloride	Kimix	2741 K39/0605
Sodium hydroxide	Kimix	11-10/01 K15/0311
SDS	AnalaR BDH	44244
T4 rapid DNA ligation kit	Thermo-Scientific	K1422
TEMED	Merck	10732
Trichloroacetic Acid	Merck	SAAR6110500EM
Tris	Melford	B2005
Tryptone	Kimix	B60678
Tween 20	Merck	8.22184.05000
Yeast extract	Kimix	LP0021

APPENDIX B

B1. General Stock Solutions and Buffers

Most buffers and solutions were autoclaved at 121 °C for 20 minutes using a Vertical Type Steam Sterilizer Speedy Autoclave HL-340 (Gemmy Industrial Corp, Taiwan)

90% acetone: 90% (v/v) acetone in distilled water.

70% ethanol: 70% (v/v) absolute ethanol in distilled water.

10% APS: 10% (w/v) APS in distilled water. The solution was freshly prepared before use.

1% blocking solution: 1% (w/v) Elite fat free instant milk powder in TBS.

Bradford reagent: 1 part of Bradford protein assay dye reagent concentrate diluted with 4 parts of distilled water.

20 mg/ml BSA stock solution: 20 mg/ml BSA in 1X PBS.

100 mM acetosyringone: 100 mM acetosyringone (3′,5′-Dimethoxy-4′-hydroxyacetophenine) in DMSO.

1 M MES: 1 M (w/v) MES 2-(N-Morpholino)ethanesulfonic acid in distilled water adjusted to pH 5.6 with 2 M NaOH.

Induction medium: 10 mM (v/v) MgCl2; 10 mM (v/v) MES pH 5.6 and 150 µM (v/v) acetosyringone in distilled water.

Linker buffer: 50 mM (v/v) Tris-HCl pH 8.0, 100 mM (v/v) NaCl and 1 mM EDTA (v/v) in distilled water.

10% CTAB/ 0.7 mM NaCl: 10% NaCl and 0.7. mM CTAB in distilled water.

0.5 M EDTA stock solution: 0.5 M (w/v) EDTA (Ethylenediaminetetraacetic acid) in distilled water.

1 M Tris/HCl stock solution: 1 M (w/v) Tris/HCl in distilled water, pH 8.0.

1X TE buffer: 10 mM Tris/HCl and 1 mM EDTA in distilled water.

Bradford dye reagent: 0.1% (w/v) CBB Commasie Brilliant blue G-250, 25% (v/v) Methanol, and 42.5% (v/v) Phosphoric acid.

10% SDS buffer: 10% (w/v) SDS in distilled water.

5 M NaCl: 5 M (w/v) NaCl in distilled water.

Chloroform/Isoamylalcohol (24.1): 24 parts (v/v) chloroform in 1 part isoamylalcohol.

10x TBE buffer: 89 mM (w/v) Tris Base, 89 mM (w/v) boric acid, and 2 mM (w/v) EDTA in distilled water.

1x TBE buffer: 1 part (v/v) 10x TBE stock in 9 parts of distilled water.

50 mg/ml Kanamycin: 50 mg/ml (w/v) in sterile distilled water

100 mg/ml Ampicillin: 100 mg/ml (w/v) in sterile distilled water

34 mg/ml Rifampicin: 34 mg/ml (w/v) in 100% methanol.

10 µM primer solution: 1 part (v/v) 100 mM primer stock solution in 9 parts of sterile distilled water.

10% Glycerol solution: 10% (v/v) glycerol in distilled water.

15% Glycerol solution: 15% (v/v) glycerol in distilled water.

0.8% agarose gel solution: 0.8% (w/v) agarose in distilled water.

1% agarose gel solution: 1% (w/v) agarose in distilled water.

1 M $MgCl_2$ stock solution: 1 M (w/v) $MgCl_2$ in distilled water.

100 mM $MgCl_2$ solution: 100 mM (v/v) $MgCl_2$ in distilled water.

100 mM CaCl2/ 15% glycerol: 100 mM (w/v) $CaCl_2$ and 15% (v/v) glycerol in distilled water.

25% TCA: 25% (w/v) TCA (Trichloroacetic acid) in distilled water.

10x PBS: NaCl 80 g/L, KCl 2 g/L, Na_2HP0_4 14.4 g/L, KH_2PO_4 2.4 g/L.

4x Tris-SDS Resolving gel solution pH 8.8: 1.5 M (w/v) Tris HCl, 0.8% (v/v) 10% SDS in distilled water.

4x Tris-SDS Stacking gel solution pH 6.8: 0.5 M (w/v) Tris HCl, 0.8% (v/v) 10% SDS in distilled water.

Transfer buffer: 25 mM (w/v) Tris Base, 192 mM (w/v) Glycine and 20% (v/v) Methanol in distilled water.

TBS (Tris Buffered saline) pH 7.5: 50 mM (w/v) Tris Base and 150 mM (w/v) NaCl in distilled water.

TTBS pH 7.5: 50 mM (w/v) Tris Base, 0.5ml/L (v/v) Tween 20 and 150 mM (w/v) NaCl in distilled water.

Coomassie Brilliant Staining solution: 0.25% (w/v) Coomassie Blue R-250, 45% methanol, and 10% acetic acid

Coomassie destaining solution: 5% methanol, 10% acetic acid in water.

B2. Bacterial growth medium

Growth media for all bacterial culture was autoclaved at 121 °C for 20 minutes using a Vertical Type Steam Sterilizer Speedy Autoclave HL-340 (Gemmy Industrial Corp, Taiwan).

LB (Luria-Bertani) broth: Yeast extract 5 g/L, Bacto-Tryptone 10 g/L, NaCl 10 g/L, Glucose 1 g/L, and $MgCl_2$ 1 g/L in distilled water.

LB-agar medium: Yeast extract 5 g/L, Bacto-Tryptone 10 g/L, NaCl 10 g/L, Glucose 1 g/L, Bacto-agar 15 g/L and $MgCl_2$ 1 g/L in distilled water.

YEP broth: Bacto-peptone 10 g/L, Yeast extract 10 g/L and NaCl 5 g/L in distilled water.

YEP agar medium: Bacto-peptone 10 g/L, Yeast extract 10 g/L, Bacto-agar 15 g/L and NaCl 5 g/L in distilled water.

YPG broth: Yeast extract 7 g/L, Bacto-peptone 7 g/L and Glucose 7 g/L in distilled water.

YPGA medium: Yeast extract 7 g/L, Bacto-peptone 7 g/L, Bacto-agar 15 g/L and Glucose 7 g/L in distilled water.

SOC medium: Trypone 20 g/L, Yeast extract 5 g/L, NaCl 10 g/L, Glucose 1 g/L, $MgCl_2.6H_20$ 1 g/L.

NBG (Nutrient broth/ 10% glycerol): Nutrient broth 8 g/L (w/v) and 10% glycerol (v/v) in distilled water.

XAM3 medium pH 6.5-8): 10 mM (w/v) $NH_4H_2PO_4$, 2.87 mM (w/v) K_2HPO_4, 5 mM (w/v) $MgCl_2.6H_2O$, Bacto-Casamino acids 0.3 g/L (w/v), Bromocresol purple 0.7 ml/L (v/v) [1.5% (w/v) stock in ethanol], 10 µM (v/v) $FeSO_4$, 15 mM (v/v) D(+)xylose, and 20 mM (v/v) L(+)arabinose in distilled water.

1 M D(+)xylose: 1 M (w/v) D(+)xylose in distilled water.

1 M L(+)arabinose: 1 M (w/v) L(+)arabinose in distilled water.